significant difference in CFR change after carvedilol therapy between patients with significant improvement in follow-up LVEF and those without it. The absence of a significant restoration in CFR after short-term therapy may imply a poor chance of improvement in LV function. On the other hand, a larger change in CFR may indicate a higher chance of it. Therefore, one possible clinical implication of this study is that alteration of CFR after short-term of β-blocker therapy is helpful to predict the response to therapy or the further improvement in LV function during therapy.

Study limitations

The study population was relatively small because of our strict screening criteria to exclude the many factors that might influence coronary flow and because of the strict management of patients during follow-up. It may be more of a logical study design if we divide the study groups based on improvement in CFR rather than that in LVEF. However, we could not determine an adequate cutoff value of CFR change for the prediction of a significant improvement in LVEF because of the small study population. A larger prospective study is needed to show the practical use of CFR for the prediction of recovery of LVEF after carvedilol therapy. Finally, in this study, the dosage of carvedilol used was relatively lower than that used in Western countries. However, the difference in the effective dose between patients in Japan and Western countries has been reported.[6] The recommended dose of carvedilol shown by the Multicenter Carvedilol Heart Failure Dose Assessment (MUCHA) trial[6] in Japan was 5 to 20 mg/d, whereas that shown by the US Multicenter Oral Carvedilol Heart Failure Assessment (MOCHA) trial[1] was 12.5 to 50 mg/d. Therefore, the difference in the effective dose between patients in Japan and Western countries should be considered.

In conclusion, this study demonstrates that improvement in CFR after short-term administration of carvedilol relates to that in LV systolic function at follow-up in patients with IDC. The present observations suggest the potential predictive value of early improvement in coronary circulation for late recovery of LV function during β-blocker therapy in patients with IDC.

References

1. Bristow MR, Gilbert EM, Abraham WT, et al. Carvedilol produces dose-related improvements in left ventricular function and survival in subjects with chronic heart failure. MOCHA Investigators. Circulation 1996;94:2807-16.
2. Packer M, Bristow MR, Cohn JN, et al. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group. N Engl J Med 1996;334:1349-55.
3. Packer M, Coats AJ, Fowler MB, et al. Effect of carvedilol on survival in severe chronic heart failure. N Engl J Med 2001;344:1651-8.
4. Packer M, Antonopoulos GV, Berlin JA, et al. Comparative effects of carvedilol and metoprolol on left ventricular ejection fraction in heart failure: Results of a meta-analysis. Am Heart J 2001;141:899-907.
5. Poole-Wilson PA, Swedberg K, Cleland JG, et al. Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the Carvedilol or Metoprolol European Trial (COMET): randomized controlled trial. Lancet 2003;362:7-13.
6. Hori M, Sasayama S, Kitabatake A, et al. Low-dose carvedilol improves left ventricular function and reduces cardiovascular hospitalization in Japanese patients with chronic heart failure: the Multicenter Carvedilol Heart Failure Dose Assessment (MUCHA) trial. Am Heart J 2004;147:324-30.
7. Bristow MR. Beta-adrenergic receptor blockade in chronic heart failure. Circulation 2000;101:558-69.
8. Opherk D, Schwarz F, Mall G, et al. Coronary dilatory capacity in idiopathic dilated cardiomyopathy: analysis of 16 patients. Am J Cardiol 1983;51:1657-62.
9. Cannon III RO, Cunnion RE, Parrillo JE, et al. Dynamic limitation of coronary vasodilator reserve in patients with dilated cardiomyopathy and chest pain. J Am Coll Cardiol 1987;10:1190-200.
10. Treasure CB, Vita JA, Cox DA, et al. Endothelium-dependent dilation of the coronary microvasculature is impaired in dilated cardiomyopathy. Circulation 1990;81:772-9.
11. Inoue T, Sakai Y, Morooka S, et al. Coronary flow reserve in patients with dilated cardiomyopathy. Am Heart J 1993;125:93-8.
12. Neglia D, Parodi O, Gallopin M, et al. Myocardial blood flow response to pacing tachycardia and to dipyridamole infusion in patients with dilated cardiomyopathy without overt heart failure. A quantitative assessment by positron emission tomography. Circulation 1995;92:796-804.
13. van den Heuvel AF, van Veldhuisen DJ, van der Wall EE, et al. Regional myocardial blood flow reserve impairment and metabolic changes suggesting myocardial ischemia in patients with idiopathic dilated cardiomyopathy. J Am Coll Cardiol 2000;35:19-28.
14. Skalidis EI, Parthenakis FI, Patrianakos AP, et al. Regional coronary flow and contractile reserve in patients with idiopathic dilated cardiomyopathy. J Am Coll Cardiol 2004;44:2027-32.
15. Camici PG, Rimoldi OE. Myocardial blood flow in patients with hibernating myocardium. Cardiovasc Res 2003;57:302-11.
16. Sugioka K, Hozumi T, Takemoto Y, et al. Early recovery of impaired coronary flow reserve by carvedilol therapy in patients with idiopathic dilated cardiomyopathy: a serial transthoracic Doppler echocardiographic study. J Am Coll Cardiol 2005;45:318-9.
17. Hall SA, Cigarroa CG, Marcoux L, et al. Time course of improvement in left ventricular function, mass, and geometry in patients with congestive heart failure treated with beta-adrenergic blockade. J Am Coll Cardiol 1995;25:1154-61.
18. Fukuchi K, Yasumura Y, Kiso K, et al. Gated myocardial SPECT to predict response to beta-blocker therapy in patients with idiopathic dilated cardiomyopathy. J Nucl Med 2004;45:527-31.
19. Hasegawa S, Kusuoka H, Maruyama K, et al. Myocardial positron emission computed tomographic images obtained with fluorine-18 fluoro-2-deoxyglucose predict the response of idiopathic dilated cardiomyopathy patients to β-blockers. J Am Coll Cardiol 2004;43:224-33.
20. Lang RM, Bierig M, Devereux RB, et al. Recommendations for chamber quantification: a report from the American Society of Echocardiography's guidelines and standards committee and the chamber quantification writing group, developed in conjunction

with the European Association of Echocardiography, a branch of the European Society of Cardiology. J Am Soc Echocardiogr 2005;18:1440-63.

21. Hozumi T, Yoshida K, Akasaka T, et al. Noninvasive assessment of coronary flow velocity and coronary flow velocity reserve in the left anterior descending coronary artery by Doppler echocardiography: comparison with invasive technique. J Am Coll Cardiol 1998; 32:1251-9.
22. Hozumi T, Eisenberg M, Sugioka K, et al. Change in coronary flow reserve on transthoracic Doppler echocardiography after a single high-fat meal in young healthy men. Ann Intern Med 2002;136: 523-8.
23. Neglia D, Michelassi C, Trivieri MG, et al. Prognostic role of myocardial blood flow impairment in idiopathic left ventricular dysfunction. Circulation 2002;105:186-93.
24. Bottcher M, Czernin J, Sun K, et al. Effect of beta 1 adrenergic receptor blockade on myocardial blood flow and vasodilatory capacity. J Nucl Med 1997;38:442-6.
25. Billinger M, Seiler C, Fleisch M, et al. Do beta-adrenergic blocking agents increase coronary flow reserve? J Am Coll Cardiol 2001;38:1866-71.
26. Lorenzoni R, Rosen SD, Camici PG. Effect of alpha 1-adrenocepter blockade on resting and hyperemic myocardial blood flow in normal humans. Am J Physiol 1996;271(4 Pt 2):H1302-6.

Circ J 2007; **71:** 327–330

Higher Serum Tenascin-C Levels Reflect the Severity of Heart Failure, Left Ventricular Dysfunction and Remodeling in Patients With Dilated Cardiomyopathy

Fumio Terasaki, MD; Hiroshi Okamoto, MD*; Katsuya Onishi, MD**; Akira Sato, MD†; Hiroaki Shimomura, MD; Bin Tsukada, MD; Kyoko Imanaka-Yoshida, MD††; Michiaki Hiroe, MD‡; Toshimichi Yoshida, MD††; Yasushi Kitaura, MD; Akira Kitabatake, MD* and Study Group for Intractable Diseases by a Grant from the Ministry of Health, Labor and Welfare of Japan

Background Tenascin-C (TN-C), an extracellular matrix glycoprotein, is specifically expressed at high levels during embryonic development, but not in the adult heart. TN-C reappears at sites of inflammatory tissue remodeling or wound healing under various pathologic conditions, such as acute myocardial infarction, acute myocarditis, and some cases of cardiomyopathy. Therefore, the expression of TN-C might be useful for detecting the clinical characteristics of, and ventricular remodeling in, dilated cardiomyopathy (DCM).

Methods and Results Circulating serum TN-C levels in 107 patients with DCM were measured using an ELISA kit. Clinical data were also assessed by Pearson's or Spearman's correlation analysis to estimate correlations between variables. Serum TN-C levels in DCM patients were higher than those in normal controls ($p<0.001$). TNC levels showed a significantly positive correlation with New York Heart Association functional class ($p<0.001$), B-type natriuretic peptide level ($p<0.001$), cardiothoracic ratio on chest X-ray ($p<0.01$), left ventricular end-diastolic diameter ($p<0.05$) and left ventricular end-systolic diameter ($p<0.01$), and a significantly negative correlation with left ventricular ejection fraction ($p<0.01$).

Conclusions The findings suggest that increased serum TN-C levels indicate the severity of heart failure, left ventricular dysfunction and remodeling in patients with DCM. (*Circ J* 2007; **71:** 327–330)

Key Words: Dilated cardiomyopathy; Heart failure; Tenascin-C; Ventricular remodeling

Dilated cardiomyopathy (DCM) refers to a spectrum of heterogeneous myocardial disorders that are characterized by ventricular dilation and depressed myocardial contractility in the absence of abnormal loading conditions or ischemic heart disease[1,2] The prognosis of DCM has improved since angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers and β-blockers have been widely used as treatment; however, despite current optimal medical management, heart failure remains a progressive disease with significant morbidity and mortality. Eventually, some cases need cardiac resynchronization or surgical interventions such as heart transplantation or left ventriculoplasty[3] Therefore, risk stratification in patients with DCM is receiving increasing interest, and is crucial for selection of the best treatment strategy. In particular, the diagnostic and prognostic value of B-type natriuretic peptide (BNP) and N-terminal pro-BNP have been established in heart failure patients[4–6]

Tenascin-C (TN-C), an extracellular matrix glycoprotein[7–10] plays important roles in the development of the myocardium, heart valves and coronary vessels in early-stage embryos but is not detected in adults[11] However, it is re-expressed under pathological conditions such as acute myocardial infarction (AMI),[12,13] myocardial hibernation,[14] myocardial injury,[15] and active myocarditis,[16–18] which are closely associated with tissue injury and inflammation. Furthermore, while TN-C molecules are deposited in the local extracellular spaces, soluble forms are also released into the blood stream. For example, serum TN-C levels are significantly elevated during the acute stage after AMI, and therefore may act as a novel inflammatory marker of left ventricular (LV) remodeling and the prognosis after AMI.[19]

We hypothesized that the TN-C levels might be useful for evaluating LV remodeling in DCM, so we measured the serum concentrations in patients with DCM, with reference to clinical characteristics and cardiac function.

Methods

Study population

A total of 107 patients (82 men, 25 women; mean age 61±14 years) with DCM, and 20 normal volunteers (14 men, 6 women; mean age 49±15 years) were enrolled. Patients with DCM were admitted or followed up at Osaka Medical College Hospital, Hokkaido University Hospital,

(Received August 24, 2006; revised manuscript received December 1, 2006; accepted December 5, 2006)

*Department of Internal Medicine (III), Osaka Medical College, Takatsuki, *Department of Cardiology, Hokkaido University, Graduate School of Medicine, Sapporo, **Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, Tsu, †Department of Cardiology, Yokosuka Kyosai Hospital, Yokosuka, ††The Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Tsu and ‡Department of Nephrology and Cardiology, International Medical Center of Japan, Tokyo, Japan*

Mailing address: Fumio Terasaki, MD, Department of Internal Medicine (III), Osaka Medical College, 2-7 Daigaku-machi, Takatsuki 569-8686, Japan. E-mail: in3012@poh.osaka-med.ac.jp

Table 1 Clinical Characteristics of Patients With Dilated Cardiomyopathy (n=107)

Age (years)	*61±14*
Men	*82 (77%)*
New York Heart Association functional class	
I	*16 (15%)*
II	*48 (45%)*
III	*31 (29%)*
IV	*12 (11%)*
Medication	
Angiotensin-converting enzyme inhibitors	*61 (57%)*
Angiotensin II receptor antagonists	*34 (32%)*
Calcium channel antagonists	*12 (11%)*
β-blockers	*67 (63%)*
Diuretics	*75 (70%)*
Digitalis	*44 (41%)*
Systolic blood pressure (mmHg)	*116±21*
Diastolic blood pressure (mmHg)	*68±12*
Heart rate (beats/min)	*74±13*
Left ventricular end-diastolic diameter (mm)	*63±9*
Left ventricular end-systolic diameter (mm)	*51±11*
Left ventricular ejection fraction (%)	*37±14*
Cardiothoracic ratio (%)	*57±8*

Data are means ±SDs or number (%).

