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Activation of Ecto-5′-Nucleotidase in the Blood and Hearts of Patients With Chronic Heart Failure

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ABSTRACT

Background: Because plasma levels of adenosine are increased in patients with chronic heart failure (CHF), we examined adenosine concentrations in the plasma and heart and assessed the activity of ecto-5′-nucleotidase in the plasma and ventricular myocardium in patients with CHF.

Methods and Results: We studied 36 patients with CHF (New York Heart Association Class I/II/III/IV, 9/8/12/7). Twenty-five subjects without CHF were used as controls. Both plasma adenosine levels and ecto-5′-nucleotidase activity were significantly higher in patients with CHF (219 ± 28 nmol/L and 0.72 ± 0.03 nmoL/mg protein/min, respectively) than in control subjects (71 ± 8 nmol/L and 0.54 ± 0.02 nmoL/mg protein/min, respectively). Plasma adenosine levels sampled from the coronary sinus were significantly higher than from the aorta in patients with CHF, but these differences were not observed in control subjects. Ecto-5′-nucleotidase protein levels were markedly increased in the ventricular myocardium in patients with CHF.

Conclusions: These increases in ecto-5′-nucleotidase in the plasma and myocardium may contribute to increased plasma and cardiac adenosine levels. The increased ecto-5′-nucleotidase activity and adenosine levels in blood may become an index of the presence or severity of CHF. (*J Cardiac Fail 2008;14:426–430*)

Key Words: Adenosine, ecto-5′-nucleotidase, heart failure.

Adenosine is believed to be cardioprotective not only against ischemia and reperfusion injury, but also heart failure via 1) attenuation of release of catecholamines and β-adrenoreceptor–mediated myocardial hypercontraction and Ca^{2+} overload, 2) increased coronary blood flow, and 3) inhibition of platelet and leukocyte activation.[1–4] We have previously reported that plasma adenosine levels were increased in patients with chronic heart failure (CHF) and that the magnitude of the increase was correlated with the severity of CHF.[5] However, it remains unknown whether ecto-5′-nucleotidase, 1 of the enzymes responsible for adenosine production, is activated in the plasma and ventricular myocardium of patients with CHF. Indeed, there is accumulating evidence that a number of neurohormonal factors including catecholamines, renin-angiotensin, and cytokines are involved in the pathophysiology of CHF, which can activate ecto-5′-nucleotidase and lead to increased plasma adenosine levels.[6,7] Considering that adenosine produced in the heart can directly modulate the pathophysiology of failing heart, this evidence led us to hypothesize that the activity of ecto-5′-nucleotidase in the plasma and myocardium is activated in patients with CHF.

To test this hypothesis, we examined adenosine concentrations not only in the plasma but also in the heart, and also investigated the activity of ecto-5′-nucleotidase in the plasma and ventricular myocardium in patients with CHF.

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We also used DNA microarrays to perform gene expression profiling in human failing myocardium.

Methods

The study protocol complies with the Declaration of Helsinki and was approved by the institutional ethics committees in human investigation and conforms to the principals of the Osaka Graduate School of Medicine, National Cardiovascular Center, and Hayama Heart Center. All subjects gave written informed consent to for participation in the study.

Protocol I: Measurements of Plasma Adenosine Levels and Ecto-5′-Nucleotidase Activity in Subjects With and Without CHF

We studied 36 patients with CHF (male/female 26/10; mean age 50 ± 2 years; New York Heart Association [NYHA] Class I/II/III/IV, 9/8/12/7). None of the CHF patients had liver, kidney, or blood diseases. We also enrolled subjects with chest pain symptoms without significant coronary stenosis (male/female 17/8, mean age 49 ± 2 years). Results of medical history, physical examination, electrocardiogram, chest x-ray, and echocardiogram were negative for cardiovascular disease in these 25 control subjects. The etiologic basis of CHF in the present study included ischemic heart disease (21 patients), dilated cardiomyopathy (9 patients), and valvular heart disease (6 patients). Patients with recent myocardial infarction, postinfarct angina, and significant aortic stenosis were excluded from the study. Gender distributions (male/female), age, left ventricular ejection fraction assessed by echocardiography, plasma levels of norepinephrine, and medical treatment regimens for the enrolled subjects were described in Table 1, and the study was conducted while receiving these medications. The plasma for measurement of adenosine levels and ecto-5′-nucleotidase activity was sampled after subjects rested in the supine position for at least 15 minutes after an overnight fast. Plasma adenosine levels were determined by radioimmunoassay as previously reported.[8] In the previous study, we found that the day-to-day difference and daily variation in plasma adenosine levels were minimal.[5] The plasma adenosine levels were not correlated with age in healthy subjects. Daily plasma adenosine levels were not influenced by fasting or timing of blood collection.

Table 1. The Characteristics of the Subjects Enrolled in Protocol I

	Control	CHF
n (male/female)	25 (17/8)	36 (26/10)
Age (y)	49 ± 2	50 ± 2
NYHA Class (I/II/III/IV)	—	9/8/12/7
Original diseases		
IHD/DCM/valvular diseases	—	21/9/6
Systolic blood pressure (mm Hg)	132 ± 5	128 ± 9
Diastolic blood pressure (mm Hg)	68 ± 7	63 ± 5
Heart rate (bpm)	78 ± 6	84 ± 6
LVEF (%)	70 ± 6	48 ± 4*
Plasma norepinephrine level (pg/mL)	82 ± 6	251 ± 37*
Drugs		
ACEI/ARB	3 (12%)	35 (97%)
Diuretics	1 (4%)	32 (89%)
B-blockers	4 (16%)	6 (17%)

CHF, chronic heart failure; NYHA, New York Heart Association; IHD, ischemic heart diseases; DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

*$P < .05$ vs. control, Values are mean ± SEM.

Protocol II: Measurement of Cardiac Adenosine Levels and Ecto-5′-Nucleotidase Protein Levels in the Myocardium in Patients With and Without CHF

To assess cardiac levels of adenosine, we sampled blood simultaneously from the aorta and coronary sinus using a catheterization technique, under fluoroscopy, in 6 non-CHF (male/female 5/1, mean age 51 ± 7 years) patients and 5 CHF patients (male/female 4/1, mean age 54 ± 10 years). Ventricular myocardial biopsy samples were obtained from 2 CHF patients who underwent cardiac surgery under treatment with angiotensin-converting enzyme inhibitors and β-blockers. Ventricular myocardial specimens were obtained as controls at autopsy from 2 subjects who died of noncardiac diseases.

Ecto-5′-nucleotidase protein levels in myocardial specimens from patients with and without CHF were determined by Western blot analysis, using anti-ecto-5′-nucleotidase antibodies (Peptide Institute, Inc, Osaka, Japan). Densitometric analysis was performed using Scanning Imager (Molecular Dynamics). Each Western blot was repeated at least 3 times using different tissue samples in each experimental group.

Subcellular Fractionation and Protein Separation

Myocardial specimens were separated into membrane and cytosolic fractions by use of the following technique. Myocardial tissue was homogenized with a Potter-Elvehjem homogenizer (30 strokes) for 5 minutes in 10 volumes of ice-cold 10 mmol/L HEPES-potassium hydroxide buffer (pH 7.4) containing 0.25 mol/L sucrose, 1 mmol/L $MgCl_2$, and 1 mmol/L mercaptoethanol at 0°C. The crude homogenate was strained through a double-layered nylon sieve and homogenized again for 1 minute. For the preparation of membrane and cytosolic fractions, the homogenate was centrifuged at 1000g for 10 minutes, and the supernatant was centrifuged at 200,000g for 1 hour. After this procedure, we regarded the pellet and supernatant fractions as the membrane and cytosolic fractions, respectively. The membrane and cytosolic fractions were dialyzed at 4°C for 4 hours against 10 mmol/L HEPES-potassium hydroxide (pH 7.4) containing 1 mmol/L $MgCl_2$, 1 mmol/L mercaptoethanol, and 0.01% activated charcoal and were divided into aliquots that were frozen immediately and stored at −80°C.

Measurement of Adenosine Levels and Ecto-5′-Nucleotidase Activity

Measurement of plasma adenosine levels was done as previously described.[8] Briefly, 1 mL of blood was drawn into a syringe containing 0.5 mL of 0.02% dipyridamole, 100 μL of 2′-deoxycoformycin (0.1 mg/mL), and 500 mM EDTA to block both the uptake of adenosine by red blood cells and its degradation. Samples were centrifuged and the adenosine concentration in the supernatant was determined by radioimmunoassay.[9] Ecto-5′-nucleotidase activity was measured by an enzymatic assay[10] and reported as nanomoles per milligram of protein per minute. Protein concentration was measured by the method of Lowry et al with bovine serum albumin as a standard. 5′-Nucleotidase activity of membrane and cytosolic fractions was defined as ecto-5′-nucleotidase and cytosolic 5′-nucleotidase activity, respectively. When cytosolic 5′-nucleotidase activity was measured, AMP-CP (50 μmol/L) was added to prevent contamination of ecto-5′-nucleotidase.

Protocol III: Gene Expression Profiles in Human Failing Hearts

Tissue samples from failing human hearts were obtained from 12 patients (male/female 10/2, mean age 55 ± 5 years) who had undergone partial left ventriculectomy (the Batista or Dor procedure) for end-stage heart failure at Hayama Heart Center. All heart tissues were stored in RNA Later (Ambion, Austin, TX). Total RNA was extracted from human heart tissues using TRIzol reagent (Invitrogen Corp, Carlsbad, CA), according to the manufacturer's protocol. Integrity of RNA was verified with the RNA 6000 Nano LabChip Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, CA). Because of the difficulty in acquiring nonfailing heart tissue in Japan, we obtained total RNA from nonfailing myocardium of Mongolian patients from the Biochain Institute, Inc. DNA microarray analysis was performed using the Affymetrix GeneChip expression analysis protocol. Biotinylated cRNA was generated and applied to Affymetrix oligonucleotide array GeneChip Human Genome U95 sets (Affymetrix, Santa Clara, CA). Expression differences between nonfailing and failing hearts were analyzed by MAS version 4.0 (Affymetrix, Santa Clara, CA).

