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Prepare Cells to Repair the Heart: Mesenchymal Stem Cells for the Treatment of Heart Failure

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Key Words

Mesenchymal stem cell · Heart failure · Neovascularization · Tissue regeneration · Translational research · Gene expression · Paracrine effect

Abstract

Heart failure is one of the most important cardiovascular diseases, with high mortality, and invasive treatment such as mechanical circulatory support and cardiac transplantation is sometimes required for severe heart failure. Therefore, the development of less invasive and more effective therapeutic strategies is desired. Cell therapy is attracting growing interest as a new approach for the treatment of heart failure. As a cell source, various kinds of stem/progenitor cells such as bone marrow cells, endothelial progenitor cells, mesenchymal stem cells (MSC) and cardiac stem cells have been investigated for their efficacy and safety. Especially, bone marrow-derived MSC possess multipotency and can be easily expanded in culture, and are thus an attractive therapeutic tool for heart failure. Recent studies have revealed the underlying mechanisms of MSC in cardiac repair: MSC not only differentiate into specific cell types such as cardiomyocytes and vascular endothelial cells, but also secrete a variety of paracrine angiogenic and cytoprotective factors. It has also been suggested that endogenous MSC as well as exogenously transplanted MSC migrate and participate in cardiac repair. Based on these findings, several clinical trials have just been started to evaluate the safety and efficacy of MSC for the treatment of heart failure.

Introduction

Heart failure is a major cardiovascular health problem worldwide. About 5.2 million patients in the US have heart failure, and the lifetime risk of developing chronic heart failure for both men and women is 1 in 5 [1]. Coronary artery disease is the most common cause of heart failure, followed by idiopathic dilated cardiomyopathy and valvular heart disease [2, 3]. Myocardial infarction causes necrosis of the myocardium, followed by infiltration of inflammatory cells. Then a scar forms, leading to the loss of cardiac function, ventricular remodeling and progressive dysfunction, and, finally, congestive heart failure [4–6]. Drugs commonly used for the treatment of chronic heart failure include loop diuretics, angiotensin-converting enzyme inhibitors, β-adrenergic receptor blockers, aldosterone antagonists, angiotensin II receptor blockers and digitoxin; however, patients with severe heart failure require invasive treatment such as mechan-

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Table 1. Characteristics of cells used for the treatment of heart failure

	Ease of harvest	Expandability	Differentiation into cardiomyocytes	Ethical issues	Arrhythmogenesis
Embryonic stem cells		√	√	√	
Cardiomyocytes			√	√	
Skeletal myoblasts	√	√	√		√
Cardiac stem cells		√	√		
Endothelial progenitor cells	√	√			
Mononuclear cells	√				
Mesenchymal stem cells	√	√	√		

ical circulatory support, continuous inotropic infusion, or cardiac transplantation [7–9]. Therefore, there is a need to develop more effective, less invasive therapeutic strategies for heart failure.

Cell therapy for heart failure has the potential to restore cardiac function by inducing neovascularization, and regenerating and protecting cardiomyocytes [4]. Bone marrow-derived mononuclear cells (MNC) and endothelial progenitor cells (EPC) have been applied for ischemic cardiovascular disease in human studies [10–12]. Recently, mesenchymal stem cells (MSC), a subpopulation of MNC, have emerged as a new therapeutic cell source because they possess multipotency and can be easily expanded in culture [13]. This article reviews cell therapy for heart failure, and focuses on the therapeutic potential of MSC for heart failure.

Cell Sources for Cardiac Repair

Several kinds of cells have the potential to be applied for cardiac repair; embryonic stem (ES) cells and somatic stem or progenitor cells (skeletal myoblasts, MNC, MSC, EPC, cardiac stem cells) (table 1). Because ethical problems may limit the immediate application of ES cells, we herein describe the features and characteristics of somatic cells.

Skeletal myoblasts, which are derived from cultured satellite cells, were the first to be tested in clinical trials. Satellite cells are muscle progenitor cells which normally mediate the regeneration of skeletal muscle [14]. They can be easily expanded in culture in an undifferentiated state, and are highly resistant to tissue ischemia. In vivo studies have demonstrated that the administration of myoblasts improved cardiac function [15–18]. However, in early clinical studies, myoblast transplantation was associated with sustained ventricular tachycardia [19], which requires defibrillator implantation and/or amiodarone therapy.

Bone marrow cells include several types of stem/progenitor cells such as hematopoietic stem cells and MSC, although the vast majority of bone marrow cells are hematopoietic cells [20, 21]. Because of the extensive clinical experience in bone marrow transplantation for hematological diseases over the past decades and the fact that large numbers of bone marrow cells can be easily obtained, the study of bone marrow cell transplantation for treating heart failure has moved quickly from small animals to human studies. Direct or intracoronary injection of bone marrow cells appears to be safe and beneficial for the treatment of acute myocardial infarction and chronic ischemic heart disease [11, 22–25]. However, it is not clear whether the optimal bone marrow cell population for transplantation is hematopoietic stem cells, MSC, or abundant committed cells. The major goal of studies using bone marrow cells will be to identify the most effective cell population from these complex mixtures.