Mie University Hospital, and Yokosuka Kyosai Hospital, Japan, between January 2001 and March 2005. The clinical diagnosis of DCM was made according to the World Health Organization/International Society and Federation of Cardiology task force[1] and the clinical characteristics are shown in Table 1. Written informed consent was given by all patients and volunteers, and the study protocol was approved by the institutional review board.

Clinical Information

Age, gender, systolic and diastolic blood pressures (mmHg), heart rate (beats/min) and New York Heart Association (NYHA) functional class were evaluated as essential information. Cardiothoracic ratio (CTR) was examined on chest X-ray. LV end-diastolic diameter (LVDd) and end-systolic diameter (LVDs), LV ejection fraction (LVEF) were measured by standard echocardiography.

Assay of Serum TN-C Levels by ELISA

Blood samples were centrifuged at 15,000G for 15min, and the resulting supernatants were stored at –80°C until

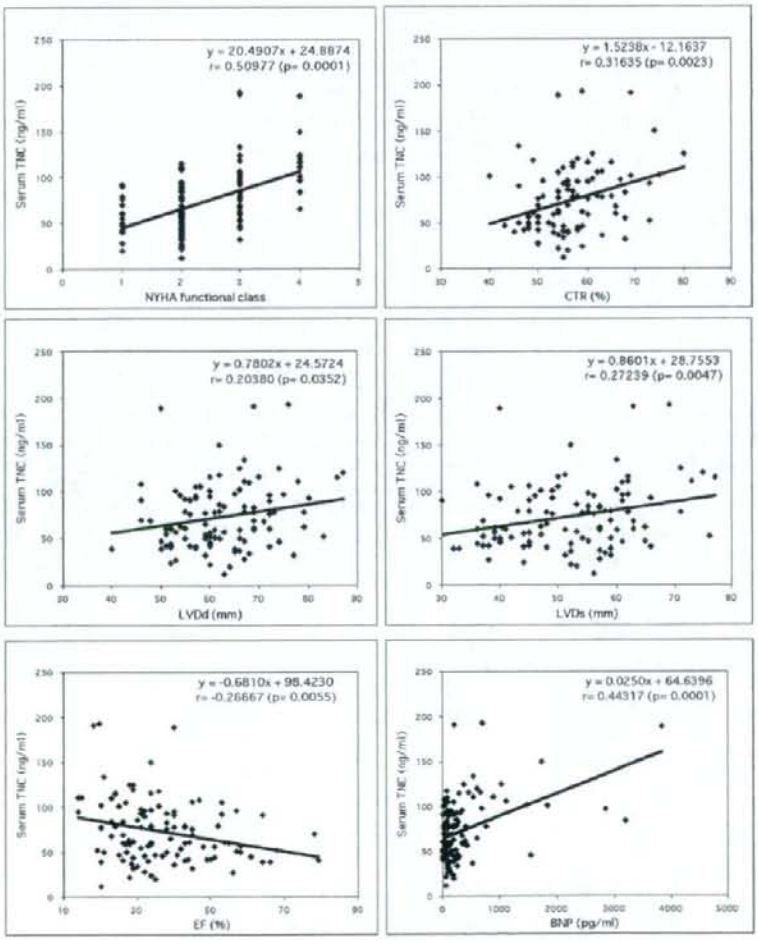

Fig 1. Associations of serum tenascin-C (TN-C) concentration with clinical information. BNP, B-type natriuretic peptide; CTR, cardiothoracic ratio; EF, ejection fraction; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; NYHA, New York Heart Association.

analysis. Serum levels of TN-C with the large subunit containing the C domain of FNIII repeats were determined using an ELISA kit with 2 monoclonal antibodies, 4F10TT and 19C4MS (IBL, Gunma, Japan), as previously described[20]

Biochemical Analysis

Plasma BNP concentrations were measured using a specific immunoradiometric commercial assay kit (Shionogi, Japan). Serum C-reactive protein (CRP) concentrations were measured by latex agglutination immunophotometric assay.

Statistical Analysis

Data are expressed as means±SD for continuous variables, and as numbers (percentages) for categorical variables. Continuous variables were analyzed by the unpaired Student's t-test. Pearson's or Spearman's correlation analysis was performed to estimate correlations between variables. A p-value <0.05 was considered statistically significant.

Results

Serum TN-C levels were significantly higher in patients with DCM than in normal volunteers (73.3±35.1 vs 30.9±8.8 ng/ml, p<0.001). Serum TN-C levels increased according to NYHA functional class (r=0.51, p=0.0001). Furthermore, the levels were negatively correlated with LVEF (r=0.27, p=0.006), and positively correlated with LVDd (r=0.20, p=0.04), LVDs (r=0.27, p=0.005), CTR (r=0.32, p=0.002) and plasma BNP level (r=0.44, p=0.0001) (Fig 1). However, serum CRP levels did not correlate with CTR, NYHA functional class, LVEF, LVDd, and LVDs on echocardiography, plasma BNP levels, and serum TNC levels.

Discussion

In the present study, serum TN-C levels were significantly higher in 107 patients with DCM than in 20 normal volunteers. Among the DCM patients, serum TN-C levels showed significant positive correlation with NYHA functional class, CTR, LVDd, LVDs and plasma BNP levels, and negative correlation with LVEF, indicating that the level reflects the severity of heart failure, LV dysfunction and remodeling. Our multicenter study with a larger DCM population has confirmed previous preliminary results obtained from 31 cases of DCM[21] and further showed a clear association between higher TN-C levels and LV remodeling, as well as LV dysfunction.

Several lines of evidence suggest that inflammation plays a pathogenic role in the development and progression of congestive heart failure, influencing cardiac contractility and myocardial remodeling[22–24] In fact, pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, are known to be upregulated in the myocardium and blood stream of DCM patients, and their high expression levels correlate to progression of refractory heart failure leading to morbidity and mortality[25,26] It is postulated that these cytokines expressed in the failed myocardium may lead to the necrosis/apoptosis and hypertrophy of cardiomyocytes, and alter the nature of the supporting connective tissue by activating matrix metalloproteinases.

As previously reported, TN-C is specifically expressed in the myocardial inflammatory lesions of myocarditis[16–18] and AMI[12,15] Furthermore, serum TN-C levels are elevated in AMI patients, reflecting local expression of TN-C in the infarcted myocardium[19] Therefore, increased serum TN-C levels in DCM patients suggest the involvement of inflammation in the myocardium. Supporting this possibility, we have recently found that most of the myocardium in DCM patients shows varying degrees of inflammation, and that expression of TN-C is enhanced in the areas of active inflammation with local tissue remodeling (unpublished data). Furthermore, after AMI, patients with high serum levels of TN-C are at higher risk of major adverse cardiac events, which suggests that the expression level of TN-C reflects remodeling activity[19] Therefore, TN-C expression might also reflect long-term cardiac function and prognosis in DCM patients. In this study, serum CRP levels were not associated with the parameters of LV size, function or serum TN-C levels (p=0.0942), which suggests that circulating CRP levels may not reflect the severity of heart failure, LV remodeling or myocardial inflammation in patients with DCM.

The origin of the elevated level of circulating TN-C in DCM is a matter of interest. It has been reported that expression of TN-C is observed in endomyocardial biopsy specimens of patients with DCM[27] which strongly suggests that the serum TN-C arises from heart tissue. In the pathological myocardium, results obtained from experimental animal models, as well as human myocarditis samples, have identified that the major source of TN-C is interstitial fibroblasts[12,16,18] Various cytokines, growth factors, hypoxia, mechanical stress, acidosis, and angiotensin II, which are important mediators of myocardial injury and inflammation during progression of heart failure in DCM, stimulate cardiac fibroblasts to synthesize TNC in vitro[28] In addition, endothelial cells and vascular smooth muscle cells of various organs have the potential to synthesize TN-C under these same stimulations[10,29] The elevated levels of circulating soluble inflammatory mediators in heart failure patients might also stimulate endothelial cells of, for example, the liver, or lung, to secrete TN-C into the blood stream.

Study Limitations

Evaluation of the relationship between serum TN-C levels and the prognosis or adverse cardiac events was not conducted satisfactorily, in part because of the relatively short follow-up period. Further study is warranted to examine whether circulating TN-C levels reflect morbidity or mortality in DCM patients. Inflammatory cytokines, such as TNF-α and IL-6, were not measured in enough patients in this study, partly because of the limitations of a multicenter study.

Conclusions

Our present data suggest that increased serum TN-C levels are associated with the severity of heart failure and the LV dysfunction and remodeling in patients with DCM. Further research is needed to discover whether TN-C levels are a promising biomarker for determining prognosis and evaluating therapeutic efficacy in patients with DCM.

Acknowledgments

This study was supported in part by a research grant for intractable diseases from the Ministry of Health, Labor and Welfare of Japan.