Statistical Analysis

Data are presented as means ± SEM. Differences in plasma adenosine levels and ecto-5′-nucleotidase activity were assessed by analysis of variance. When analysis of variance yielded a significant result, Bonferroni's post hoc test was applied. The relationship between plasma adenosine levels and ecto-5′-nucleotidase activity was investigated by Pearson's coefficient test. The difference between the aortic and coronary sinus levels of adenosine was assessed by a paired *t*-test. A level of $P < .05$ was considered to be statistically significant.

Results

Plasma Adenosine Levels and Ecto-5′-Nucleotidase Activity in Patients With CHF

Plasma adenosine levels were increased in patients with CHF compared with control subjects (219 ± 28 versus 71 ± 8 nmol/L) (Fig. 1A), and progressively increased as the NYHA class increased. Ejection fraction was 70 ± 6% in control subjects and 48 ± 4% ($P < .05$) in CHF patients. The left ventricular ejection fraction of patients with CHF was relatively high compared with severe CHF patients. However, we chose patients with CHF whose cardiologist had diagnosed based on clinical symptoms and physical examination. Patients may also have had right ventricular dysfunction and diastolic dysfunction. Ejection fraction was not correlated with plasma adenosine levels in patients with CHF ($r = 0.12$, P = NS). The plasma norepinephrine level was also increased in patients with CHF (251 ± 37 pg/mL) compared with control subjects (82 ± 6 pg/mL) and was correlated with plasma adenosine levels in patients with CHF ($r = 0.47$, $P < .05$).

Figure 1B shows that plasma ecto-5′-nucleotidase activity was significantly increased in patients with CHF compared with control subjects (0.72 ± 0.03 versus 0.54 ± 0.02 nmoL/mg/min, $P < .001$). As shown in Fig. 2, the plasma adenosine level was significantly correlated with ecto-5′-nucleotidase activity (n = 36, $r = 0.56$, $P < .01$).

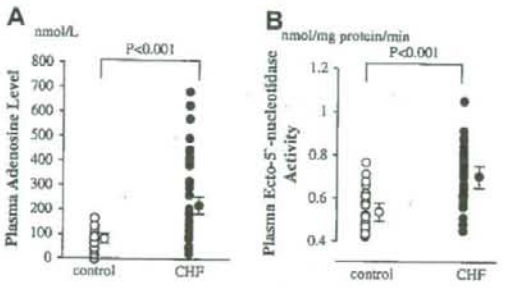

Fig. 1. (A) Plasma adenosine levels in patients with chronic heart failure (CHF) and control subjects. (B) Plasma ecto-5′-nucleotidase activity in controls and patients with CHF. Plasma ecto-5′-nucleotidase activity was significantly increased in patients with CHF compared with control subjects.

Cardiac Adenosine Levels and Ecto-5′-Nucleotidase Activity in Patients With CHF

Plasma adenosine levels sampled from either the aorta or coronary sinus in patients with CHF were significantly increased compared with those in control subjects. Furthermore, plasma levels of adenosine sampled from the coronary sinus (445 ± 65 nmol/L) were significantly higher than those from the aorta (221 ± 34 nmol/L) in patients with CHF, but these differences were not observed in control subjects (Fig. 3). Western blot analysis showed that protein levels of ecto-5′-nucleotidase from ventricular myocardium were markedly increased in CHF patients compared with control subjects (Fig. 4).

Gene Expression Profiles in Failing Human Hearts

DNA microarray analysis of failing myocardium revealed that about 3% of genes were upregulated more than 3-fold compared with nonfailing myocardium. Approximately 1% of genes were downregulated lower than

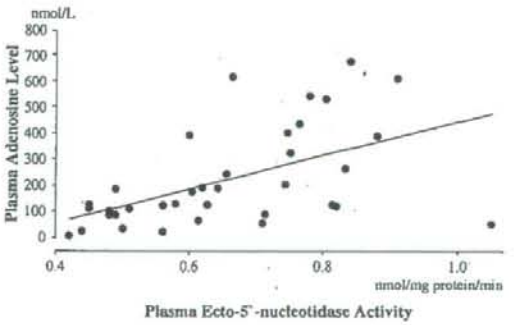

Fig. 2. Relationship between plasma adenosine levels and ecto-5′-nucleotidase in patients with chronic heart failure (CHF). There was a significant correlation between plasma adenosine levels and ecto-5′-nucleotidase in patients with CHF ($r = 0.56$, $P < .01$).

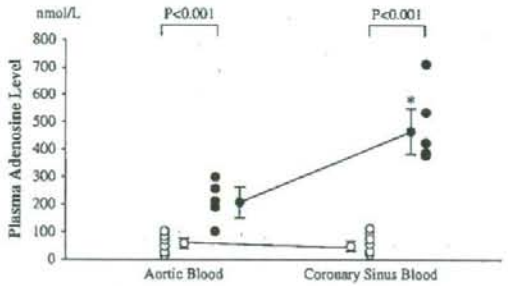

Fig. 3. The difference between aortic and coronary sinus levels of adenosine in controls (open circles) and patients with chronic heart failure (CHF) (closed circles). Cardiac adenosine levels were increased in patients with CHF (*$P < .01$).

1/3-fold in failing myocardium. Among the genes that were significantly modulated, we checked the expression levels of both cytosolic and ecto-5′-nucleotidase. Although the level of cytosolic-5′-nucleotidase in failing hearts changed 85 ± 15% compared with nonfailing hearts, ecto-5′-nucleotidase in failing hearts was upregulated 189 ± 11% compared with nonfailing hearts.

Discussion

We have demonstrated here that plasma ecto-5′-nucleotidase activity and ventricular myocardium protein levels were increased in patients with CHF. The increase in ecto-5′-nucleotidase in the plasma and myocardium may explain the increase in plasma and cardiac adenosine levels in patients with CHF.

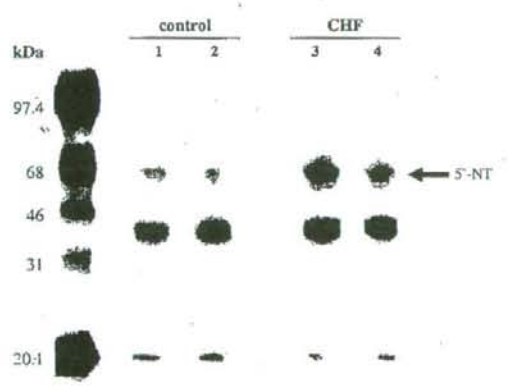

Fig. 4. Immunoblotting of ecto-5′-nucleotidase from 2 patients with chronic heart failure (CHF) and 2 control subjects. The arrow indicates the molecular weight of ecto-5′-nucleotidase. Protein levels of plasma ecto-5′-nucleotidase was increased in patients with CHF. Lane 1, 2: control subjects; Lane 3, 4: patients with CHF. 5′-NT indicates ecto-5′-nucleotidase.

The Cellular Mechanisms for the Increased Ecto-5′-Nucleotidase Activity in Patients With CHF

Several neurohormonal factors, including catecholamines, renin-angiotensin, and cytokines, are involved in the pathophysiology of CHF.[11–13] Activation of protein kinase C from either norepinephrine or angiotensin II activates ecto-5′-nucleotidase, and cytokines increase the transcriptional and protein levels of ecto-5′-nucleotidase,[6,7] which may lead to increased plasma adenosine levels. The increase in cardiac adenosine levels in patients with CHF may be due to increased metabolic activity in the failing myocardium. Indeed, in this study and our previous study, plasma levels of norepinephrine and ecto-5′-nucleotidase were elevated in patients with CHF and were closely correlated with the plasma adenosine level.[5] Because the endogenous level of norepinephrine increases as CHF progresses, and endogenous norepinephrine increases the activity of ecto-5′-nucleotidase, the increased norepinephrine level may contribute to an increase in adenosine production in patients with CHF.

In this study, we observed that the protein levels and activity of ecto-5′-nucleotidase in both plasma and myocardium were increased in patients with CHF, and that plasma levels of adenosine in blood sampled from the coronary sinus were significantly higher than those from the aorta. These results suggest that the enhanced activity of ecto-5′-nucleotidase in the ventricular myocardium possibly contributes to the increase in adenosine levels in the heart as well as in the systemic circulation in patients with CHF. Adenosine is well-known to be formed from adenosine triphosphate through adenosine diphosphate and adenosine monophosphate. There are reports that myocardial adenosine triphosphate concentration is decreased in the failing hearts.[14,15] The precise underlying mechanisms remains uncertain, Shen et al found that the myocardial total purine pool decreased and the loss of the total purine pool resulted in the reduction of ATP in rapid pacing-induced heart failure in dogs.[14] Notably, in this report, the myocardial AMP contents, a substrate for adenosine production, were unchanged during the progression of heart failure, suggesting that the activation of ecto-5′-nucleotidase explain the increased adenosine production. These findings were consistent with our data that the increases in plasma and cardiac adenosine levels and ecto-5′-nucleotidase were observed in patients with CHF.

The Impact of Increased Adenosine Levels and Ecto-5′-Nucleotidase Activity in Patients With CHF

Adenosine, which is produced in both cardiomyocytes and endothelial cells, inhibits the release of catecholamines, β-adrenoceptor-mediated myocardial hypercontraction, and Ca^{2+} overload via A1 receptors and also increases coronary blood flow and inhibits the release of renin and the production of tumor necrosis-α in experimental models.[1,16] These observations suggest that increased plasma adenosine levels may be associated with decreased severity of CHF. However, in this study, we observed increased plasma adenosine

levels as CHF worsened. These results are consistent with previous reports from our group and others.[5,17] The role of increased adenosine levels was considered to be cardioprotective, because adenosine has been reported to attenuate the sympathetic nervous system, renin-angiotensin system, and cytokine systems. The pathophysiology of CHF includes decreased coronary vascular reserve and cardiovascular endothelial cell function from low cardiac output and damage or dysfunction of the myocardium and cardiovascular endothelial cells from the effects of catecholamines, renin-angiotensin, cytokines, and superoxides. In the present study, ecto-5′-nucleotidase activity in plasma and myocardium increased in patients with CHF. The increase in ecto-5′-nucleotidase activity may inhibit the adverse effects of these substances via autocrine and paracrine mechanisms. Because adenosine also has a vasodilatory effect,[2] the increase in plasma levels may contribute to the augmentation of blood supply not only to the heart but also to the skeletal muscle. Interestingly, the increase in plasma adenosine level in patients with CHF after treatment with either dipyridamole or dilazep was reported to contribute to the improvement in NYHA functional classification, ejection fraction, and peak oxygen uptake.[18] Moreover, a study by Loh et al indicated that patients with an AMP deaminase mutation, in whom plasma adenosine levels were increased because of a failure to deaminate AMP by AMP deaminase, have a better prognosis than those without mutations.[19] Furthermore, we have recently reported that the gene expression of adenosine deaminase, adenosine A2a receptors, A2b receptors, and A3 receptors were downregulated in failing hearts compared with nonfailing hearts.[20] Although further investigation is needed, these results suggest that the metabolism of adenosine is involved in the pathophysiology of CHF.