References

1. Richardson P, McKenna W, Bristow M, Maisch B, Mautner B,

O'Connell J, et al. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies. *Circulation* 1996; **93:** 841–842.
2. Amiya E, Tanabe K, Ikari Y, Nakajima Y, Hara K. Prolonged QRS duration and severity of mitral regurgitation are unfavorable prognostic markers of heart failure in patients with nonischemic dilated cardiomyopathy. *Circ J* 2006; **70:** 57–62.
3. Kanzaki Y, Terasaki F, Okabe M, Hayashi T, Toko H, Shimomura H, et al. Myocardial inflammatory cell infiltrates in cases of dilated cardiomyopathy as a determinant of outcome following partial left ventriculectomy. *Jpn Circ J* 2001; **65:** 797–802.
4. Tsutamoto T, Wada A, Maeda K, Hisanaga T, Maeda Y, Fukai D, et al. Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure. *Circulation* 1997; **96:** 509–516.
5. Groenning BA, Raymond I, Hildebrandt PR, Nilsson JC, Baumann M, Pedersen F. Diagnostic and prognostic evaluation of left ventricular systolic heart failure by plasma N-terminal pro-brain natriuretic peptide concentrations in a large sample of the general population. *Heart* 2004; **90:** 297–303.
6. Wang TJ, Larson MG, Levy D, Benjamin EJ, Leip EP, Omland T, et al. Plasma natriuretic peptide levels and the risk of cardiovascular events and death. *N Engl J Med* 2004; **350:** 655–663.
7. Chiquet-Ehrismann R, Tucker RP. Connective tissues: Signalling by tenascins. *Int J Biochem Cell Biol* 2004; **36:** 1085–1089.
8. Chiquet-Ehrismann R, Chiquet M. Tenascins: Regulation and putative functions during pathological stress. *J Pathol* 2003; **200:** 488–499.
9. Jones FS, Jones PL. The tenascin family of ECM glycoproteins: Structure, function, and regulation during embryonic development and tissue remodeling. *Dev Dyn* 2000; **218:** 235–259.
10. Jones PL, Jones FS. Tenascin-C in development and disease: Gene regulation and cell function. *Matrix Biol* 2000; **19:** 581–596.
11. Imanaka-Yoshida K, Matsumoto K, Hara M, Sakakura T, Yoshida T. The dynamic expression of tenascin-C and tenascin-X during early heart development. *Differentiation* 2003; **71:** 291–298.
12. Imanaka-Yoshida K, Hiroe M, Nishikawa T, Ishiyama S, Shimojo T, Ohta Y, et al. Tenascin-C modulates adhesion of cardiomyocytes to extracellular matrix during tissue remodeling after myocardial infarction. *Lab Invest* 2001; **81:** 1015–1024.
13. Willems IE, Arends JW, Daemen MJ. Tenascin and fibronectin expression in healing human myocardial scars. *J Pathol* 1996; **179:** 321–325.
14. Frangogiannis NG, Shimoni S, Chang S, Ren G, Dewald O, Gersch C, et al. Active interstitial remodeling: An important process in the hibernating human myocardium. *J Am Coll Cardiol* 2002; **39:** 1468–1474.
15. Tamaoki M, Imanaka-Yoshida K, Yokoyama K, Nishioka T, Inada H, Hiroe M, et al. Tenascin-C regulates recruitment of myofibroblasts during tissue repair after myocardial injury. *Am J pathol* 2005; **167:** 71–80.
16. Imanaka-Yoshida K, Hiroe M, Yasutomi Y, Toyozaki T, Tsuchiya T, Noda N, et al. Tenascin-C is a useful marker for disease activity in myocarditis. *J Pathol* 2002; **197:** 388–394.
17. Sato M, Toyozaki T, Odaka K, Uehara T, Arano Y, Hasegawa H, et al. Detection of experimental autoimmune myocarditis in rats by ^{111}In monoclonal antibody specific for tenascin-C. *Circulation* 2002; **106:** 1397–1402.
18. Morimoto S, Imanaka-Yoshida K, Hiramitsu S, Kato S, Ohtsuki M, Uemura A, et al. The diagnostic utility of tenascin-C for evaluation of activity of human acute myocarditis. *J Pathol* 2005; **205:** 460–467.
19. Sato S, Aonuma K, Imanaka-Yoshida K, Yoshida T, Isobe M, Kawase D, et al. Serum tenascin-C might be a novel predictor of left ventricular remodeling and prognosis after acute myocardial infarction. *J Am Coll Cardiol* 2006; **47:** 2319–2325.
20. Hasegawa M, Hirata H, Sudo A, Kato K, Kawase D, Kinoshita N, et al. Tenascin-C concentration in synovial fluid correlates with radiographic progression of knee osteoarthritis. *J Rheumatol* 2004; **31:** 2021–2026.
21. Aso N, Tamura A, Nasu M. Circulating tenascin-C levels in patients with idiopathic dilated cardiomyopathy. *Am J Cardiol* 2004; **94:** 1468–1470.
22. Mann DL. Activation of inflammatory mediators in heart failure. *In*: Mann DL, editor. Heart failure: A companion to Braunwald's heart disease. Philadelphia: WB Saunders; 2004; 159–180.
23. Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP, et al. Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha. *Circ Res* 1997; **81:** 627–635.
24. Deswal A, Petersen NJ, Feldman AM, Young JB, White BG, Mann DL. Cytokines and cytokine receptors in advanced heart failure: An analysis of the cytokine database from the Vesnarinone trial (VEST). *Circulation* 2001; **103:** 2055–2059.
25. Aukrust P, Ueland T, Lien E, Bendtzen K, Muller F, Andreassen AK, et al. Cytokine network in congestive heart failure secondary to ischemic or idiopathic cardiomyopathy. *Am J Cardiol* 1999; **83:** 376–382.
26. Matsumori A, Yamada T, Suzuki H, Matoba Y, Sasayama S. Increased circulation cytokines in patients with cardiomyopathy and myocarditis. *Br Heart J* 1994; **72:** 561–566.
27. Tamura A, Kusachi S, Nogami K, Yamanishi A, Kajikawa Y, Hirohata S, et al. Tenascin expression in endomyocardial biopsy specimens in patients with dilated cardiomyopathy: Distribution along margin of fibrotic lesions. *Heart* 1996; **75:** 291–294.
28. Imanaka-Yoshida K, Hiroe M, Yoshida T. Interaction between cell and extracellular matrix in heart disease: Multiple roles of tenascin-C in tissue remodeling. *Histol Histopathol* 2004; **19:** 517–525.
29. Ballard VL, Sharma A, Duignan I, Holm JM, Chin A, Choi R, et al. Vascular tenascin-C regulates cardiac endothelial phenotype and neovascularization. *FASEB J* 2006; **20:** 717–719.

Circ J 2007; **71:** 1545–1550

Enhanced Expression of Myeloid-Related Protein Complex (MRP8/14) in Macrophages and Multinucleated Giant Cells in Granulomas of Patients With Active Cardiac Sarcoidosis

Fumio Terasaki, MD; Masatoshi Fujita, MD*; Hiroaki Shimomura, MD; Bin Tsukada, MD; Koji Otsuka, MD; Kaoru Otsuka, MD; Takashi Katashima, MD; Masaki Ikemoto, PhD*; Yasushi Kitaura, MD

Background The myeloid-related protein complex (MRP8/14) is expressed in activated human macrophages and reported to be involved in the inflammatory process. The expression of MRP8/14 in patients with cardiac sarcoidosis and idiopathic dilated cardiomyopathy (DCM) was investigated.

Methods and Results Serum MRP8/14 levels were measured in 35 patients with sarcoidosis and 23 patients with DCM. Sera from 30 normal volunteers served as controls. Additionally, the expression profiles of MRP8/14 in the myocardium from 12 patients with active cardiac sarcoidosis and 10 DCM patients were examined immunohistochemically. Serum MRP8/14 levels were significantly higher in patients with sarcoidosis than in normal controls [515±549 (SD) ng/ml vs 230±115 ng/ml, p=0.0019]. In the sarcoidosis group, serum MRP8/14 levels in patients with definite cardiac involvement (n=10) were significantly higher than in those without (n=25) (974±878 ng/ml vs 332±204 ng/ml, p=0.0227) and they were also higher than in DCM patients (vs 252±108 ng/ml, p=0.0026). Immunohistochemically, MRP8/14 was specifically positive in the cytoplasm of macrophages and multinucleated giant cells in the myocardial granulomas.

Conclusions MRP8/14 may be involved in the pathogenesis of sarcoid granulomas. The measurement of serum MRP8/14 levels is useful for the diagnosis of sarcoidosis, and their higher levels suggest the cardiac involvement. (*Circ J* 2007; **71:** 1545–1550)

Key Words: Cardiomyopathy; Inflammation; Myeloid-related protein; Pathology; Sarcoidosis

The myeloid-related protein (MRP) complex, MRP8/14 (S100A8/A9), consisting of 2 subunits with low molecular mass (10.8 and 13.2 kDa), is a member of the S100 family and contains 2 calcium-binding sites per molecule.[1–4] The 2 subunits are expressed in activated human granulocytes and macrophages in the inflammatory lesions.[4–8] MRP8 (S100A8) and MRP14 (S100A9) are non-covalently associated to quickly form a heterodimer, MRP8/14 complex, in a calcium-dependent manner.[4,5,9–11] Translocation of the complexes from the cytosol to membrane is induced under increased intracellular calcium concentrations and correlates with the inflammatory action of granulocytes and macrophages.

The MRP8/14 complex is reported to be present in a subset of monocytes/macrophages in acute inflammatory lesions.[11] Ikemoto et al have recently reported that serum MRP8/14 complex levels increase as a result of inflammation associated with transplant rejection.[12]

It is assumed that MRP8/14 is heavily involved in the pathogenesis of acute inflammation. To date, however, there have been few reports on the clinical importance of MRP8/14 as a marker of inflammatory reaction. The present study was based on our hypothesis that MRP8/14 could serve as a useful marker for active inflammation associated with monocyte/macrophage infiltration.

Sarcoidosis is a systemic disorder of unknown cause that is characterized by the presence of non-caseating granulomas in multiple organs.[13] Cardiac involvement is one of the major determinants of morbidity and mortality in patients with sarcoidosis. Thus, early diagnosis and estimation of disease activity are critically important in the treatment of cardiac sarcoidosis. However, these are often difficult, particularly in cases without any definite extra-cardiac organ involvement.[14–16] Actually, it is occasionally experienced that the correct diagnosis of cardiac sarcoidosis is done by the histological examination of myocardial specimens after autopsy or heart surgery.[17] Furthermore, in the course of disease progression, it is sometimes quite difficult to distinguish it from idiopathic dilated cardiomyopathy (DCM).

In contrast, activated macrophages (epithelioid cells) are the main components of sarcoid granulomas that play a crucial role in the pathogenesis and pathophysiology of sarcoidosis via various inflammatory cytokine networks.[18,19] Thus, MRP8/14 is likely to be involved in the development of the granulomatous tissue; however, its in situ expression in the sarcoid heart has not been clarified. Elucidation of the MRP8/14 expression would be a great help for the estimation of disease activity in cardiac sarcoidosis.

(Received January 15, 2007; revised manuscript received May 7, 2007; accepted June 12, 2007)

*Department of Internal Medicine III, Osaka Medical College, Takatsuki, *Department of Laboratory Science, School of Health Sciences, Faculty of Medicine, Kyoto University, Kyoto, Japan*

Mailing address: Fumio Terasaki, MD, Department of Internal Medicine III, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki 569-8686, Japan. E-mail: in3012@poh.osaka-med.ac.jp

Table 1 Clinical Characteristics of Patients With DCM and Cardiac Sarcoidosis

Characteristic	DCM (n=23)	Cardiac sarcoidosis (n=10)	p value
Age (years)	58.2±14.5	52.7±11.9	NS
Men	18 (78%)	3 (30%)	0.0069
NYHA functional class			
I+II	11 (48%)	3 (30%)	NS
III+IV	12 (52%)	7 (70%)	NS
Medication			
ACEI/ARB	22 (96%)	4 (40%)	0.0001
Calcium channel antagonists	4 (17%)	0 (0%)	NS
β-blockers	17 (74%)	3 (30%)	0.0221
Diuretics	17 (74%)	6 (60%)	NS
Digitalis	9 (39%)	3 (30%)	NS
Spironolactone	9 (39%)	4 (40%)	NS
Electrocardiogram			
Complete AVB	0 (0%)	4 (40%)	0.0006
VT	3 (13%)	3 (30%)	NS
Systolic blood pressure (mmHg)	116±13	98±17	0.0041
Diastolic blood pressure (mmHg)	71±8	63±12	NS
Heart rate (beats/min)	70±5	82±21	0.02
LV end-diastolic diameter (mm)	62.5±7.8	63.8±12.3	NS
LV end-systolic diameter (mm)	50.1±9.5	53.1±15.2	NS
LVEF (%)	40.9±14.2	34.6±16.9	NS
Brain natriuretic peptide (pg/ml)	240±412	519±490	NS
ACE (U/L)	9.2±3.6	19.0±10.7	<0.0001
C-reactive protein (mg/dl)	0.1±0.1	2.9±5.8	0.031

Data are presented as means±SDs or numbers (%).
DCM, dilated cardiomyopathy; NYHA, New York Heart Association; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin-II receptor blockers; AVB, atrioventricular block; VT, ventricular tachycardia; LV, left ventricular; LVEF, LV ejection fraction; ACE, angiotensin-converting enzyme.

Therefore, in the present study, we measured the serum levels of MRP8/14, and examined the expression of MRP 8/14 with special reference to its in situ localization, in the myocardium from patients with active cardiac sarcoidosis.

Methods

Patients and Materials

Study Population Thirty-five patients with histologically diagnosed sarcoidosis [15 men and 20 women; 53±16 (mean±SD) years] and 23 patients with DCM (18 men and 5 women; 58±15 years) were included in the present serum MRP8/14 analysis study. In the sarcoidosis group, there were 10 patients with definite cardiac involvement (cardiac sarcoidosis). Sera from 30 normal volunteers served as controls. The clinical characteristics of the DCM and cardiac sarcoidosis patients are shown in Table 1. Written informed consent was obtained from all patients and volunteers, and the study protocol was approved by the institutional review board.

Clinical Information Age, gender, systolic and diastolic blood pressures (mmHg), heart rate (beats/min) and New York Heart Association functional class were evaluated as the essential information. Left ventricular end-diastolic diameter and end-systolic diameter, left ventricular ejection fraction were measured using standard echocardiography. The existence of complete atrio-ventricular blocks and ventricular tachycardia were evaluated using 24-h ambulatory electrocardiogram monitoring.

Assay of Serum MRP8/14 Levels by ELISA Blood samples were centrifuged at 15,000G for 15 min, and the resulting supernatants were stored at –80°C until analysis. We measured serum MRP8/14 levels using a sandwich enzyme-linked immunosorbent assay system as previously described.[12]

Biochemical Analysis

Plasma brain natriuretic peptide concentrations were measured using a specific immunoradiometric commercial assay kit (Shionogi, Osaka, Japan). Serum C-reactive protein (CRP) concentrations were measured using a latex agglutination immunophotometric assay. Serum angiotensin-converting enzyme (ACE) levels were measured using the Kasahara method.[20]

Histopathologic and Immunohistochemical Examination

Twelve patients with active cardiac sarcoidosis were included in this examination (6 men and 6 women; mean age, 50±10 years). Nine of the 12 patients underwent the left ventriculoplasty or mitral valvuloplasty between September 1997 and January 2006 at Shonan Kamakura General Hospital, Hayama Heart Center or Osaka Medical College Hospital, Japan, due to the diagnosis of DCM or unclassified cardiomyopathy. These patients were first diagnosed with active cardiac sarcoidosis postoperatively following histological examination of the resected myocardium. The remaining 3 patients were diagnosed with cardiac sarcoidosis during cardiac biopsy or autopsy (Table 2). Ten age-matched patients with DCM who underwent the left ventriculoplasty served as controls. Approval was obtained from the institutional review boards at each hospital, and all patients undergoing operation gave informed consent for inclusion in the present study. In 7 cases of left ventriculoplasty, a portion of the left ventricular wall was resected. In 2 cases of the mitral valvuloplasty, an open biopsy of the left ventricular wall was conducted. A portion of each sample was immediately fixed in buffered formalin. Each specimen was embedded in paraffin and sections were stained

Table 2 Characteristics of Cardiac Sarcoidosis Patients Included in the Histopathologic Study

Case	Age (years)	Gender	Mode of detection	Date	Clinical diagnosis	LVDd (mm)	LVEF (%)	Duration of heart disease (years)	History of cardiac involvement
1	60	M	LVP	1997.9.1	DCM	88	24	9	MVP (MR) at the time of onset
2	63	M	LVP	1999.7.6	DCM	85	21	9	PMI (IIIºAVB) 6 years after onset
3	51	F	LVP	1999.7.30	Unclassified CM	62	29	8	PMI (IIIºAVB) at the time of onset
4	59	F	LVP	1999.10.12	DCM	68	18	5	PMI (IIºAVB) 1 year after onset
5	51	F	LVP	2001.10.2	DCM	68	35	10	LV aneurysm at the time of onset
6	36	M	LVP	2001.12.6	DCM	73	8	0.2	(–)
7	45	M	LVP	2006.1.18	DCM	81	11	7	PMI (IIIºAVB) at the time of onset
8	49	F	MVP	2002.7.29	Unclassified CM	66	24	0.3	(–)
9	55	M	MVP	2004.4.28	DCM	69	26	1.5	VT at the time of onset
10	51	F	Biopsy	1998.11.30	DCM	56	33	3	LV aneurysm, VT at the time of onset
11	31	M	Autopsy	1985.9.27	Sarcoidosis	76	31	0.7	(–)
12	43	F	Autopsy	1991.10.2	DCM	71	22	1.3	VT at the time of onset

LVDd, LV end-diastolic dimension; LVP, left ventriculoplasty; MVP, mitral valvuloplasty; MR, mitral regurgitation; PMI, permanent pacemaker implantation; CM, cardiomyopathy. Other abbreviations see in Table 1.

with hematoxylin-eosin and observed under a light microscope.

The rabbit monoclonal anti-human MRP8/14 antibody was utilized to determine the localization of the MRP8/14.[12] Mouse monoclonal antibodies against both CD45 (a marker for lymphocytes) and CD68 (a marker for macrophages) (DAKO Japan, Kyoto, Japan) were used to identify types of infiltrating inflammatory cells. Immunoreactivity was examined using the avidin-biotin peroxidase complex method (ScyTek Laboratories, UT, USA). Finally, the reactions were optimized with diaminobenzidine chromogen and were counterstained with hematoxylin.

Statistical Analysis

Data are expressed as means±SD for continuous variables, and as numbers (percentages) for categorical variables. Spearman's correlation analysis was performed to estimate correlations between variables. Multiple comparisons among more than 3 groups were performed using the Steel-Dwass's test. Comparison between 2 groups was performed by Wilcoxon rank sum test or the unpaired Student's t-test. A p value of <0.05 was considered statistically significant.

Results

Clinical Information

Between the DCM and cardiac sarcoidosis groups, statistical differences were observed in gender; treatment with ACE inhibitors/angiotensin II receptor blockers, and β blockers; the presence of a complete atrio-venricular block; systolic blood pressure; heart rate; and serum levels of ACE and CRP (Table 1).

Measurements of Serum MRP8/14 Levels

Serum MRP8/14 levels were significantly higher in patients with sarcoidosis than those in normal controls (515±549 vs 230±115 ng/ml, p=0.0019) and tended to be higher than those in DCM (vs 252±108 ng/ml, p=0.1224) (figure not shown). In the sarcoidosis group, serum MRP 8/14 levels were 974±838 ng/ml in patients with definite cardiac involvement (n=10), being significantly higher than 332±204 ng/ml (p=0.0227) in patients without cardiac involvement (n=25) (Fig 1). Serum MRP8/14 levels in cardiac sarcoidosis patients were also significantly higher than in DCM patients (vs 252±108 ng/ml, p=0.0026) and normal controls (vs 230±115 ng/ml, p=0.0020) (Fig 1). All 10 cases with definite cardiac involvement in the present study presented DCM-like clinical features. In the sarcoidosis group, there was no significant correlation between the serum MRP8/14 and ACE levels (data not shown).

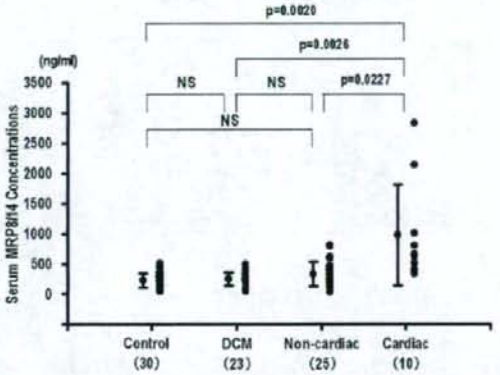

Fig 1. Serum myeloid-related protein (MRP) 8/14 levels in the sarcoidosis patients with definite cardiac involvement (n=10) were 974±838 ng/ml, being significantly higher than 332±204 ng/ml (p=0.0227) in patients without cardiac involvement (n=25). Serum MRP8/14 levels in cardiac sarcoidosis patients were also significantly higher than in dilated cardiomyopathy (DCM) patients (vs 252±108 ng/ml, p=0.0026) and in normal controls (vs 230±115 ng/ml, p=0.0020).

Histopathologic and Immunohistochemical Examination

General Features Large non-caseating sarcoid granulomas were observed in sections from all sarcoidosis patients. They were mainly composed of lymphocytes, macrophages, multinucleated giant cells and interstitial components. Lymphocytes were mostly positive for CD45, while macrophages and giant cells were positive for CD68. Cardiomyocytes adjacent to the sarcoid granulomas were occasionally found to be degenerative or necrotic (Figs 2a,b).

In DCM hearts, moderate to severe interstitial fibrosis and fatty infiltration were generally observed. Adjacent to interstitial fibrosis, the cardiomyocytes occasionally exhibited cytoplasmic vacuolization, scarcity of myofibrils, atrophy

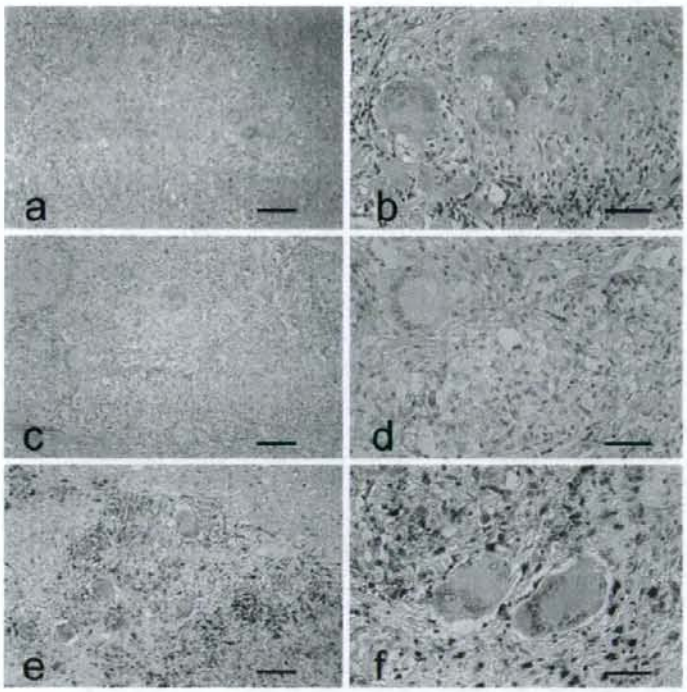

Fig 2. Histological and immunohistochemical findings in the specimens obtained from the myocardium of active cardiac sarcoidosis. Large non-caseating sarcoid granulomas are observed. They are mainly composed of lymphocytes, macrophages, multinucleated giant cells and interstitial components. Cardiomyocytes adjacent to the sarcoid granulomas are degenerative or necrotic (a, b). The myeloid-related protein 8/14 is strongly positive in the cytoplasm of macrophages and mildly positive in giant cells in the granulomas (e, f) (a, b: hematoxylin and eosin staining; c, d: negative control; bar indicates 100μm in a, c, e, and 50μm in b, d, f).

or hypertrophy in size and nuclear alterations.

Expression of MRP8/14

In DCM hearts, expression of the MRP8/14 was generally negative or weak. Weak MRP8/14-positive staining was occasionally observed in mononuclear interstitial cells. In the myocardium of cardiac sarcoidosis patients, the MRP8/14 was specifically and strongly positive in the cytoplasm of macrophages, and mildly positive in multinucleated giant cells in the granulomas (Figs 2e, f).

Discussion

Recent studies have demonstrated the significance of the expression of cytokines in the pathogenesis of sarcoidosis.[15,18] Moller et al described that sarcoidosis is an antigen-driven type 1 helper T cell (Th1)-mediated delayed-type hypersensitivity, dominated by the expression of IL-12 and IFN-γ.[19,21] Preferential expression of Th1 cytokines has been reported in bronchoalveolar lavage cells and fluids in patients with pulmonary sarcoidosis.[21,22] Thus, in the Th1 cytokine networks in sarcoid granulomas, activated macrophages (epithelioid cells) play a crucial role in the pathogenesis and pathophysiology of sarcoidosis.

In the present study, strong expression of the MRP8/14 in macrophages was demonstrated in the immunohistochemistry in granulomas of cardiac sarcoidosis, suggesting that the MRP8/14, produced by macrophages and giant cells, may play an important role in the pathogenesis of granulomas in active cardiac sarcoidosis. To our knowledge, this is the first report describing the in situ localization of MRP8/14 in the sarcoid heart.

It has been shown that increased serum concentration of MRP8/14 is an useful biomarker of disease activity in inflammatory disorders, such as inflammatory bowel disease, inflammatory muscle disease, rheumatoid arthritis, Kawasaki disease and transplant rejection.[12,23–32] Patients with diabetes mellitus have high plasma levels of MRP8/14.[33] Most recently, it has been reported that increasing plasma concentrations of MRP8/14 among healthy individuals can predict the risk of future cardiovascular events.[34]

In the present study, the serum MRP8/14 levels were significantly higher in patients with sarcoidosis than in normal controls. Thus, the serum MRP8/14 levels may be indicative of the activity of granulomatous inflammation in sarcoidosis. Furthermore, in the sarcoidosis group, the serum MRP8/14 levels were significantly higher in patients with definite cardiac involvement than in patients without. It is likely that the high serum MRP8/14 levels are due to the inflammatory response associated with cardiac sarcoidosis. The reason why the serum MRP8/14 levels were higher in patients with cardiac involvement is not clear. One possible reason may be that the number of activated macrophages–that is, amount of active sarcoid granulomas–was much larger in the heart than in other organs such as eye and skin lesions.

Three of 10 cardiac sarcoidosis patients in the serum MRP8/14 analysis study were included in the histopathologic and immunohistochemical study of the myocardium after cardiac surgery (Cases 6, 8 and 10 in Table 2). Two of them (Cases 6 and 8) showed extremely high serum MRP8/14 levels (Fig 1). In both cases, heart failure progressed acutely in a few months and histological examination revealed severe and active sarcoid granulomas in the myocardium, that could be called fulminant cardiac sarcoidosis. Immunohistochemistry showed extremely enhanced ex-

pression of MRP8/14 in macrophages and giant cells in the granulomas, which may be related to higher serum MRP 8/14 levels in these cases.