Study Limitation

In this study, we could not rule out the possibility that combinations of drugs or the effects of drugs on underlying pathophysiology of CHF influenced the plasma adenosine level. Furthermore, in our previous study, we observed no influence of drugs for CHF on plasma adenosine levels.[5] In this study, there was not a significant difference in plasma adenosine levels between patients with and without either angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. Therefore, it is not likely that angiotensin-converting enzyme inhibitors or angiotensin receptor blocker influence the levels of adenosine. However, we could not rule out the possibility of drug effects on adenosine levels in patients with CHF. Further investigation is needed to clarify this issue.

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Oxidative Stress and Mitochondrial DNA Damage in Heart Failure

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Recent experimental and clinical studies have suggested that oxidative stress is enhanced in heart failure. The production of oxygen radicals is increased in the failing heart while antioxidant enzyme activities are preserved. Mitochondrial electron transport is an enzymatic source of oxygen radical generation and also a target against oxidant-induced damage in the failing myocardium. Chronic increases in oxygen radical production in the mitochondria can lead to a catastrophic cycle of mitochondrial DNA (mtDNA) damage, as well as functional decline, further oxygen radical generation, and cellular injury. Reactive oxygen species induce myocyte hypertrophy, apoptosis, and interstitial fibrosis by activating matrix metalloproteinases. These cellular events play an important role in the development and progression of maladaptive cardiac remodeling and failure. Therefore, oxidative stress and mtDNA damage are good therapeutic targets. Overexpression of peroxiredoxin-3 (Prx-3), mitochondrial antioxidant, or mitochondrial transcription factor A (TFAM) could ameliorate the decline in mtDNA copy number in failing hearts. Consistent with alterations in mtDNA, the decrease in oxidative capacities is also prevented. Therefore, the activation of Prx-3 or TFAM expression could ameliorate the pathophysiological processes seen in myocardial failure. Inhibition of oxidative stress and mtDNA damage could be novel and potentially effective treatment strategies for heart failure. (*Circ J* 2008; **Suppl A:** A-31 – A-37)

Key Words: DNA; Heart failure; Mitochondria; Oxidative stress; Remodeling

Heart failure is a leading cause of morbidity and mortality in industrialized countries.[1] It is also a growing public health problem, mainly because of aging of the population and the increase in the prevalence of heart failure in the elderly. Previous basic, clinical, and population sciences have advanced the modern treatment of heart failure, but despite extensive studies, the fundamental mechanisms responsible for the development and progression of left ventricular (LV) failure have not yet been fully elucidated.

Reactive oxygen species (ROS), such as superoxide anions ($\cdot O_2^-$) and hydroxy radicals ($\cdot OH$), cause the oxidation of membrane phospholipids, proteins, and DNA[2] and have been implicated in a wide range of pathological conditions including ischemia–reperfusion injury,[3] neurodegenerative diseases,[4] and aging.[5] Under physiological conditions, their toxic effects can be prevented by such scavenging enzymes as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase, as well as by other non-enzymatic antioxidants. However, when the production of ROS becomes excessive, oxidative stress might have a harmful effect on the functional and structural integrity of biological tissue.

ROS cause contractile failure and structural damage in the myocardium. The importance of oxidative stress is increasingly emerging, with respect to a pathophysiological mechanism of the LV remodeling responsible for heart failure progression.

Direct Evidence of Oxidative Stress in Heart Failure

Recent experimental and clinical studies have suggested that the generation of ROS increases in heart failure.[6–9] Lipid peroxides and 8-iso-prostaglandin F2α, which are the major biochemical markers of ROS generation, have been shown to be elevated in the plasma and pericardial fluid of patients with heart failure and also positively correlated with severity.[6,9]

Using electron spin resonance (ESR) spectroscopy combined with the nitroxide radical, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl, a definitive and direct demonstration of enhanced generation of ROS in the failing myocardium has been obtained.[10] The $\cdot O_2^-$ is a primary radical that could lead to the formation of other ROS, such as H_2O_2 and $\cdot O_2^-$, in the failing myocardium. The $\cdot OH$ could arise from electron exchange between $\cdot O_2^-$ and H_2O_2 via the Harber-Weiss reaction. In addition, $\cdot OH$ is generated by the reduction of H_2O_2 in the presence of endogenous iron by means of the Fenton reaction. The generation of $\cdot OH$ implies a pathophysiological significance of ROS in heart failure, because $\cdot OH$ radicals are the predominant oxidant species causing cellular injury.

Decreased antioxidant capacity could further aggravate ROS accumulation in heart failure; however, the activities of SOD, catalase, and GSHPx are not decreased in the failing heart,[11] indicating that oxidative stress in heart failure is primarily related to enhancement of pro-oxidant generation rather than to a decline in antioxidant defenses.

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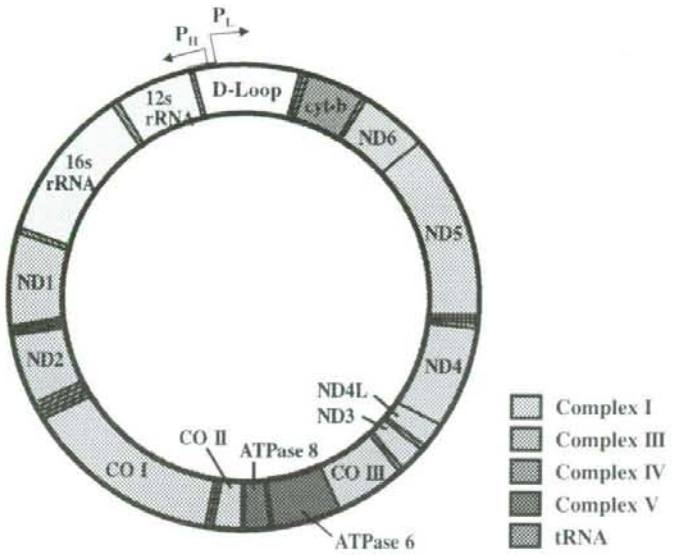

Fig 1. Map of the mitochondrial genome. The 16.3-kb mouse mitochondrial genome is shown with the 13 mRNA, 2 rRNA (12S and 16S), and 21 tRNA coding genes. mRNA genes are the areas labeled with the codes of the corresponding electron transport chain complexes I, III, IV, and V. P_H and P_L are the promoters of heavy (H) and light (L) strand transcription, respectively.

Mitochondria as a Source of Oxidative Stress

The cellular sources of ROS generation within the heart include cardiac myocytes, endothelial cells, and neutrophils. Within cardiac myocytes, ROS can be produced by several mechanisms, including mitochondrial electron transport, NADPH oxidase, and xanthine dehydrogenase/xanthine oxidase.

The heart has the highest oxygen uptake rate within the human body, consuming about 0.1 ml O_2/g min at basal rates. To meet the demand for synthesis of ATP by oxidative metabolism, cardiac myocytes have the highest volume density of mitochondria, which produce ROS through 1-electron carriers in the respiratory chain. Under physiological conditions, the small quantities of ROS formed during mitochondrial respiration can be detoxified by the endogenous scavenging mechanisms of myocytes.

Using ESR spectroscopy with 5,5'-dimethyl-1-pyrroline-N-oxide as a spin trap, the inhibition of electron transport at the sites of complex I and complex III in the normal submitochondrial particles results in a significant production of $\cdot O_2^-$.[12] Mitochondria in heart failure produce more $\cdot O_2^-$ than normal mitochondria in the presence of NADH, indicating that mitochondrial electron transport could be the predominant source of such $\cdot O_2^-$ production. Furthermore, the failing mitochondria are associated with a decrease in complex enzyme activity. Therefore, mitochondria are an important source of ROS in the failing heart, indicating a pathophysiological link between mitochondrial dysfunction and oxidative stress,[13] as has been reported in other disease conditions including aging and neurodegenerative diseases.

Even though mitochondrial electron transport is considered to play an important role in ROS production in heart failure, we can not completely exclude the possibility that other enzymatic sources of ROS generation, such as vascular endothelial cells (via xanthine oxidase and/or NADPH oxidase) and activated leukocytes (via NADPH oxidase), also contribute to oxidative stress.[14] In fact, Bauersachs et al have demonstrated that vascular NAD(P)H oxidase is activated in heart failure.[15] This enzyme system is the major source of ROS in both the endothelium and vascular smooth muscle, which are able to generate ROS in response to angiotensin II and thus stimulate the expression of NAD(P)H oxidase. Plasma renin activity, as well as tissue angiotensin-converting enzyme activity, is activated in heart failure. Therefore, enhanced formation of angiotensin II may lead to oxidative stress via this enzyme system.

Consequences of Oxidative Stress in Heart Failure

Oxidative Stress and Mitochondrial DNA (mtDNA) Damage

Mitochondria have their own genomic system, mtDNA, a closed-circular double-stranded DNA molecule of approximately 16.5 kb (Fig 1). MtDNA contains 2 promoters, the light-strand and heavy-strand promoters (LSP and HSP, respectively), from which transcripts are produced and then processed to yield the individual mRNAs encoding 13 subunits of the oxidative phosphorylation, including 7 subunits (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) of rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I), 1 subunit (cytochrome b) of ubiquinol-cytochrome c oxidoreductase (complex III), 3 subunits (COI, COII, and COIII) of cytochrome-c oxidase (complex IV), and 2 subunits (ATPases 6 and 8) of complex V, together with 22 tRNAs and 2 rRNA (12S and 16S) subunits.[16,17] Transcription from the LSP also produces RNA primer, which is necessary for initiating mtDNA replication. Mitochondrial function is controlled by the mtDNA, as well as factors that regulate mtDNA transcription and/or replication.[18] This raises the possibility that mitochondrial gene replication, and thus the mtDNA copy number and/or mitochondrial gene transcription, are impaired in heart failure. Indeed, heart failure is frequently associated with qualitative and quantitative defects in mtDNA.[19–22] Recently, the decline in mitochondrial function and mtDNA copy number was shown to play a major role in the development of the heart failure that

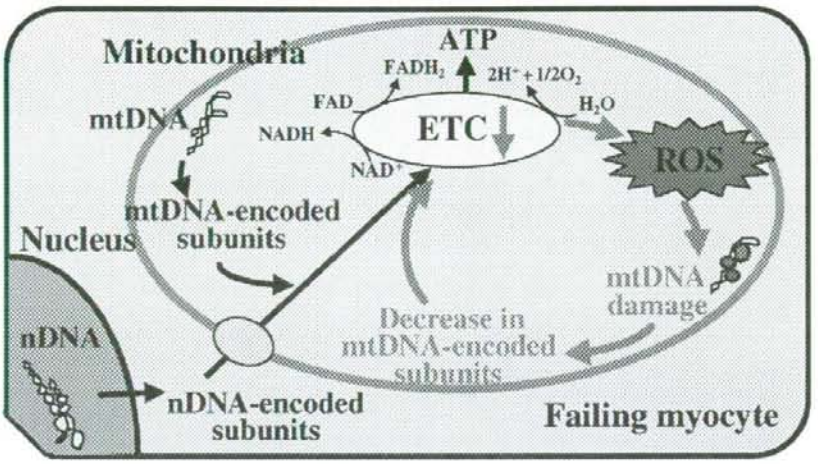

Fig 2. Schematic representation of the intimate link between reactive oxygen species (ROS), mitochondrial DNA (mtDNA) damage, and respiratory chain dysfunction in the mitochondria. Mitochondrial ROS generation may lead to a catastrophic cycle of mitochondrial functional decline, further ROS generation, and cellular injury.

occurs after myocardial infarction (MI)[12,23]

ROS can damage mitochondrial macromolecules either at or near the site of their formation. Therefore, in addition to the role of mitochondria as a source of ROS, the mitochondria themselves can be damaged by ROS. The mtDNA could be a major target for ROS-mediated damage for several reasons. First, mitochondria do not have the complex chromatin organization consisting of histone proteins that may serve as a protective barrier against ROS. Second, mtDNA has limited repair ability against DNA damage. Third, a large part of the $\cdot O_2^-$ formed inside the mitochondria can not pass through the membranes and hence, ROS damage may be contained largely within the mitochondria. In fact, mtDNA accumulates significantly higher levels of the DNA oxidation product, 8-hydroxydeoxyguanosine, than nuclear DNA.[24] As opposed to nuclear-encoded genes, mitochondrial-encoded gene expression is largely regulated by the copy number of mtDNA.[25] Therefore, mitochondrial injury is reflected by mtDNA damage, as well as by a decline in the mitochondrial RNA (mtRNA) transcripts, protein synthesis, and mitochondrial function.[26,27] We have shown that increased generation of ROS is associated with mitochondrial damage and dysfunction in the failing heart, characterized by increased lipid peroxidation in the mitochondria, decreased mtDNA copy number, a decrease in the number of mtRNA transcripts, and reduced oxidative capacity because of low complex enzyme activities.[23] A chronic increase in ROS production is associated with mitochondrial damage and dysfunction, which can lead to a catastrophic cycle of mitochondrial functional decline, further ROS generation, and cellular injury (Fig 2). MtDNA defects may thus play an important role in the development and progression of myocardial remodeling and failure.

A number of pathogenic mtDNA base substitution mutations, such as missense mutations and mtDNA rearrangement mutations (deletions and insertions), have been identified in patients with mitochondrial diseases.[22] An accumulation of the deleted forms of mtDNA in the myocardium frequently results in cardiac hypertrophy, conduction block, or heart failure.[28] Furthermore, there is now a consensus view that mutations in mtDNA and abnormalities in mitochondrial function are associated with common forms of cardiac disease, such as ischemic heart disease[29] and dilated cardiomyopathy.[30] In those conditions, however, the strict causal relationships between abnormalities in mtDNA and cardiac dysfunction have yet to be fully elucidated.[31] Even though the mechanisms by which mtDNA damage arises have not been clarified, ROS have been proposed as the primary contributing factor. We have provided direct evidence that mtDNA defects occur not only in a limited, small subset of mitochondrial diseases, but also in a more common form of heart failure phenotype occurring after MI.

Oxidative Stress and Myocardial Damage

ROS have direct effects on cellular structure and function, and may be integral signaling molecules in myocardial remodeling and failure. ROS result in a phenotype characterized by hypertrophy and apoptosis in isolated cardiac myocytes.[32] ROS have also been shown to activate matrix metalloproteinase (MMP) in cardiac fibroblasts.[33] Myocardial MMP activity is increased in the failing heart.[32,34] Furthermore, an MMP inhibitor has been shown to limit early LV dilatation in a murine model of MI.[35] We have shown significant improvement in survival after MI in MMP-2 knockout mice, which was mainly attributable to inhibition of early cardiac rupture and the development of subsequent LV dysfunction.[36] Because MMP can be activated by ROS,[37] a proposed mechanism of LV remodeling is activation of MMPs secondary to increased ROS production. Sustained MMP activation might influence the structural properties of the myocardium by providing an abnormal extracellular environment with which the myocytes interact. We have demonstrated that the $\cdot OH$ scavenger, dimethylthiourea, inhibits the activation of MMP-2 in association with the development of LV remodeling and failure.[38] These findings raise the interesting possibility that increased ROS after MI may be a stimulus for myocardial MMP activation, which

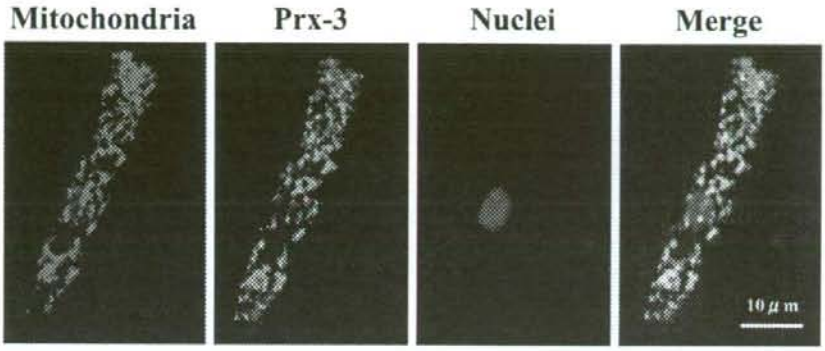

Fig 3. Cardiac myocytes isolated from transgenic mice were doubly stained with MitoTracker dye (red), a rat Prx-3-specific antibody (green), and nucleus (blue). Immunoreactivity for Prx-3 can be seen in the cytoplasm of cardiac myocytes. The merged images show Prx-3 colocalized with the mitochondria (yellow). Scale bar=10 μm.

then play an important role in the development of heart failure.

Oxidative Stress and Skeletal Muscle Damage

Limited exercise capacity is a major symptom in patients with heart failure[39] and is independent of the degree of cardiac dysfunction.[40] Increased oxidative stress has been shown to be related to the limitation of exercise capacity in patients with heart failure.[41] We have demonstrated that ROS are increased in skeletal muscle in patients with heart failure after MI and that they originate from $\cdot O_2^-$ produced by mitochondrial oxidase.[42] Recently, Kinugawa et al clarified the relationship between $\cdot O_2^-$ and the limitation of exercise capacity by using heterozygous manganese superoxide anion dismutase (SOD2) gene–knockout mice, in which SOD2, a family of enzymes that catalyze the dismutation of $\cdot O_2^-$, is reduced by 30–80%, increasing $\cdot O_2^-$ production in the mitochondria, associated with altered mitochondrial function.[43] The whole-body oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) at rest were increased in $SOD2^{+/-}$. The work (vertical distance run × body weight) to exhaustion was decreased in $SOD2^{+/-}$. When the maximum $\dot{V}O_2$ and $\dot{V}CO_2$ were corrected to the per work unit, they were increased in $SOD2^{+/-}$. Tempol normalized the basal $\dot{V}O_2$ and $\dot{V}CO_2$ and improved the work to exhaustion and corrected $\dot{V}O_2$ and $\dot{V}CO_2$ in $SOD2^{+/-}$. There was a decrease in the SOD2 protein level and a concomitant increase in lucigenin-detectable $\cdot O_2^-$ production in skeletal muscle from $SOD2^{+/-}$. Therefore, exercise capacity was reduced in conditions in which $\cdot O_2^-$ was increased, and this was associated with a greater increase in whole-body oxygen consumption.

Amelioration of Oxidative Stress, MtDNA Damage, and Heart Failure

GSHPx

The first line of defense against ROS-mediated cardiac injury comprises several antioxidant enzymes including SOD, catalase, and GSHPx. Among these, GSHPx is an important enzyme that performs several vital functions. It is a key antioxidant that catalyses the reduction of H_2O_2 and hydroperoxides. GSHPx not only scavenges H_2O_2, but also prevents the formation of other more toxic radicals such as $\cdot OH$. GSHPx possesses a higher affinity for H_2O_2 than catalase. Furthermore, it is present in relatively high amounts within the heart, especially in the cytosolic and mitochondrial compartments.[44] These lines of evidence imply the primary importance of GSHPx as a defense mechanism within the heart, compared witho catalase. Moreover, GSHPx is expected to exert greater protective effects against oxidative damage than SOD because greater dismutation of $\cdot O_2^-$ by SOD may result in increased H_2O_2. Therefore, compared with SOD or catalase, GSHPx is thought to be more effective in protecting cells, tissues, and organs against oxidative damage.[45]

GSHPx overexpression inhibits the development of LV remodeling and failure after MI, and so might contribute to improved survival.[46] These findings not only extend the previous observational study that used antioxidants, but also reveal the major role of ROS in the pathophysiology of myocardial remodeling. The effects were associated with attenuation of myocyte hypertrophy, apoptosis, and interstitial fibrosis.[46] Similarly, overexpression of GSHPx attenuates myocardial remodeling and preserves diastolic function in the diabetic heart.[47] Therefore, therapies designed to interfere with oxidative stress by using GSHPx could be beneficial in preventing heart failure.