In some patients in the present study, biopsies of the cervical and mediastinal lymphnodes were conducted. The immunohistochemistry revealed enhanced expression of MRP8/14 in macrophages and multinucleated giant cells in the sarcoid granulomas in these lymph nodes. Thus, it is likely that enhanced expression of MRP8/14 is observed in other sarcoidosis tissues such as lung, lymph node or skin lesions. It should be kept in mind that MRP8/14 is not a heart-specific protein, and the higher serum levels do not necessarily represent active sarcoid granulomas only in the heart.

In all cardiac sarcoidosis patients of the serum MRP8/14 analysis study, the blood sampling was performed at the time of histopathological examinations, such as endomyocardial biopsy or cardiac surgery before steroid therapy. Hirono et al have reported that the serum MRP8/14 levels were upregulated during the early stages of acute Kawasaki disease, and decreased after intravenous immune globulin therapy.[31] In sarcoidosis patients, it may also be possible that serum levels of MRP8/14 could become useful markers of disease activity.

In the clinical characteristics, ACE serum levels were significantly higher in the cardiac sarcoidosis group than that in DCM group. The serum level of ACE may be a useful marker to differentiate cardiac sarcoidosis from DCM. In contrast, there was no significant correlation between the serum MRP8/14 and ACE levels in the sarcoidosis group (data not shown). The mechanisms of upregulation of MRP8/14 and ACE may be different in sarcoidosis; however, the reason for this is not clear. The evaluation of the superiority of either MRP8/14 or ACE as a biomarker of sarcoidosis will be an interesting study in the future.

All cases with cardiac involvement in the present study presented DCM-like clinical features. It is not easy to confirm the diagnosis of cardiac sarcoidosis, particularly when there is no apparent extra-cardiac involvement. Furthermore, during the course of disease progression, it is often difficult to distinguish it from DCM. Actually, as in cases in the present study, the diagnosis of cardiac sarcoidosis occasionally missed, even in a country like Japan, where this condition seems to be relatively prevalent.[35] Most diagnostic tests, including endomyocardial biopsy, suffer from low sensitivity, low specificity or both.[16] The most accurate diagnostic strategy for this condition is currently unknown. Thus, a valuable diagnostic marker would be extremely useful and is anticipated. Because serum MRP8/14 levels were significantly higher in patients with cardiac sarcoidosis than in DCM patients, and MRP8/14 expression is limited to areas with well-formed, active noncaseating granulomas in the myocardium, it may be suggested that enhanced expression of this protein could either confirm the diagnosis of cardiac sarcoidosis or estimate disease activity, particularly when there is no apparent extra-cardiac involvement. All cardiac sarcoidosis patients included in the present study have already deteriorated to relatively advanced cardiac dysfunction. Thus, the results of the present study may apply to the detection of serious cardiac involvement in sarcoidosis. Further investigations of the measurement of serum MRP8/14 for the early detection of cardiac involvement in sarcoidosis will be necessary.

In conclusion, the MRP8/14, produced by macrophages and multinucleated giant cells, is involved in the pathogenesis of sarcoid granulomas. The measurement of serum MRP8/14 levels may be useful for the diagnosis of sarcoidosis, and their higher levels may suggest the cardiac involvement.

Study Limitations

In the present study of serum MRP8/14 measurements, the sarcoidosis group without definite cardiac involvement (n=25) included patients with eye, skin, lung or lymph nodes lesions. Most of them were relatively stable patients who had been followed up at an out-patient clinic, with or without the history of steroid therapy, suggesting that their sarcoid lesions might be inactive. In contrast, most patients with definite cardiac involvement were in progressive conditions with probable active sarcoid lesions before steroid treatment. That may be one reason why there was a significant difference in serum MRP8/14 levels between the 2 groups. Further investigations with larger numbers of patients are warranted, including the information on pre- and post-steroid therapy to confirm the value of serum MRP8/14 measurements.

Acknowledgments

The authors express deep gratitude to Taiko Horii, MD, Tadashi Isomura, MD and Hisayoshi Suma, MD for their contribution by providing the valuable myocardial tissue and clinical data. The present study was supported in part by a research grant for intractable diseases from the Ministry of Health, Labor and Welfare of Japan.

References

1. Kerkhoff C, Klempt M, Sorg C. Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). *Biochim Biophys Acta* 1998; **1448:** 200–211.
2. Van den Bos C, Roth J, Koch HG, Hartmann M, Sorg C. Phosphorylation of MRP14, an S100 protein expressed during monocytic differentiation, modulates Ca^{2+}-dependent translocation from cytoplasm to membranes and cytoskeleton. *J Immunol* 1996; **156:** 1247–1254.
3. Vogl T, Propper C, Hartmann M, Strey A, Strupat K, van den Bos C, et al. S100A12 is expressed exclusively by granulocytes and acts independently from MRP8 and MRP14. *J Biol Chem* 1999; **274:** 25291–25296.
4. Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8, 14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J Biol Chem* 1991; **266:** 7706–7713.
5. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via novel, tubulin-dependent pathway. *J Biol Chem* 1997; **272:** 9496–9502.
6. Odink K, Cerletti N, Bruggen J, Clerc RG, Tarcsay L, Zwadlo G, et al. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* 1987; **330:** 80–82.
7. Dorin JR, Novak M, Hill RE, Brock DJH, Secher DS, Van Heyningen V. A clue to the basic defect in cystic fibrosis from cloning the CF antigen gene. *Nature* 1987; **326:** 614–617.
8. Lagasse E, Clerc RG. Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol Cell Biol* 1988; **8:** 2402–2410.
9. Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hager G, Karas M, Sorg C. Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. *J Biol Chem* 1991; **266:** 13462–13467.
10. Vogl T, Roth J, Sorg C, Hillenkamp F, Strupat K. Calcium-induced noncovalently linked terramers of MRP8 and MRP14 detected by ultraviolet matrix-assisted laser desorption. Ionization mass spectrometry. *J Am Soc Mass Spectrom* 1992; **10:** 1124–1130.
11. Bhardwaj R, Zotz C, Zwadlo-Klarwasser G, Roth J, Goebeler M, Mahnke K, et al. The calcium-binding proteins MRP8 and MRP14 form a membrane-associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions. *Eur J Immunol* 1992; **22:** 1891–1897.

12. Ikemoto M, Tanaka T, Takai Y, Murayama H, Tanaka K, Fujita M. New ELISA system for myeloid-related protein complex (MRP8/14) and its clinical significance as a sensitive marker for inflammatory responses associated with transplant rejection. *Clin Chem* 2003; **49:** 594–600.
13. Newman LS, Rose CS, Maier LA. Sarcoidosis. *N Engl J Med* 1997; **336:** 1224–1234.
14. Sekiguchi M, Yazaki Y, Isobe M, Hiroe M. Cardiac sarcoidosis: Diagnostic, prognostic, and therapeutic considerations. *Cardiovasc Drugs Ther* 1996; **10:** 495–510.
15. Shammas RL, Movahed A. Sarcoidosis of the heart. *Clin Cardiol* 1993; **16:** 462–472.
16. Uemura A, Morimoto S, Hiramitsu S, Kato Y, Ito T, Hishida H. Histologic diagnostic rate of cardiac sarcoidosis: Evaluation of endomyocardial biopsies. *Am Heart J* 1999; **138:** 299–302.
17. Fujita M, Sasayama S, Hoshino T, Sakurai T, Nonogi H. Myocardial sarcoidosis with severe congestive heart failure. *Jpn Circ J* 1987; **51:** 436–438.
18. Fuse K, Kodama M, Okura Y, Ito M, Aoki Y, Hirono S, et al. Levels of serum interleukin-10 reflect disease activity in patients with cardiac sarcoidosis. *Jpn Circ J* 2000; **64:** 755–759.
19. Moller DR. Cells and cytokines involved in the pathogenesis of sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1999; **16:** 24–31.
20. Kasahara Y, Ashihara Y. Colorimetry of angiotensin-I converting enzyme activity in serum. *Clin Chem* 1981; **27:** 1922–1925.
21. Moller DR, Forman JD, Liu MC, Noble PW, Greenlee BM, Vyas P, et al. Enhanced expression of IL-12 associated with Th1 cytokine profiles in active pulmonary sarcoidosis. *J Immunol* 1996; **156:** 4952–4960.
22. Minshall EM, Tsicopoulos A, Yasruel Z, Wallaert B, Akoum H, Vorng H, et al. Cytokine mRNA gene expression in active and non-active pulmonary sarcoidosis. *Eur Respir J* 1997; **10:** 2034–2039.
23. Sorg C. The calcium binding proteins MRP8 and MRP14 in acute and chronic inflammation. *Behring Inst Mitt* 1992; 126–137.
24. Foell D, Frosch M, Sorg C, Roth J. Phagocyte-specific calcium-binding S100 proteins as clinical laboratory markers of inflammation. *Clin Chim Acta* 2004; **344:** 37–51.
25. Lugering N, Stoll R, Schmid KW, Kucharzik T, Stein H, Burmeister G, et al. The myeloic related protein MRP8/14 (27E10 antigen)-usefulness as a potential marker for disease activity in ulcerative colitis and putative biological function. *Eur J Clin Invest* 1995; **25:** 659–664.
26. Lugering N, Stoll R, Kucharzik T, Schmid KW, Rohlmann G, Burmeister G, et al. Immunohistochemical distribution and serum levels of the Ca(2+)-binding proteins MRP8, MRP14 and their heterodimeric form MRP8/14 in Crohn's disease. *Digestion* 1995; **56:** 406–414.
27. Seeliger S, Vogl T, Engels IH, Schroder JM, Sorg C, Sunderkotter C, et al. Expression of calcium-binding proteins MRP8 and MRP14 in inflammatory muscle diseases. *Am J Pathol* 2003; **163:** 947–956.
28. Frosch M, Vogl T, Seeliger S, Wulffraat N, Kuis W, Viemann D, et al. Expression of myeloid-related proteins 8 and 14 in systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheum* 2003; **48:** 2622–2626.
29. Liao H, Wu J, Kuhn E, Chin W, Chang B, Jones MD, et al. Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. *Arthritis Rheum* 2004; **50:** 3792–3803.
30. Viemann D, Strey A, Janning A, Jurk K, Klimmek K, Vogl T, et al. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. *Blood* 2005; **105:** 2955–2962.
31. Hirono K, Foell D, Xing Y, Miyagawa-Tomita S, Ye F, Ahlmann M, et al. Expression of myeloid-related protein-8 and -14 in patients with acute Kawasaki disease. *J Am Coll Cardiol* 2006; **48:** 1257–1264.
32. Burkhardt K, Radespiel-Troger M, Rupprecht HD, Goppelt-Struebe M, Riess R, Renders L, et al. An increase in myeloid-related protein serum levels precedes acute renal allograft rejection. *J Am Soc Nephrol* 2001; **12:** 1947–1957.
33. Bouma G, Lam-Tse WK, Wierenga-Wolf AF, Drexhage HA, Versnel MA. Increased serum levels of MRP-8/14 in type 1 diabetes induce an increased expression of CD11b and an enhanced adhesion of circulating monocytes to fibronectin. *Diabetes* 2004; **53:** 1979–1986.
34. Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, et al. Platelet expression profiling and clinical validation of myeloid-related protein-14 as a novel determinant of cardiovascular events. *Circulation* 2006; **113:** 2278–2284.
35. Iwai K, Sekiguchi M, Hosoda Y, DeRemee RA, Tazelaar HD, Sharma OP, et al. Racial difference in cardiac sarcoidosis incidence observed at autopsy. *Sarcoidosis* 1994; **11:** 26–31.

European Journal of Heart Failure (2009) **11**, 229–237
doi:10.1093/eurjhf/hfn049

Suppression of inflammation in rat autoimmune myocarditis by S100A8/A9 through modulation of the proinflammatory cytokine network

Kaoru Otsuka[1], Fumio Terasaki[1]*, Masaki Ikemoto[2], Shuichi Fujita[1], Bin Tsukada[1], Takashi Katashima[1], Yumiko Kanzaki[1], Koichi Sohmiya[1], Tatsuji Kono[1], Haruhiro Toko[3], Masatoshi Fujita[2], and Yasushi Kitaura[1]

[1]Department of Internal Medicine III, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki 569-8686, Japan; [2]Human Health Sciences, Kyoto University Graduate School of Medicine, Japan; and [3]Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Japan

Received 31 July 2008; revised 11 September 2008; accepted 20 November 2008; online publish-ahead-of-print 16 January 2009

Aims S100A8/A9 is expressed in activated monocytes/macrophages and assumed to be heavily involved in the pathogenesis of acute inflammation. Although several studies have asserted that S100A8/A9 has a proinflammatory function, the exact biological function of S100A8/A9 is yet to be described. We examined the anti-inflammatory effects of S100A8/A9 on experimental autoimmune myocarditis (EAM) in rats.

Methods and results Experimental autoimmune myocarditis was induced in Lewis rats by immunization with porcine cardiac myosin. The recombinant (R-) S100A8/A9 was injected intraperitoneally into EAM rats. R-S100A8/A9 attenuated the severity of myocarditis, as evidenced by echocardiographic and histological findings. In addition, we found that not only the mRNA expression of proinflammatory cytokines [interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α] in the myocardium, but also their serum concentrations were suppressed in EAM rats treated with R-S100A8/A9. Nuclear factor-kappa B expression in inflammatory cells was also suppressed in the treated rats. To elucidate the mechanistic function of S100A8/A9 on proinflammatory cytokines *in vivo*, we used an ELISA on the supernatant of homogenized heart tissue treated with R-S100A8/A9. The findings revealed high-affinity binding of R-S100A8/A9 with IL-1β, IL-6, and TNF-α in the myocardium, suggesting the trapping of proinflammatory cytokines by R-S100A8/A9.

Conclusion S100A8/A9 attenuates EAM through modulation of the proinflammatory cytokine network.

Keywords S100A8/A9 • Autoimmune myocarditis • Cytokines • Inflammation

Introduction

S100A8/A9, consisting of two low molecular mass subunits (10.6 and 13.5 kDa), is a member of the S100 family and contains two calcium-binding sites per molecule.[1,2] The two subunits are expressed in activated human granulocytes and macrophages in the inflammatory process.[2,3] In activated human neutrophils and macrophages, S100A8 and S100A9 are non-covalently associated to rapidly form the heterodimer S100A8/A9 in a calcium-dependent manner.[2–4] Translocation of the heterodimer from cytoplasm to membrane is induced in the presence of increased intracellular calcium concentration and correlates with the inflammatory action of activated granulocytes and/or macrophages. This protein is known to be involved in several infections and immune diseases, including advanced arthritis, transplant rejection, and sarcoidosis,[5–7] suggesting that it plays an important role in the pathogenesis of the inflammatory process. Although several studies have suggested that S100A8/A9 has a proinflammatory function, we recently demonstrated that S100A8/A9 indirectly suppresses the overproduction of nitrous oxide in activated neutrophils and/or macrophages in rats with lipopolysaccharide-induced liver injury and revealed the binding of S100A8/A9 with proinflammatory

* Corresponding author. Tel: +81 72 683 1221, Fax: +81 72 684 6598, Email: in3012@poh.osaka-med.ac.jp

cytokines *in vitro*.[8] However, the presence of the S100A8/A9-proinflammatory cytokine complexes was not confirmed *in vivo* in that study.

Experimental autoimmune myocarditis (EAM) in rat models is characterized by severe myocardial damage with inflammatory cell infiltration. The EAM model has been widely used as a disease model of human myocarditis,[9] and experimental data have documented that macrophages play a pivotal role in the inflammatory process of EAM in rats.[10]

To verify our hypothesis that S100A8/A9 has an anti-inflammatory effect and to clarify the mechanistic function of S100A8/A9 on proinflammatory cytokines *in vivo*, we treated the EAM rat model with newly prepared recombinant S100A8/A9.

Methods

Preparation of recombinant S100A8/A9

Expression of cDNA for S100A8 or S100A9 in *Escherichia coli* cells

cDNAs with the histidine tag sequence for the human S100A8 and S100A9 subunits were synthesized using PCR.[11] The resulting cDNAs were inserted into pCold I vectors (Takara Bio, Shiga, Japan). The two different expression vectors were then separately transfected into *E. coli* cells. The S100A8 or S100A9 cDNA-transfected *E. coli* cells were cultivated in Miller's LB Broth for 3 h at 37°C. When the absorbance of the culture medium at 600 nm ranged between 0.5 and 0.8, the culture bottle was quickly cooled to 15°C and cultivated for 24 h after adding a final concentration of 1 mmol/L IPTG. The cultivated cells were harvested and then frozen at −80°C until use.

Purification of S100A8 and S100A9 by Ni-agarose affinity column

The *E. coli* cells expressing S100A8 or S100A9 were suspended in 200 mL of binding buffer and then treated by an ultrasonic generator for 10 min at 4°C. After centrifugation at 17 400 *g* for 20 min at 4°C, the supernatant obtained was applied to a Ni-agarose affinity column equilibrated with the binding buffer. After washing the column with washing buffer, S100A8 and S100A9 were eluted from the column with the elution buffer. The fractions containing S100A8 or S100A9 were concentrated to an adequate volume using an Amicon Ultra centrifugal filter device (MW 5000; Millipore, Billerica, MA, USA), and their protein concentrations were obtained by measuring absorbance at 280 nm.

Synthesis and purification of S100A8/A9

Equal molar concentrations of S100A8 and S100A9 were mixed and the mixture was poured into an MW 5000 and incubated overnight in 2.0 mol/L Tris–NaOH solution (pH 12.0) at 4°C. It was dialyzed against 0.1 M Tris–HCl buffer (pH 10.0) containing 300 mM NaCl for 3–4 h at 4°C. After confirming the protein band of recombinant S100A8/A9 by SDS–PAGE, S100A8/A9 was partially purified on a gel filtration column (Sephacryl S-300 HR). Major fractions containing S100A8/A9 were pooled and concentrated to an adequate volume using the same filter device. The above procedures were repeated until the product was adequately concentrated.

Animals and immunization

Animal experimental protocols were approved by the Institutional Animal Care and Use Committee, Osaka Medical College. Male Lewis rats (7 weeks old; body weight 200–250 g) were purchased from Japan SLC (Shizuoka, Japan). Before initiating the experiments, they were kept in cavity for 1 week with free access to food and water. The rats were immunized subcutaneously twice with 0.7 mg purified porcine cardiac myosin (Sigma Chemical Co., St Louis, MO, USA) in an equal volume of complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* H37RA (Difco, Sparks, MD, USA) on Days 0 and 7.[12]

Administration of recombinant S100A8/A9

Immunized rats were randomly assigned to two groups: Group S (recombinant S100A8/A9, $n = 20$) and Group C (saline as vehicle, $n = 20$). Recombinant S100A8/A9 (1 mg/day) or saline was injected intraperitoneally into the immunized rats each day from Days 8 to 13. On Day 14 or 21, 10 rats in each group (S_{14}, S_{21}, C_{14}, and C_{21}) were sacrificed under ether anaesthesia. Rats that were neither immunized nor received S100A8/A9 were used as normal controls (N_{14} and N_{21}, $n = 5$, respectively).

Echocardiography

Rats were lightly anaesthetized with pentobarbital sodium (1 mg/kg body weight i.p.) on Day 14 or 21. Echocardiography was performed with an echocardiographic apparatus equipped with a 10-MHz transducer (Vivid Five, General Electric-Vingmed, Milwaukee, WI, USA). Two-dimensional targeted M-mode echocardiograms were obtained along the short axis of the left ventricle at the level of the papillary muscles. Left ventricular dimensions at end-diastole (LVDd) and end-systole (LVDs) were measured. Left ventricular ejection fraction (LVEF) was calculated as follows: $[(LVDd^3 - LVDs^3)/LVDd^3] \times 100$.

Histological assessment

For the microscopic evaluation, apex, mid-ventricular, and basal level slices were stained with haematoxylin–eosin. The entire heart and the regions affected by myocarditis (i.e. regions showing inflammation with inflammatory cells and myocardial necrosis) were examined as described previously,[10,13,14] using a computer-assisted analyzer (Scion Image Beta 4.03; Scion Corp, Frederick, MD, USA). The area ratio (percentage value of affected area/entire area) was calculated by two investigators (Y.K. and T.K.) who were blinded to slide identification; the inter- and intra-observer variance was <5%.

Immunohistochemistry

Anti-human S100A8/A9 (rabbit) antibody[6] was used to determine the localization of endogenous S100A8/A9 in Group C_{14}, with anti-rabbit immunogloblins/FITC swine polyclonal antibody (Dako, Denmark) as secondary antibody. Mouse monoclonal antibodies against CD68 were used as a marker for macrophages (AbD serotec, Raleigh, NC, USA), with labelled goat anti-mouse IgG antibody (10 μg/mL, Invitrogen, Carlsbad, CA, USA) as secondary antibody. The resulting fluorescent signal was detected by a fluorescence microscopy.

Rabbit monoclonal antibodies against nuclear factor-kappa B (NF-κB) p65 (Cell Signaling Technology, Danvers, MA, USA) were used to evaluate the activation of NF-κB. The primary antibodies were reacted to sections and immunoreactivity was evaluated using the avidin–biotin horseradish peroxidase complex method (ScyTek Laboratories, Logan, UT, USA). The reactions were optimized with diaminobenzidine chromogen.

Measurement of mRNA levels for proinflammatory cytokines in the heart

RNA isolation and cDNA preparation

RNA was extracted from the excised LV using the RNeasy Mini Kit (Qiagen). The concentration and purity of RNA was determined by measuring the optical density at 260 and 280 nm prior to use. Total RNA (1 μg) from each sample was used for reverse transcription. First strand cDNA was synthesized with random primers and Moloney murine leukaemia virus reverse transcriptase (SuperScript III; Invitrogen).

Quantitative RT–PCR analysis

Quantitative RT–PCR analysis was performed using the LightCycler Real-Time PCR System (Roche Molecular Biochemicals, Indianapolis, IN, USA) to detect the mRNA expression of interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α. The expression of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was measured as the internal control. Assays were designed using the Roche Universal Probe Library (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). For each reaction, the LightCycler Taqman Master Kit (Roche Molecular Biochemicals) was used according to the manufacturer's instructions. PCR cycling was conducted under the following conditions: preheating for 1 cycle at 95°C for 10 min, amplification for 45 cycles at 95°C for 10 s and 60°C for 25 s, and final cooling to 40°C. mRNA levels were quantified and normalized against levels of GAPDH. The averaged and normalized levels of mRNA in each control group were expressed as 1.0.

Measurement for serum concentrations of proinflammatory cytokines

Serum concentrations of IL-1β, IL-6, and TNF-α were determined by enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Detection of S100A8/A9–proinflammatory cytokine complexes in heart tissue

The S100A8/A9–proinflammatory cytokine complexes were detected as follows: Heart tissue from EAM rats treated with recombinant S100A8/A9 (Group S_{14}) was quickly homogenized after sacrifice and centrifuged at 7700 *g* at 4°C. Supernatant was assayed by ELISA in which anti-S100A8/A9 monoclonal antibody was used as the first antibody. S100A8/A9 of the complexes reacted first to the antibody. Subsequently, biotinylated anti-rat IL-1β, IL-6, and TNF-α IgGs were added to the wells and incubated for 1 h at room temperature. After washing with washing buffer, 100 μL of diluted streptavidin–horseradish peroxidase conjugate was added and further incubated for 30 min at room temperature. After washing, colour development of the plate was achieved by adding 100 μL of colour reagent (30 mg o-PD/10 μL of 30% hydrogen peroxide/20 mL of citrate buffer, pH 5.0), and terminated by adding 100 μL of 0.75 mol/L sulfuric acid. The concentrations of the S100A8/A9–IL-1β, IL-6, and TNF-α complexes were obtained using a standard curve for S100A8/A9.

Statistical analysis

Data are expressed as the mean ± SE for continuous variables and as numbers (%) for categorical variables. Multiple comparisons among three groups were performed by ANOVA followed by Scheffe's test (SPSS; Chicago, IL, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Infiltration of macrophages and S100A8/A9

We examined the localization of endogenous S100A8/A9 in the inflamed myocardium of EAM rats by a double staining method using the fluorescent immunohistochemistry. Most of the infiltrating mononuclear cells were positive for CD68, and all cells positive for S100A8/A9 were positive for CD68 (*Figure 1*).