Peroxiredoxin-3 (Prx-3)

We have recently demonstrated that the overexpression of a mitochondrial antioxidant, Prx-3, a member of peroxiredoxin family that can scavenge H_2O_2 in cooperation with thiol and peroxynitrite (Fig 3), protects the heart against post-MI remodeling and failure in mice. It reduces LV cavity dilatation and dysfunction, as well as myocyte hypertrophy, interstitial fibrosis, and apoptosis of the noninfarcted myocardium. These beneficial effects of Prx-3 gene overexpression are associated with attenuation of oxidative stress, mtDNA decline, and dysfunction.[48] The specific localization of Prx-3 in the mitochondria suggests that mitochondrial oxidative stress plays an important role in the development and progression of heart failure, and that the antioxidant localized specifically within the mitochondria provides a primary line of defense against this disease process.

Mitochondrial Transcription Factor A (TFAM)

TFAM is a nuclear-encoded protein that binds upstream

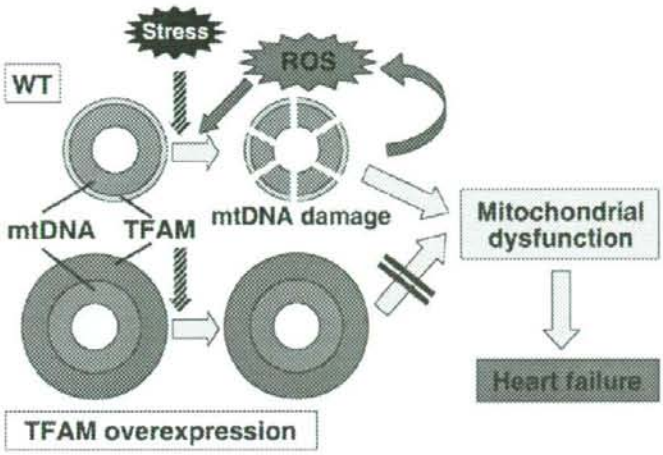

Fig 4. Proposed mechanisms byh which mitochondrial transcription factor A (TFAM) overexpression prevents mitochondrial DNA (mtDNA) damage, oxidative stress, and myocardial remodeling and failure. In wild-type mice, TFAM directly interacts with mtDNA to form nucleoids. Stress such as ischemia causes mtDNA damage, which increases the production of reactive oxygen species and thus leads to a catastrophic cycle of mitochondrial electron transport impairment, further ROS generation, and mitochondrial dysfunction. TFAM overexpression may protect mtDNA from damage by directly binding and stabilizing mtDNA and increase the steady-state levels of mtDNA, which ameliorates mitochondrial dysfunction and thus the development and progression of heart failure.

of the LSP and HSP of mtDNA and promotes transcription of mtDNA. TFAM not only regulates mtDNA transcription and replication[49] but also maintains mtDNA copy number. In fact, *Tfam* knockout mice, which have a 50% reduction in their transcript and protein levels, show a 34% reduction in mtDNA copy number, 22% reduction in the mitochondrial transcript levels, and partial reduction in the cytochrome *c* oxidase levels in the heart.[50] Moreover, cardiac-specific disruption in *Tfam* in mice results in dilated cardiomyopathy in association with a reduced amount of mtDNA and mitochondrial transcripts.[51] The transfection of antisense plasmids in culture, designed to reduce the expression of *TFAM*, effectively decreased the levels of mitochondrially encoded transcripts.[19] In contrast, forced overexpression of *TFAM* produced the opposite effect.[52] These lines of evidence obtained from knockout mice have established a critical role for TFAM in the regulation of mtDNA copy number and mitochondrial function, as well as maintenance of the physiological function of the heart in vivo. In addition, a reduction in *TFAM* expression has been demonstrated in several forms of cardiac failure.[20,23,3,54]

By using transgenic mice that overexpress human *TFAM*, we examined whether TFAM could protect the heart from mtDNA deficiencies and attenuate LV remodeling and failure after MI.[55] *TFAM* overexpression could ameliorate the decline in mtDNA copy number and preserve it at a normal level in post-MI hearts. *TFAM* overexpression might increase the steady-state level of mtDNA by directly stabilizing mtDNA. Consistent with alterations in mtDNA, the decrease in oxidative capacities seen in MI was also prevented. Moreover, TFAM played an important role in myocardial protection against remodeling and failure.

Several factors may be attributed to the protective effects conferred by *TFAM* overexpression against myocardial remodeling and failure. First, *TFAM* overexpression prevented a decrease in mtDNA copy number and mitochondrial electron transport function, which may contribute to decreased myocardial oxidative stress, which in turn could contribute to the amelioration of cardiac hypertrophy, apoptosis, and interstitial fibrosis.[55] A recent study by Ekstrand et al demonstrated that the overexpression of human *TFAM* in the mouse increased mtDNA copy number.[56] These lines of evidence imply the primary importance of TFAM as a regulatory mechanism of mtDNA copy number. TFAM has been shown to directly interact with mtDNA to form nucleoids.[57,58] Therefore, in transgenic mice increased TFAM may increase the steady-state levels of mtDNA by directly binding and stabilizing mtDNA (Fig 4). Second, *TFAM* overexpression may induce mitochondrial biogenesis, although this is thought to be unlikely because the number and size of the mitochondria assessed by electron microscopy were unaltered.

The results obtained from human *TFAM* transgenic mice differ from those from the inducible, cardiac-specific overexpression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) transgene in adult mice, which leads to a modest increase in mitochondrial number and the development of reversible cardiomyopathy.[59] PGC-1α is a transcriptional coactivator and acts upstream of TFAM, and also has the capacity to increase mtDNA levels as well as mitochondrial mass in both cultured cells and transgenic mice.[60,61] The reason for the discrepant results between the PGC-1α and TFAM transgene overexpression studies remains unsolved, but may be related to the complex regulatory mechanisms of mitochondrial biogenesis and function by PGC-1α and its downstream factors, including nuclear respiratory factors 1 and 2 and TFAM.[62,63]

MtDNA decline and mitochondrial defects are now well recognized in a variety of diseases, such as neurodegenerative diseases, diabetes mellitus, cancer, and even aging. Therefore, with further knowledge about the mechanisms of TFAM for maintaining mtDNA copy number and mitochondrial function, it may eventually be possible to develop novel strategies for the treatment of such diseases based on manipulation of TFAM.

Conclusions

To improve the prognosis of patients with heart failure, novel therapeutic strategies based on new insights into the pathophysiology of myocardial remodeling and failure need to be developed. The approach to regulating mitochondrial oxidative stress and mtDNA damage may contribute to the establishment of effective treatment strategies for patients with heart failure. Oxidative stress is involved not only in heart failure, but also in various other diseases, including

atherosclerosis, hypertension, and aging. Therefore, therapeutic strategies to modulate this maladaptive response should definitely become a target for future extensive investigation and could have broad application.

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Soluble Receptor for Advanced Glycation End Products (RAGE) is a Prognostic Factor for Heart Failure

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ABSTRACT

Background: We recently reported that serum levels of pentosidine, one of the well-defined advanced glycation end products (AGE), was an independent prognostic factor for heart failure. Receptor for AGEs (RAGE) is expressed in a variety of tissues, and RAGE has a C-truncated secretory isoform of the receptor protein, termed *soluble RAGE*. In the present study, we measured serum soluble RAGE levels in patients and examined whether serum soluble RAGE predicts prognosis in patients with heart failure.
Methods and Results: Serum soluble RAGE concentration was measured in 160 patients with heart failure by a competitive enzyme-linked immunosorbent assay. Patients were prospectively followed during a median follow-up period of 872 days with end points of cardiac death or rehospitalization. Serum soluble RAGE level increased with advancing New York Heart Association functional class. Serum soluble RAGE level was also higher in patients with cardiac events than in event free patients. From the receiver operating characteristic curve analysis, the cutoff value of serum soluble RAGE level was determined as 1220 pg/mL. Kaplan-Meier analysis clearly demonstrated that the high soluble RAGE group had a significantly higher incidence of cardiac events than occurred in the low serum soluble RAGE group ($P = .0004$). In the multivariate Cox proportional hazard analysis, soluble RAGE and serum pentosidine were independent risk factors for cardiac events (soluble RAGE: HR 1.90, 95% CI 1.16-3.09, $P = .010$; pentosidine: HR 1.59, 95% CI 1.11-2.29, $P = .012$).
Conclusions: Serum soluble RAGE level is an independent prognostic factor for heart failure, and this novel marker may be useful for risk stratification of patients with heart failure. (*J Cardiac Fail 2008;14:133–139*)
Key Words: Advanced glycation end products (AGE), receptor for AGE (RAGE), soluble RAGE, heart failure, prognosis.

Congestive heart failure is an important cause of morbidity and mortality. In patients with heart failure, excess free-radical generation may arise from many sources.[1] Markers of oxidative stress such as thiobarbituric acid reactive substances and 8-isoprostaglandin F2-α are elevated in the blood and pericardial fluid, and levels of these markers correlate with the functional severity of heart failure.[2,3] Advanced glycation end products (AGE) are generated nonenzymatically by glycation and oxidation of proteins.[4] Receptor for AGEs (RAGE) is expressed in a variety of tissues including endothelial cells, vascular smooth cells, and cardiac myocytes.[5,6] It has been reported that interaction of AGE with RAGE causes activation of intracellular signaling, gene expression, production of pro-inflammatory cytokines, and free radicals.[7,8] We have recently reported that serum levels of pentosidine, one of the well-defined AGE, is associated with the severity of heart failure and an independent prognostic factor for heart failure.[9]

RAGE has a C-truncated secretory isoform of the receptor protein, termed *soluble RAGE*, that may neutralize the AGE-mediated damage by acting as a decoy.[10–12] In an animal experiment, administration of soluble RAGE results in significantly decreased neointimal expansion after arterial injury and decreases smooth muscle cell proliferation, migration, and expression of extracellular matrix proteins.[13]

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However, serum soluble RAGE has not been previously examined in patients with heart failure, and the clinical significance is still unclear.[14,15] In the present study, we measured serum soluble RAGE levels in patients with heart failure and examined whether levels of serum soluble RAGE are related to the disease severity and prognosis of patients with heart failure.

Methods

Study Subjects

We measured serum concentration of soluble RAGE in 160 patients (95 male and 65 female, mean age 69 ± 12 years) admitted to the Yamagata University Hospital for the treatment of worsening heart failure or for diagnosis and pathophysiologic investigations of heart failure or for therapeutic evaluation of heart failure. Some of the patients were overlapped with a previous study from our institution.[9] The diagnosis of heart failure was based on a history of dyspnea and symptomatic exercise intolerance with signs of pulmonary congestion or peripheral edema or documentation of left ventricular enlargement or dysfunction by chest X-ray, echocardiography, or left ventriculography.[16] We excluded patients with acute coronary syndrome occurring within the 3 months preceding admission, inflammatory disease, autoimmune disease, malignant disease, and renal dysfunction (creatinine >2 mg/dL). Written informed consent was obtained from all patients, and the study protocol was approved by Institutional Review Board on human research.

The etiologies of heart failure were identified as dilated cardiomyopathy in 47 patients (29%), ischemic heart failure in 42 patients (26%), valvular heart disease in 35 patients (22%), tachycardia-induced heart failure in 17 patients (11%), hypertensive heart disease in 16 patients (10%), and hypertrophic cardiomyopathy in 3 patients (2%). Severity of New York Heart Association (NYHA) class was assessed by independent proficient cardiologists at initial inclusion in the study. There were 33 (21%), 63 (39%), 51 (32%), and 13 patients (8%) with NYHA Class I, II, III, and IV, respectively.

Hypertension, diabetes mellitus, hyperlipidemia, and current smoking were identified in 87 (54%), 39 (24%), 32 (20%), and 36 (23%) patients, respectively. Hypertension was defined as elevated systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or when patients had taken antihypertensive drugs. Diabetes mellitus was defined as an increased fasting plasma glucose concentration of ≥126 mg/dL, glycosylated hemoglobin of ≥6.5%, or when patients undergo treatment with insulin or hypoglycemic agents. Hyperlipidemia was defined by total cholesterol ≥220 mg/dL, triglyceride ≥150 mg/dL, high-density lipoprotein <40 mg/dL, or current use of antihyperlipidemic drugs. Current smoking was defined by self-report.

Echocardiographic Studies

We performed conventional 2-dimensional echocardiographic studies using standard techniques. Left ventricular end-diastolic volume (LVEDV) and left ventricular ejection fraction (EF) was calculated based on Simpson's rule.

Measurements of Soluble RAGE and Pentosidine

The serum soluble RAGE concentration in patients with heart failure were measured using a commercially available enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems, Minneapolis, Minnesota) in duplicate according to the manufacturer's protocol. Serum pentosidine level was measured as reported previously.[9]

End Points and Follow-up

Median follow-up period was 872 days (range 17–1095 days). Events were centrally adjudicated using medical records, autopsy reports, death certificates, and witness statements. The end points, which were judged independently by researchers, were 1) cardiac death, defined as death from worsening heart failure or sudden cardiac death and 2) rehospitalization with worsening heart failure.[16,17] Sudden cardiac death was defined as death without definite premonitory symptoms or signs and was established by the attending physician. Patients were contacted after the initial presentation by telephone interview performed by trained researchers.

Statistical Analysis

Soluble RAGE, pentosidine, and B-type natriuretic peptide (BNP) are reported as median and interquartile range. Other values are expressed as mean ± SD. A P value less than .05 was considered statistically significant. Significance between 2 groups was determined by Mann-Whitney test for continuous variables and chi-square test for discrete variables. The Cox proportional hazard regression model was used to determine which variables were associated with cardiac events. The variables with P values less than .05 in the univariate Cox regression analysis were entered into the multivariate Cox regression analysis. Kaplan-Meier survival analysis was used to compare cardiac event-free rates among 2 groups stratified by levels of serum soluble RAGE concentration and analyzed by a log-rank test. All analyses were performed using a Stat View statistical software package (version 5.0, SAS Institute Inc).

Results

In baseline clinical characteristics of study subjects, serum creatine was 0.88 ± 0.28 mg/dL, serum pentosidine was 32.0 (22.8–44.8) ng/mL, and plasma BNP was 305 (68–741) pg/mL. LVEDV was 149 ± 57 mL, and EF was 48 ± 19%. Median serum soluble RAGE level was 1066 pg/mL and interquartile range was 669–1733 pg/mL. As shown in Fig. 1, serum soluble RAGE level was increased with advancing NYHA functional class (Class I: 659 [480–1022]; Class II: 1108 [668–1626]; Class III: 1372 [842–2422]; Class IV: 1363 [1015–2634] pg/mL, P <.0001).

Serum soluble RAGE levels were not different between patients with and without diabetes mellitus (1113 [685–1749] vs. 1028 [667–1729] pg/mL, P = .943), hypertension (1113 [715–1664] versus 1021 [68–1768] pg/mL, P = .774) and hyperlipidemia (1074 [659–1787] versus 1066 [669–1733] pg/mL, P = .998).

During follow-up periods (median 872 days, range 17–1095 days), there were 48 cardiac events including 11 cardiac deaths and 37 rehospitalizations from worsening of heart failure. Table 1 shows comparisons of clinical characteristics between patients with cardiac events and event-free patients. Patients with cardiac events were older

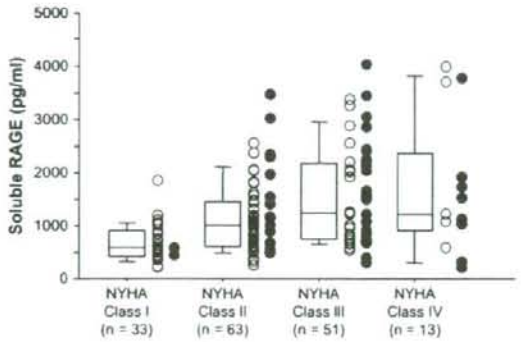

Fig. 1. Association between concentration of serum soluble RAGE and severity of New York Heart Association (NYHA) functional class. Serum soluble RAGE levels were increased as severity of NYHA functional class advanced. Box plots show median and 25 to 75 percentiles. Bar graphs show 10 to 90 percentiles. Closed circle and open circles show patients with cardiac event and event free patients, respectively.

(P = .044) and had more severe NYHA functional class (P < .0001) than did event-free patients. Serum soluble RAGE level, plasma BNP level, serum pentosidine level, and LVEDV were significantly higher in patients with cardiac events than event-free patients.

We examined receiver operating characteristic curve analysis and determined the cutoff value of serum soluble RAGE levels to predict cardiac events. As shown in Fig. 2, the cutoff value of serum soluble RAGE was 1220 pg/mL (sensitivity 0.60; specificity 0.69). Next, patients with heart failure were divided into 2 groups based on the cutoff value of serum soluble RAGE level (1220 pg/mL). High and low soluble RAGE level groups included 64 and 96 patients, respectively. Comparisons of clinical characteristics between two groups are shown in Table 2. In high soluble RAGE level group, NYHA functional class was more severe (P = .0006). In high soluble RAGE group, BNP was higher and EF was lower than in low soluble RAGE group. Age, gender, etiology of heart failure, prevalence of hypertension, diabetes mellitus, and hyperlipidemia were not different between 2 groups. Use of β-blockers and loop diuretics was significantly higher in high soluble RAGE group than in low soluble RAGE group. During follow-up periods, cardiac events were more frequently occurred in high soluble RAGE group than in low soluble RAGE group (45% versus 20%, P = .0006). Positive and negative predictive values of our determined cutoff value were 0.45 and 0.80, respectively. As shown in Fig. 3, Kaplan-Meier analysis clearly demonstrated that the high soluble RAGE group had a significantly higher incidence of cardiac events than occurred in the low serum soluble RAGE group (P = .0004 by a log-rank test).

We evaluated correlation between soluble RAGE level and BNP, EF, and pentosidine by linear regression analyses

Table 1. Clinical Characteristics of Patients with Cardiac Events and Event-free patients

Variables	Cardiac events (−) (n = 112)	Cardiac events (+) (n = 48)	P Value
Age (y)	68 ± 11	72 ± 13	.044
Gender (male/female)	65/47	30/18	.598
NYHA class			
NYHA I-II	80 (71%)	16 (33%)	
NYHA III-IV	32 (29%)	32 (67%)	<.0001
Etiology			
DCM	33 (29%)	14 (29%)	
IHD	29 (26%)	13 (27%)	
VHD	23 (21%)	12 (25%)	
Tachycardia-induced	15 (13%)	2 (4%)	
HHD	10 (10%)	6 (13%)	
HCM	2 (2%)	1 (2%)	.629
Hypertension	67 (60%)	20 (42%)	.035
Diabetes mellitus	29 (26%)	10 (21%)	.495
Hyperlipidemia	22 (20%)	10 (21%)	.863
Current smoking	29 (26%)	7 (15%)	.116
Laboratory data			
Soluble RAGE (pg/mL)	937 (647–1353)	1622 (818–2463)	.001
Creatinine (mg/dL)	0.85 ± 0.27	0.94 ± 0.29	.060
Pentosidine (ng/mL)	30.2 (22.5–41.0)	41.4 (24.9–72.9)	.010
BNP (pg/mL)	148 (55–456)	750 (308–1430)	<.0001
Echocardiography			
LVEDV (mL)	140 ± 51	171 ± 64	.003
EF (%)	49 ± 19	43 ± 20	.099

NYHA, New York Heart Association; DCM, dilated cardiomyopathy; IHD, ischemic heart disease; VHD, valvular heart disease; HHD, hypertensive heart disease; HCM, hypertrophic cardiomyopathy; soluble RAGE, soluble receptor for advanced glycation end products; BNP, B-type natriuretic peptide; LVEDV, left ventricular end-diastolic volume; EF, ejection fraction.

(Fig. 4). Log (soluble RAGE) was significantly and positively correlated with log (BNP) (r = 0.48, P < .0001, Fig. 4A). Furthermore, log (soluble RAGE) was negatively correlated with EF (r = −0.25, P = 0.003, Fig. 4B).

There were 87 patients with preserved EF (EF >40%). In patients with preserved EF group, 19 patients had cardiac events. Clinical characteristics of patients with cardiac events and event-free patients with preserved EF are shown in Table 3. Soluble RAGE, BNP, and pentosidine were higher in patients with cardiac events than event-free patients in preserved EF group.