Purification of recombinant S100A8/A9

Recombinant S100A8/A9 was purified successfully. As determined by densitometry, the purity of recombinant S100A8/A9 was ~93% protein concentration (*Figure 2*). The recombinant S100A8/A9 was used in the subsequent study.

Effect of treatment with recombinant S100A8/A9 on the severity of myocarditis

Group S_{14} vs. Group C_{14}

On macroscopic observation, the hearts in Group C_{14} were enlarged and contained large, dark-blue–greyish areas with massive pericardial effusion. The hearts in Group S_{14}, however, were slightly enlarged, had small, dark-blue–greyish areas, and showed almost no pericardial effusion. Echocardiography revealed that LVEF in Group S_{14} was significantly higher than that in Group C_{14} (81 ± 2% vs. 70 ± 3%, $P = 0.017$) (*Figure 3A* and *B*). There was no significant difference in LVEF between Groups N_{14} and S_{14} (85 ± 2% vs. 81 ± 2%). The heart weight (1.02 ± 0.03 g vs. 1.21 ± 0.06 g, $P = 0.015$) as well as the heart weight/body

Figure 1 Infiltration of macrophages and endogenous S100A8/A9 in cardiac tissue of rats with EAM. Most of the infiltrating mononuclear cells were positive for CD68, and all cells positive for S100A8/A9 were positive for CD68 (merged). Bar indicates 25 μm.

weight ratio (0.41 ± 0.02% vs. 0.55 ± 0.03%, $P = 0.002$) in Group S_{14} were significantly lower than that in Group C_{14} (*Table 1*). The area ratio of myocarditis in microscopic grading was significantly lower in Group S_{14} than that in Group C_{14} (15.4 ± 4.2% vs. 37.2 ± 7.3%, $P = 0.036$) (*Figure 4A–G*).

Group S_{21} vs. Group C_{21}

Echocardiography revealed a significant difference in LVEF between Groups S_{21} and C_{21} (78 ± 2% vs. 67 ± 4%, $P = 0.049$) (*Figure 3B*). The heart weight/body weight ratio in Group S_{21} was significantly lower than that in Group C_{21} (0.39 ± 0.02% vs. 0.48 ± 0.02%, $P = 0.009$) (*Table 1*). The area of myocarditis was significantly smaller in Group S_{21} than that in Group C_{21} (16.0 ± 3.8% vs. 44.3 ± 4.4%, $P < 0.001$) (*Figure 4G*).

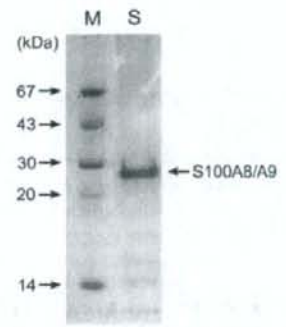

Figure 2 Purification of recombinant S100A8/A9. SDS–PAGE was performed as described in the Methods section. Lane S: purified recombinant S100A8/A9. As determined by densitometry, the purity of recombinant S100A8/A9 as a protein concentration was ~93%. Lane M: this lane contains molecular mass markers.

Effect of treatment with recombinant S100A8/A9 on mRNA expression of proinflammatory cytokines

Real-time RT–PCR analysis was performed to assess the expression of IL-1β, IL-6, and TNF-α. The mRNA expression of these proinflammatory cytokines in the myocardium was significantly depressed in Group S_{14} compared with Group C_{14}. However, between Groups S_{21} and C_{21}, there were no significant differences in the mRNA expression of IL-1β, IL-6, and TNF-α (*Figure 5A*).

Effect of treatment with recombinant S100A8/A9 on serum proinflammatory cytokine concentrations

The serum IL-1β and IL-6 concentrations in Group S_{14} markedly decreased compared with those in group C_{14} ($P = 0.008$ and $P = 0.019$, respectively). The serum TNF-α concentration in Group S_{14} tended to decrease compared with that in Group C_{14}. On Day 21, there was a significant difference only in the serum concentration of IL-1β ($P = 0.002$) between Groups S_{21} and C_{21} (*Figure 5B*).

Suppression of NF-κB expression in the heart of S100A8/A9-treated rats

To evaluate NF-κB activity, we immunohistochemically examined the expression of the nuclear p65 protein in the myocardium. This revealed that the expression of p65 was enhanced in Group

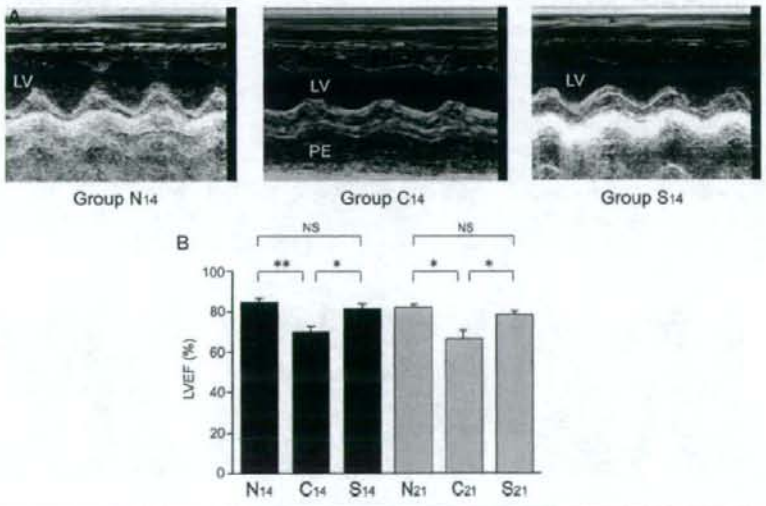

Figure 3 Echocardiographic findings. (*A*) Representative M-mode echocardiographic findings. Non-treated EAM rats (Group C_{14}) showed reduced left ventricular ejection fraction (LVEF) and pericardial effusion on Day 14. Recombinant S100A8/A9 administration from Days 8 to 13 (Group S_{14}) improved LVEF and suppressed pericardial effusion. (*B*) EF (%) in Groups N_{14}, C_{14}, S_{14}, N_{21}, C_{21}, and S_{21}. *$P < 0.05$; **$P < 0.01$.

Table 1 Effect of S100A8/A9 on heart weight, body weight, and heart/body weight ratio

	Day 14			Day 21		
	Normal control (Group N_{14}, $n = 5$)	EAM (Group C_{14}, $n = 10$)	EAM with S100A8/A9 (Group S_{14}, $n = 10$)	Normal control (Group N_{21}, $n = 5$)	EAM (Group C_{21}, $n = 10$)	EAM with S100A8/A9 (Group S_{21}, $n = 10$)
Heart weight (g)	0.89 ± 0.02	$1.21 \pm 0.06^{\#}$	$1.02 \pm 0.03^{*}$	0.97 ± 0.01	$1.16 \pm 0.04^{\#}$	$0.99 \pm 0.05^{*}$
Body weight (g)	279 ± 6	$221 \pm 5^{\#\#}$	$248 \pm 6^{\#,**}$	297 ± 4	$242 \pm 4^{\#\#}$	$256 \pm 6^{\#\#}$
Heart/body weight ratio (%)	0.32 ± 0.004	$0.55 \pm 0.03^{\#\#}$	$0.41 \pm 0.02^{**}$	0.33 ± 0.004	$0.48 \pm 0.02^{\#\#}$	$0.39 \pm 0.02^{**}$

$^{\#}P < 0.05$ (vs. Group N).
$^{\#\#}P < 0.01$ (vs. Group N).
$^{*}P < 0.05$ (vs. Group C).
$^{**}P < 0.01$ (vs. Group C).

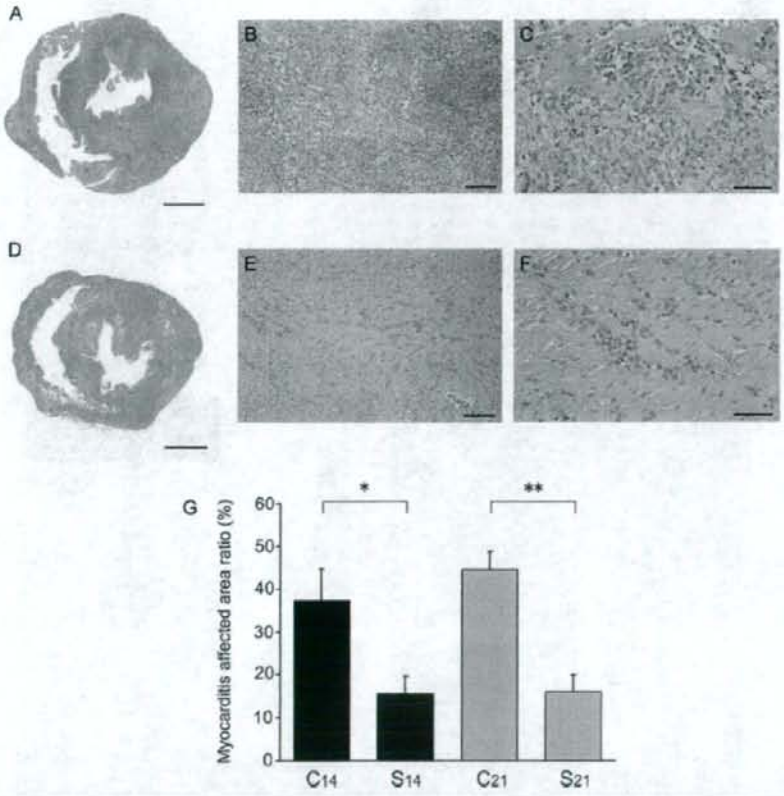

Figure 4 Representative cross-sections of heart. (*A*) Histopathological findings of specimen obtained from the midportion of the left ventricle of a vehicle rat (Group C_{14}). (*B* and *C*) Severe infiltration of relatively large mononuclear cells was observed in myocardium (Group C_{14}). (*D*) Cross-section of heart from a rat treated with recombinant S100A8/A9 (Group S_{14}). (*E* and *F*) Less severe infiltration of inflammatory cells is revealed (Group S_{14}). (*G*) Myocarditis-affected area ratio in the respective groups. Bar indicates 5 mm in (A and D), 100 μm in (B and E), and 50 μm in (C and F). $^{*}P < 0.05$; $^{**}P < 0.001$.

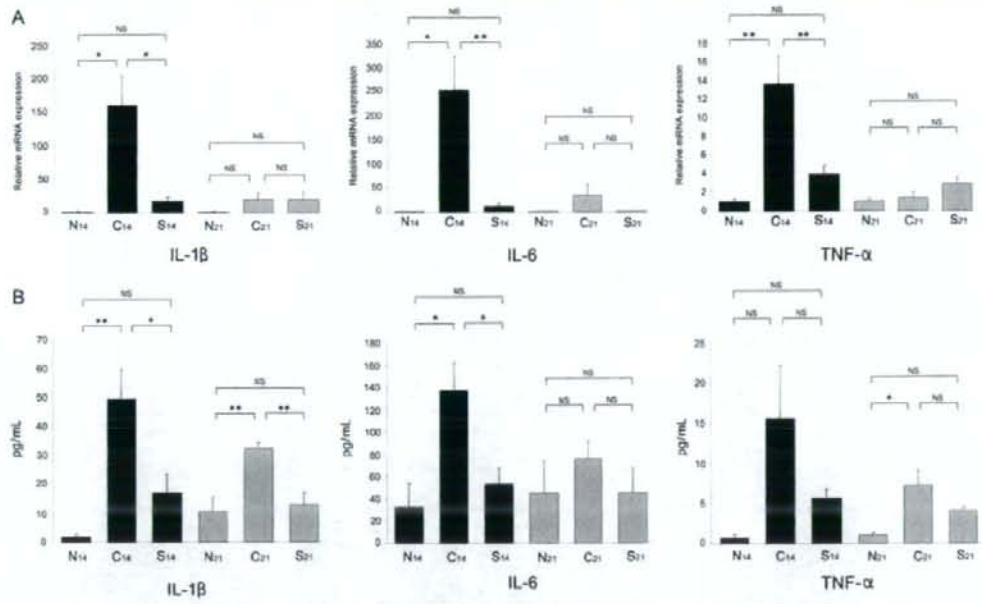

Figure 5 Effect of recombinant S100A8/A9 on proinflammatory cytokines: mRNA expression in EAM heart and serum concentrations. (*A*) Total RNA was extracted from the heart and real-time RT–PCR analysis was performed. Bar graphs show relative mRNA expression of IL-1β, IL-6, and TNF-α. $^{*}P < 0.05$, $^{**}P < 0.01$. (*B*) Serum IL-1β, IL-6, and TNF-α concentrations were determined using an ELISA method. The *Y*-axis represents the concentrations of each proinflammatory cytokine. $^{*}P < 0.05$; $^{**}P < 0.01$.