To determine risk factors for cardiac events, we examined the univariate Cox proportional hazard regression analysis (Table 4). Serum soluble RAGE, plasma BNP, and serum pentosidine were entered as log-transformed continuous variables. In the univariate analysis, log (soluble RAGE) was significantly associated with cardiac events (hazard ratio 1.64, 95% confidence interval 1.23–2.19, P = .001). Furthermore, age, NYHA functional class, hypertension, creatinine, log (BNP), log (pentosidine), and LVEDV were significantly associated with cardiac events, as shown in Table 4.

Then, those variables with a P value less than .05 in the univariate analysis were entered into the multivariate Cox

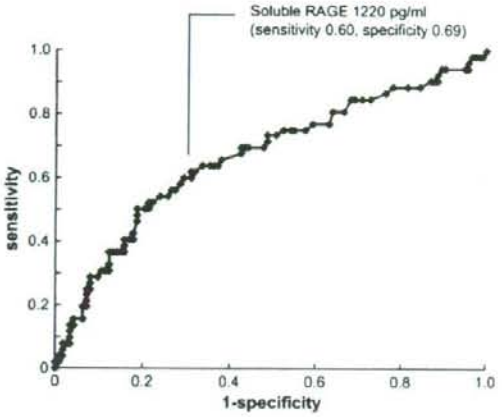

Fig. 2. Receiver operating characteristic (ROC) curve analysis. Serum soluble advanced glycation end-product receptor (RAGE) levels were evaluated for the prediction of cardiac events in patients with heat failure. Cutoff value of serum soluble RAGE was determined as 1220 pg/mL.

proportional hazard regression analysis. As shown in Table 5, soluble RAGE and pentosidine were independent predictors for cardiac events in patients with heart failure (soluble RAGE: hazard ratio 1.90, 95% confidence interval 1.16−3.09, P = .010 and pentosidine: hazard ratio 1.59, 95% confidence interval 1.11−2.29, P = .012).

Table 2. Clinical Characteristics of High and Low Soluble RAGE Groups in Patients with Heart Failure

Variables	High Soluble RAGE (>1220 pg/mL) (n = 64)	Low Soluble RAGE (≤1220 pg/mL) (n = 96)	P Value
Age (y)	69 ± 11	69 ± 12	.921
Gender (male/female)	35/29	60/36	.324
NYHA class			
NYHA I-II	28 (44%)	68 (71%)	
NYHA III-IV	36 (56%)	28 (29%)	.0006
Etiology			
DCM	22 (34%)	25 (26%)	
IHD	14 (22%)	28 (29%)	
VHD	16 (25%)	19 (20%)	
Tachycardia-induced	6 (9%)	11 (11%)	
HHD	6 (9%)	10 (10%)	
HCM	0 (0%)	3 (3%)	.499
Hypertension	36 (56%)	51 (53%)	.697
Diabetes mellitus	17 (27%)	22 (23%)	.599
Hyperlipidemia	15 (23%)	17 (18%)	.375
Current smoking	11 (17%)	25 (26%)	.189
Laboratory data			
Creatinine (mg/dL)	1.00 ± 0.32	0.80 ± 0.22	<.0001
Pentosidine	36.9 (22.3−59.8)	31.1 (22.8−40.3)	.129
BNP (pg/mL)	604 (247−1295)	122 (42−412)	<.0001
Echocardiography			
LVEDV (mL)	164 ± 50	137 ± 59	.006
EF (%)	43 ± 18	51 ± 19	.011

Abbreviations as in Table 1.

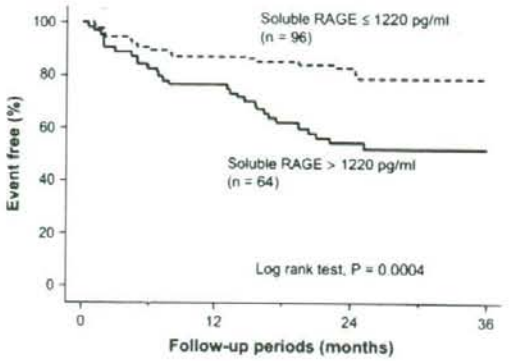

Fig. 3. Kaplan-Meier survival curve analysis between 2 groups in patients with heart failure. Patients were divided into 2 groups by the cutoff value (soluble advanced glycation end-product receptor 1220 pg/mL).

Discussion

In the present study, we showed that serum soluble RAGE level increased with advancing NYHA functional class. Serum soluble RAGE level was also higher in patients with cardiac events than in those without cardiac events. Patients with high soluble RAGE levels had higher cardiac event rates than those with low soluble RAGE levels. The multivariate Cox proportional hazard analysis demonstrated that serum soluble RAGE and pentosidine were independent prognostic factors for heart failure. Because BNP has been widely accepted as a marker of prognosis in patients with heart failure, it is noteworthy that only soluble RAGE and pentosidine, but not BNP, were independent factors to predict prognosis of heart failure in the present study.

Activation of RAGE by AGE induces activation of NADPH oxidase and production of reactive oxygen species.[18] Interaction of AGE with RAGE causes oxidative stress and activation of nuclear factor-κB via $p21^{ras}$ and the mitogen activated protein kinase signaling pathway.[19] Nuclear factor-κB modulates gene transcription and generates pro-inflammatory cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor-α.[20]

It has been reported that angiotensin II upregulates RAGE mRNA levels in endothelial cells, and administration of telmisartan decreases serum soluble RAGE levels in patients with essential hypertension.[21] Although carvedilol, one of the β-blockers, has antioxidant activity,[22] the effects of β-blocker on AGE-RAGE signaling have not been previously examined. In the present study, the use of β-blockers and loop diuretics was significantly higher in high soluble RAGE group than in low soluble RAGE group. The percentages of patients given angiotensin-converting enzyme inhibitors or angiotensin receptor blockers were not statistically different between high soluble RAGE group and low soluble RAGE group. Effects of pharmacotherapy on serum soluble RAGE levels should be further examined.

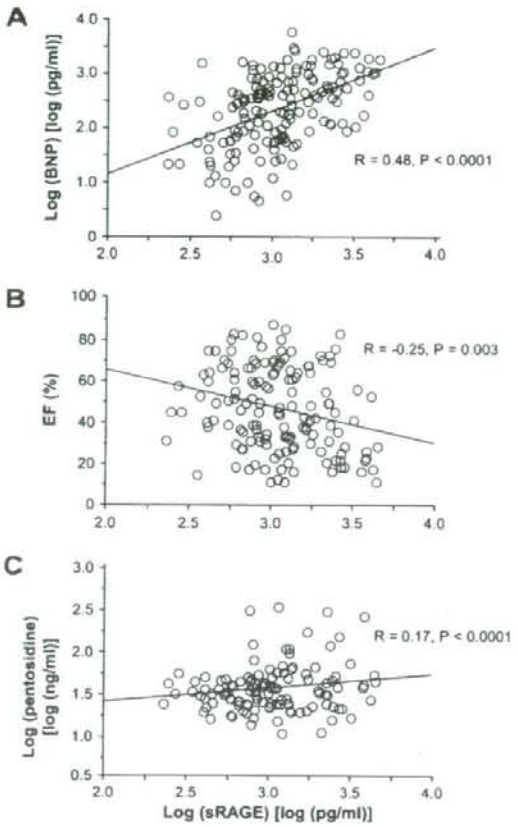

Fig. 4. Correlation between serum soluble advanced glycation end-product receptor and plasma BNP level (A), EF (B), and serum pentosidine level (C).

In the present study, we found that soluble RAGE was increased in patients with cardiac events in heart failure patients with preserved EF (Table 3). Little et al demonstrated that treatment with alagebrium chloride (ALT-711), and AGE cross-link breaker, decreased left ventricular mass and improved left ventricular diastolic filling and improved quality of life in patients with diastolic heart failure.[23] In a previous study, we reported that serum pentosidine levels were increased in patients with cardiac events and preserved EF.[9] Taken together, these data may suggest that the AGE-RAGE system is at least partially contributed to diastolic heart failure.

Sugiyama et al reported that plasma pentosidine level was significantly influenced by the quality of glycemic control and renal function.[24] In our present study, patients with renal dysfunction (creatinine >2 mg/dL) were excluded, and only 24% of patients had diabetes mellitus. Heidland et al reported that N$^{\varepsilon}$carboxymethyl lysine and AGE-associated fluorescence were decreased in patients with heart failure, and increased after heart transplantation.[25]

Table 3. Clinical Characteristics of Patients with Preserved EF

Variables	Cardiac Event (−) (n = 68)	Cardiac Events (+) (n = 19)	*P* Value
Age (y)	68 ± 12	76 ± 7	.012
Gender (male/female)	37/31	11/8	.787
NYHA class			
NYHA I-II	52 (76%)	4 (21%)	
NYHA III-IV	16 (23%)	15 (79%)	<.0001
Etiology			
DCM	10 (15%)	2 (11%)	
IHD	16 (24%)	2 (11%)	
VHD	18 (26%)	9 (47%)	
Tachycardia-induced	13 (19%)	2 (11%)	
HHD	9 (13%)	3 (15%)	
HCM	2 (3%)	1 (5%)	.489
Hypertension	41 (60%)	8 (42%)	.158
Diabetes mellitus	14 (21%)	5 (26%)	.593
Hyperlipidemia	12 (18%)	4 (21%)	.735
Current smoking	17 (25%)	2 (11%)	.177
Laboratory data			
Soluble RAGE (pg/mL)	884 (27–352)	1674 (768–2070)	.048
Creatinine (mg/dL)	0.81 ± 0.24	0.29 ± 0.27	.210
Pentosidine	29.9 (22.8–38.9)	43.0 (29.4–184.3)	.017
BNP (pg/mL)	83 (27–352)	520 (235–1220)	<.001
Echocardiography			
LVEDV (mL)	118 ± 43	128 ± 42	.375
EF (%)	60 ± 12	61 ± 12	.725

Abbreviations as in Table 1.

In contrast to this report, we previously demonstrated that serum pentosidine levels were increased in patients with NYHA Class III/IV, and pentosidine was an independent prognostic factor for heart failure.[9] Furthermore, we showed in the present study that serum soluble RAGE level

Table 4. Results of the Univariate Cox proportional Hazard Analysis

Variables	Chi-Square	HR	95% CI of HR	*P* Value
Age (per 1 y increase)	4.51	1.03	1.00–1.06	.034
Gender (female vs. male)	0.35	0.84	0.47–1.50	.554
NYHA (Class III/IV vs. Class I/II)	18.8	3.78	2.07–6.91	<.0001
Hypertension	4.19	0.55	0.31–0.98	.041
Diabetes mellitus	0.51	0.78	0.39–1.56	.477
Hyperlipidemia	0.08	1.11	0.55–2.22	.775
Smoking	2.35	0.53	0.24–1.19	.125
Laboratory data				
Log (sRAGE) (per 1 SD increase)	11.1	1.64	1.23–2.19	.001
Creatinine (per 1 SD increase)	4.00	1.30	1.01–1.68	.006
Log (pentosidine) (per 1 SD increase)	20.1	2.06	1.50–2.83	<.0001
Log (BNP) (per 1 SD increase)	25.5	2.65	1.81–3.87	<.0001
Echocardiography				
LVEDV (per 1 SD increase)	8.48	1.57	1.12–2.08	.004
EF (per 1 SD increase)	2.63	0.77	0.56–1.06	.105

Abbreviations as in Table 1.
HR, hazard ratio; CI, confidence interval.

Table 5. Results of the Multivariate Cox Proportional Hazard Analysis

Variables	Chi-Square	HR	95% CI of HR	*P* Value
Log (sRAGE) (per 1 SD increase)	6.62	1.90	1.16–3.09	.010
Log (pentosidine) (per 1 SD increase)	6.32	1.59	1.11–2.29	.012
Log (BNP) (per 1 SD increase)	2.43	1.64	0.88–3.07	.119
Creatinine (per 1 SD increase)	1.95	0.74	0.48–1.13	.162
EDV (per 1 SD increase)	0.37	1.12	0.75–1.76	.546
NYHA (Class III/IV vs. Class I/II)	0.35	1.36	0.49–3.80	.553
Age (per 1 y increase)	0.02	1.00	0.96–1.03	.904

Abbreviations as in Table 1.
HR, hazard ratio; CI, confidence interval.

increased with advancing NYHA functional class and soluble RAGE was a prognostic factor for heart failure. These data may suggest that the AGE-RAGE system is included as one of subcellular mechanisms of heart failure.

To date, clinical significance of serum soluble RAGE level is still controversial. Falcone et al have reported that low plasma soluble RAGE level is associated with the presence of coronary artery disease in nondiabetic men.[14] However, Nakamura et al have reported that serum soluble RAGE levels are significantly higher in type 2 diabetic patients than in nondiabetic subjects and positively associated with the presence of coronary artery disease.[15] It has been reported that RAGE mRNA is upregulated by the AGEs themselves, tumor necrosis factor-α, and 17β-estradiol in human vascular endothelial cells.[26] Furthermore, soluble RAGE is generated from the cleavage of cell surface RAGE by the actions of matrix metalloproteinases.[27,28] Two types of enzyme-linked immunosorbent assays are available to measure circulating RAGE. One immunoassay system[20,29,30] is specifically measures endogenous secretory RAGE. The other assay that we and others[14,15,31] used is to quantify total soluble RAGE detecting not only native secretory RAGE, but also other soluble forms resulted from the cleavage of cell surface receptor by matrix metalloproteinase. It has been reported that matrix metalloproteinase activity was increased in patients with heart failure.[32,33] Yamagishi et al have demonstrated that serum soluble RAGE levels are positively associated with circulating AGEs levels in the nondiabetic general population.[31] We have recently reported that serum levels of pentosidine, one of the well-defined AGE, is associated with the severity of heart failure.[9] In human cardiac auricles, RAGE protein expression is positively correlated with cardiac dysfunction.[34] In the present study, we demonstrated that serum soluble RAGE level was associated with NYHA functional class severity. We speculate that increased levels of AGEs induced by excessive oxidative stress and inflammation cause upregulation of RAGE expression and increase circulating serum soluble RAGE resulted from the cleavage of cell surface receptor by matrix metalloproteinase in patients with heart failure.

There are some limitations in the present study. First, it has been reported that some genetic polymorphism exist in the RAGE gene.[35] Jang et al have investigated the association between the Gly82Ser polymorphism in the RAGE gene and circulating levels of soluble RAGE in 1676 nondiabetic and nonobese Korean subjects. In this study, gene distribution was homozygous for the G allele (G/G) in 1180 subjects, heterozygous for the S allele (G/S) in 449 subjects, and homozygous for the S allele (S/S) in 47 subjects. Plasma soluble RAGE levels were significantly higher in subjects with G/G genotype (1038 ± 33 pg/mL) than in those with G/S (809 ± 19) and the S/S (428 ± 43 pg/mL) genotype.[36] Although the distribution of RAGE Gly82Ser genotype is still unknown in Japanese population, polymorphism might affect on serum soluble RAGE levels in our study population. Second, the use of angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers and β-blockers was relatively low in this study. These were data taken at admission, and there are some reasons for this low rate: 1) many patients were referred to our hospital for admission from general physicians, who were unfamiliar with recent advancement in pharmacotherapy for heart failure including β-blockers; 2) heart failure patients with the first decompensation were included in study population; 3) many of study subjects had preserved left ventricular systolic function (LVEF >40%) in the present study; and 4) numbers of patients with ischemic heart failure were relatively low (26%) in this study population compared with Western countries. However, at discharge, angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers and β-blockers were given in 92% and 67% of patients, respectively. Third, in our study population, mean LVEF was high (48 ± 19%). Dilated cardiomyopathy and ischemic heart disease were present in only 47 patients (29%) and 42 patients (26%), respectively. It is possible that, because percentages of dilated cardiomyopathy and ischemic heart disease were low compared with previous studies, the mean LVEF was relatively high in the present study.

Conclusions

Serum soluble RAGE level is related to the severity of heart failure and is an independent predictor for heart failure. Soluble RAGE may be a novel marker for risk stratification of patients with heart failure.

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Differential Effects of GM-CSF and G-CSF on Infiltration of Dendritic Cells during Early Left Ventricular Remodeling after Myocardial Infarction[1]

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Several lines of evidence suggest that the immune activation after myocardial infarction (MI) induces secondary myocardial injury. Although dendritic cells (DC) are potent regulators of immunity, their role in MI is still undetermined. We investigated the effect of DC modulation by CSF on left ventricular (LV) remodeling after MI. MI was induced by ligation of the left coronary artery in male Wistar rats. G-CSF (20 μg/kg/day, MI-G, $n = 33$), a GM-CSF inducer (romurtide, 200 μg/kg/day, MI-GM, $n = 28$), or saline (MI-C, $n = 55$) was administered for 7 days. On day 14, MI-G animals had higher LV max dP/dt and smaller LV dimensions, whereas MI-GM animals had lower LV max dP/dt and larger LV dimensions than did MI-C animals, despite similar infarct size. In MI-C, OX62$^+$ DC infiltrated the infarcted and border areas, peaking on day 7. Bromodeoxyuridine-positive DC were observed in the border area during convalescence. Infiltration by DC was decreased in MI-G animals and increased in MI-GM animals compared with MI-C ($p < 0.05$). In the infarcted area, the heat shock protein 70, TLR2 and TLR4, and IFN-γ expression were reduced in MI-G, but increased in MI-GM in comparison with those in MI-C animals. IL-10 expression was higher in MI-G and lower in MI-GM than in MI-C animals. In conclusion, G-CSF improves and GM-CSF exacerbates early postinfarction LV remodeling in association with modulation of DC infiltration. Suppression of DC-mediated immunity could be a new strategy for the treatment of LV remodeling after MI. *The Journal of Immunology*, **2008, 181: 5691–5701.**

Left ventricular (LV)[3] remodeling, an important structural event after myocardial infarction (MI), is characterized by myocardial necrosis, wall thinning, infarct expansion, collagen accumulation, and noninfarcted myocardial hypertrophy, and contributes significantly to a worse clinical outcome. These alterations are caused not only by significant loss of myocardium, but also by myocardial injury secondary to local and systemic factors such as neurohumoral activation, oxidative stress, and inflammatory response. The inflammatory response after MI is integral to the healing process and contributes to LV remodeling (1–3). However, no effective therapeutic strategy against inflammation has been established.

We previously reported that elevated concentrations of serum C-reactive protein (4), plasma IL-6 (5), and peripheral monocytosis (6) predict a worse clinical outcome after acute MI, suggesting that an immune-mediated inflammatory response may have some role during infarct healing and ventricular remodeling. Although an excessive inflammatory response after MI is associated with a poor clinical outcome (2), antiinflammatory therapy using corticosteroids (7, 8) or nonsteroidal antiinflammatory drugs (9, 10) leads to catastrophic results such as a higher incidence of infarct expansion and cardiac rupture. These findings suggest that an inflammatory reaction is a prerequisite for the healing process. As the cause of inappropriate activation of the inflammatory response after MI, an autoimmune reaction is a possible mechanism relating to LV remodeling (11–13). Abbate et al. reported infiltration of activated T cells into both infarcted and remote areas of the myocardium in patients with recent MI (1). Moreover, the presence of autoimmunity to cardiac myosin (12), actin (14), and troponin (15) is associated with an adverse clinical outcome after MI. These findings suggest that autoimmunity may contribute to secondary myocardial injury after MI.

For the activation of autoimmune responses, myocardial Ag presentation is required. Dendritic cells (DC) are potent regulators of immunity by presenting Ag, activating T cells, and by causing differentiation T cells into Th1 and Th2 cells (16–18). After tissue injury, heat shock proteins (HSP) released from necrotic cells can promote activation and maturation of DC through stimulation of TLRs (19, 20). Additionally, Cheng et al. demonstrated that Th1/Th2 imbalance participated in ventricular remodeling after MI (21). However, the role of DC during the healing process after MI has not been determined.

The development of DC from hematopoietic progenitor cells is differentially regulated by various cytokines such as GM-CSF and G-CSF. We previously reported that G-CSF treatment improved (22) and GM-CSF induction aggravated (23) early LV remodeling after MI through modification of the infarct healing process. GM-CSF induces differentiation from immature DC to myeloid DC, with a subsequent increase in Th1 cells, whereas G-CSF induces

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[3] Abbreviations used in this paper: LV, left ventricle; ALP, alkaline phosphatase; DC, dendritic cells; HSP, heat shock protein; MI, myocardial infarction.