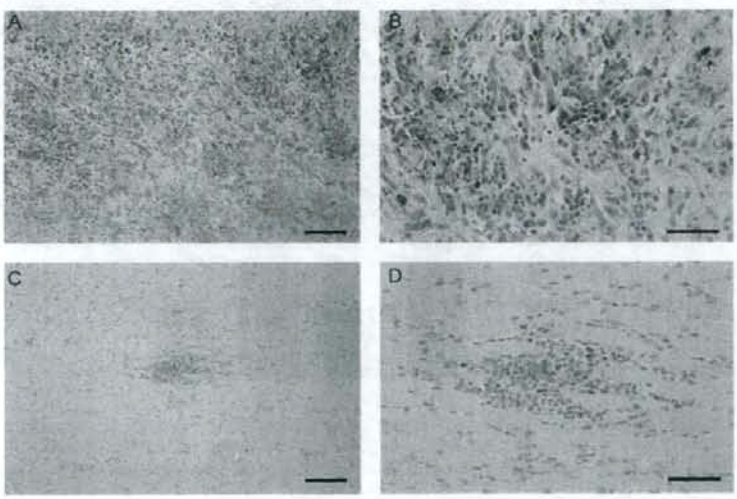

Figure 6 Representative results of immunohistochemical detection of NF-κB. Enhanced expression of NF-κB (p65) was observed in non-treated EAM heart (Group C_{14}) (*A* and *B*), while recombinant S100A8/A9 (Group S_{14}) suppressed expression (*C* and *D*). Bar indicates 100 μm in (A and C) and 50 μm in (B and D).

C_{14}, whereas in Group S_{14}, it was significantly suppressed (*Figure 6*). There was no detectable expression of p65 in naive rat hearts in Group N_{14}.

In vivo binding between S100A8/A9 and proinflammatory cytokines

To confirm the *in vivo* binding of S100A8/A9 with IL-1β, IL-6, and TNF-α, we used the ELISA test for S100A8/A9 to the supernatant of the homogenized heart tissue from EAM rats treated with recombinant S100A8/A9 (Group S_{14}). We demonstrated three kinds of S100A8/A9–proinflammatory cytokine complexes in heart tissue. As determined by ELISA, these complexes were quantitatively measured (*Figure 7*).

Discussion

We first examined the localization of endogenous S100A8/A9 in the inflamed myocardium of EAM rats. Fluorescent immunohistochemistry revealed that mononuclear cells both positive for CD68 and S100A8/A9 infiltrated into the myocardium to a great extent. This suggested that S100A8/A9 might play an important role in the pathogenesis of acute inflammation in the EAM model. However, it remains unclear how endogenous S100A8/A9 acts in this myocarditis model.

The pathogenic mechanism of the EAM model involves three sequential processes: (i) autoreactive macrophages and T lymphocytes are activated and expanded by a fragment of cardiac myosin; (ii) activated macrophages and T lymphocytes are recruited to the target organ; and (iii) effector–target interaction occurs.[14] Thus, inflammation of the EAM model consists mainly of macrophages and T lymphocytes. During the inflammatory phase, proinflammatory and Th1-type cytokines (e.g. IL-1β, IL-6, TNF-α, IFN-γ, and IL-2) are produced, which induce inflammation.[15,16] Pilot studies of the EAM model have shown that the expression of IL-1β, IL-6, and TNF-α in heart tissue had already increased on Day 14 when histological myocarditis did not reach the severest phase. Therefore, to verify our hypothesis that S100A8/A9 suppresses the inflammation of EAM by neutralizing the activity of proinflammatory cytokines, we intraperitoneally injected the recombinant S100A8/A9 into immunized EAM rats from Days 8 to 13 and sacrificed them on Day 14 or 21. On Day 14, data such as echocardiographic parameters, heart weight/body weight ratio, and histological assessment revealed that acute inflammation in recombinant S100A8/A9-treated EAM rats (Group S_{14}) was significantly suppressed compared with the vehicle group that had not received S100A8/A9 (Group C_{14}). Additionally, on Day 21, when histologically severe myocarditis was reported to be observed in EAM rats,[15] the area affected with myocarditis in the S100A8/A9-treated EAM rats (Group S_{21}) was significantly smaller than that in the vehicle group (Group C_{21}).

These data indicate that treatment with S100A8/A9 inhibits the development of acute inflammation in EAM, and are in agreement with the results of our previous study that intraperitoneal injection of S100A8/A9 suppresses liver injury induced by lipopolysaccharides in rats.[8] In the present study, the mRNA expression of IL-1β, IL-6, and TNF-α in the myocardium was dramatically

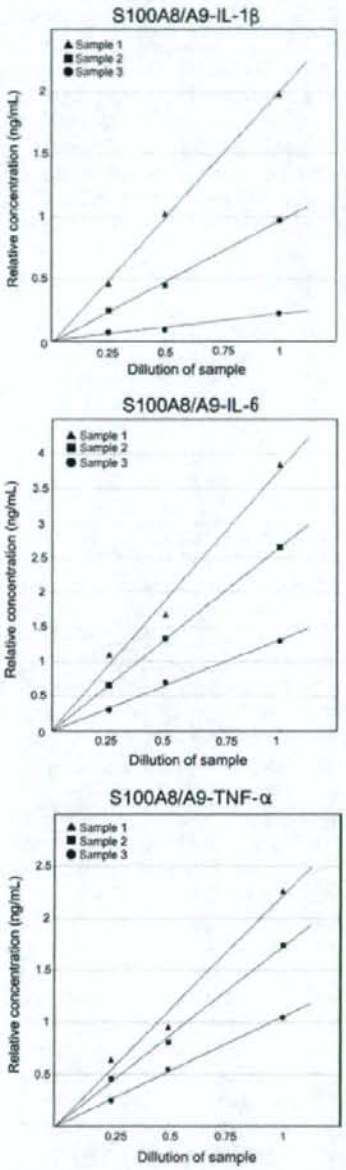

Figure 7 Evidence of binding of S100A8/A9 with proinflammatory cytokines. For detection of S100A8/A9–proinflammatory cytokine complexes, we used the ELISA plate coated with anti-S100A8/A9 monoclonal IgG (Mo2B9). Biotinylated anti-rat IL-1β, IL-6, and TNF-α IgGs were used as the second antibody. Finally, colour development was achieved by measuring horseradish peroxidase activity after incubation of streptavidin–horseradish peroxidase conjugate for 30 min. The reaction was significantly positive and quantitative, indicating the existence of S100A8/A9–cytokine complexes. This is a representative result of three samples.

suppressed in Group S_{14} compared with Group C_{14}, whereas there was no significant difference in the mRNA expression of these cytokines between Groups S_{21} and C_{21}. It has been reported that the mRNA expression of proinflammatory cytokines in the EAM model has only one peak during the inflammatory phase.[15] Thus, it may be said that mRNA levels of proinflammatory cytokines in our EAM model peaked around Day 14, and that recombinant S100A8/A9 ameliorated the peak expression of proinflammatory cytokines. On Day 21, there was no significant difference in mRNA levels of proinflammatory cytokines between Groups S_{21} and C_{21}, which may be due to the natural decrease in the mRNA expression of these cytokines in Group C_{21}.[15]

NF-κB is a rapid-response transcription factor that regulates the expression of the genes encoding cytokines, chemokines, and adhesion molecules. NF-κB exists in the cytoplasm as a heterodimer of 50-kDa (p50) and 65-kDa (p65) subunits associated with an inhibitory protein of the IκB family. When cells are stimulated, the IκB inhibitory protein is phosphorylated and dissociates from the NF-κB heterodimer, followed by translocation of free NF-κB into the nucleus.[17] In the cytokine network, in which the actions of certain cytokines are regulated by the activity of others, IL-1β and TNF-α both activate and are activated by NF-κB.[17–20] In addition, NF-κB-binding sequences have been found in the promoter regions of cytokine genes associated with inflammatory responses, including IL-6, TNF-α, IL-2, and the IL-2 receptor.[20,21] In our EAM model, immunohistochemical findings demonstrated that S100A8/A9 suppressed the activation of NF-κB as reflected by p65. Treatment with S100A8/A9 ameliorated the expression of proinflammatory cytokines and the activity of NF-κB; this suggests that S100A8/A9 has a suppressive function in the cytokine network.

In our previous study, affinity chromatography was performed using the purified S100A8/A9–Sepharose 4B column to confirm the binding of S100A8/A9 with proinflammatory cytokines.[8] A significant amount of IL-1β, IL-6, and TNF-α was eluted from the column, but anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β were not, indicating that S100A8/A9 binds with these proinflammatory cytokines *in vitro*.[8] However, the presence of the S100A8/A9–proinflammatory cytokine complexes was not documented *in vivo* in that study. Therefore, in the present study, effort was made to clarify the presence of these complexes *in vivo* using an antibody specific for S100A8/A9 in the ELISA system. It was established that in the acute phase of EAM, S100A8/A9 binds to at least three kinds of proinflammatory cytokines, IL-1β, IL-6, and TNF-α, *in vivo*. On the other hand, using anti-inflammatory cytokines IgGs as the second antibody, biotinylated anti-rat IL-4 and TGF-β IgGs, the reaction was not detected in the ELISA system (data not shown). It has been reported that extracellular S100A8/A9 interacts with binding sites on specific surface molecules, such as heparan sulfate, carboxylated glycans, and arachidonic acid.[22–24] Thus, S100A8/A9 may be a protein with multiple binding sites for many substances and may often bind to proinflammatory cytokines. The present study revealed that mRNA expression in the myocardium and also serum concentrations of proinflammatory cytokines were significantly decreased by the S100A8/A9 treatment. Possible causes of this effect may be the binding of the proinflammatory cytokines with S100A8/A9, as well as a decrease in cytokine production via the suppression of mRNA. Treatment with S100A8/A9 presents a new mechanistic approach for mitigating inflammation in the EAM model by trapping proinflammatory cytokines and altering the cytokine network.

Although the biological function of S100A8/A9 is yet to be described in detail, it has been proposed that S100A8/A9 has several functions, including antimicrobial activity, enhancement of transendothelial leucocyte migration, and induction of apoptosis.[25–27] While several studies to date have asserted that S100A8/A9 has a proinflammatory function,[28,29] few have revealed its anti-inflammatory function. The present study may shed some light on the novel anti-inflammatory function of S100A8/A9, which occurs by its binding to proinflammatory cytokines and modulating the cytokine network. Thus, treatment with S100A8/A9 is capable of neutralizing several kinds of proinflammatory cytokines, which may be unique because so-called anti-cytokine therapy generally targets a certain cytokine.

To summarize, we found that treatment with recombinant S100A8/A9 attenuated acute myocarditis in rats with EAM. At least three kinds of S100A8/A9 complexes with IL-1β, IL-6, and TNF-α were found in the inflamed organ tissues, which might have contributed to the reduction in acute inflammatory responses.

Study limitations

There are some limitations to the present study. First, the dosage of S100A8/A9 (1 mg/day) was chosen based on our previous study.[8] Myocarditis was not suppressed significantly when one-tenth of the dosage (0.1 mg/day) was given to EAM rats in the preliminary experiment. We did not attempt to use any other doses of S100A8/A9, which might have led to different results. Since only a single timing framework for S100A8/A9 administration was used in this study, the time dependency of the observed effects could not be confirmed. Further investigations on the efficacy of S100A8/A9 at different dosages and timings are therefore needed. Second, Th1/Th2 balance has been reported to play an important role in the pathogenesis of the inflammatory process in the EAM model.[13] As a counter-regulator of inflammatory cytokines, the suppressor of cytokine signalling (SOCS) family has also attracted attention.[30] However, we could not evaluate the involvement of Th1/Th2 balance and SOCS in the present study. Further studies on these factors are necessary.

Conflict of interest: none declared.

Funding

This study was supported in part by a research grant for intractable diseases from the Ministry of Health, Labour and Welfare of Japan (YK), a grants-in-aid for scientific research from the Ministry of Health, Labour and Welfare of Japan (FT), and a grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 20590567) (MI).

References

1. Kerkhoff C, Klempt M, Sorg C. Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). *Biochim Biophys Acta* 1998;**1448**:200–211.