EPC, a rare subpopulation of bone marrow cells with a similar phenotype and function to those of fetal angioblasts, have been demonstrated to be involved in neovascularization after myocardial infarction [26]. In addition, there was a significant reduction in collagen deposition and apoptosis of cardiomyocytes and an improvement in cardiac function. It has been demonstrated that granulocyte colony-stimulating factor (G-CSF) and stem cell factor can mobilize EPC [27, 28]. Clinical trials studying the ability of G-CSF to mobilize stem/progenitor cells in patients with coronary artery disease did not reach a conclusion on efficacy, while concerns have been raised on safety in relation to arterial restenosis and plaque destabilization. HMG-CoA reductase inhibitors [29], estrogens [30], exercise [31], and nonsmoking [32] are more practical than growth factor or chemokine administration to enhance the number of circulating EPC in patients. Currently, clinical trials of EPC therapy for neovascularization and myocardial re-

generation using CD34-positive cells from BM are in progress.

In the past few years, the search for stem or progenitor cells in the heart has intensified. These would be comparable to the satellite cells in skeletal muscle. Evidence is accumulating that cardiac stem cells reside at specific locations in the adult heart. Beltrami et al. [33] described the isolation of lineage-negative, c-kit-positive cells from the adult rat heart and showed that these cells were able to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells. In addition, Sca-1-positive cells were isolated from the adult mouse heart [34]. This Sca-1-positive subpopulation differentiated in vitro into cardiomyocytes in response to 5-azacytidine, and expressed several cardiac specific markers. In addition, Sca-1-positive cells differentiated into cardiomyocytes after intravenous injection. Messina et al. [35] identified a subpopulation of cardiosphere-forming cells after culturing human atrial or ventricular biopsies and embryonic, fetal or postnatal mouse hearts. The mouse cardiospheres started to beat spontaneously, whereas human cardiospheres only started to beat after co-culture with rat neonatal cardiomyocytes. Furthermore, cardiospheres expressed endothelial markers and were positive for Sca-1 and c-kit. Recently, another subpopulation of cardiac stem cells has been described [36]. These cardiac stem cells were Isl1-positive and were identified in rat, mouse and human adult hearts. Isl1-positive cardiac stem cells were positive for cardiac transcriptional factors Nkx2.5 and GATA4, while transcriptional factors associated with mature cardiomyocytes were absent. However, Sca-1 and c-kit were not expressed on these cells. When co-cultured with neonatal rat cardiomyocytes, 30% of the Isl1-positive cardiac stem cells differentiated into cardiomyocytes.

MSC: Distribution and Behavior

MSC reside not only in bone marrow [37] and adipose tissue [38], but also in other tissues such as synovium [39], periosteum [40], muscle [41], dental pulp [42], periodontal ligament [43], placenta [44] and umbilical cord blood [45]. A recent study suggests that MSC reside in virtually all postnatal organs and tissues, and may be localized to vessel walls [46]. MSC can differentiate not only into osteoblasts, chondrocytes and adipocytes, but also into cardiomyocytes and vascular endothelial cells [13, 47]. Bone marrow-derived MSC, which are most investigated, are a rare subpopulation of bone marrow cells (approximately 0.001–0.01%) [13]. It has been demonstrated that bone marrow MSC can be mobilized and differentiate into cardiomyocytes in a murine model of myocardial infarction, suggesting the importance of bone marrow MSC in cardiac regeneration [48]. Moreover, when cultured MSC were intravenously administered to rats with myocardial infarction, they were preferentially engrafted into the infarcted, but not the non-infarcted, myocardium, and a small fraction of the transplanted MSC differentiated into cardiomyocytes and vascular endothelial cells [49].

Differentiation of MSC into Myocardial Lineage

In vitro studies have demonstrated that MSC can differentiate not only into adipocytes and osteocytes, but also into cardiomyocytes and vascular endothelial cells [13, 47, 50–53]. The differentiation of MSC into cardiomyocytes in vitro has been induced in cultures containing either MSC alone treated with 5-azacytidine or a cocktail of growth factors, and in co-culture with cardiomyocytes, and has been demonstrated by evidence of spontaneous beating or cardiomyocyte specific markers [50–52, 54–61], although there is a lack of a clear definition of which markers are evidence for full differentiation into functional cells [62]. After direct injection of MSC into infarcted myocardium in a pig model, engrafted MSC differentiated toward a myogenic lineage, expressing muscle specific proteins, and attenuated cardiac function and pathologic thinning [63]. Furthermore, we and others have demonstrated that directly or intravenously injected MSC differentiated into endothelial cells, and were involved in angiogenesis as well as myogenesis in animal models of myocardial infarction [49, 64, 65].

Paracrine Effects Produced by MSC

MSC exert their effect on cardiac regeneration not only by differentiation into specific cell types, but also through paracrine actions. In vitro studies have demonstrated that MSC can secrete a variety of angiogenic, anti-apoptotic and mitogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), adrenomedullin (AM) and insulin-like growth factor-1 (IGF-1) [66, 67]. Interestingly, administration of conditioned medium obtained from MSC culture exerted cytoprotective effects on the myocardium in an animal model of myocardial infarction [68]. We have recently

demonstrated that cultured cardiomyocytes were injured in response to monocyte chemoattractant protein-1 (MCP-1), which plays an important role in myocarditis, whereas this effect was significantly attenuated by conditioned medium derived from MSC culture [69]. These results suggest a cardioprotective effect of MSC acting in a paracrine manner, demonstrating the importance of secreted factors in cardiac repair.

Gene Expression in MSC

MNC have been shown to induce therapeutic neovascularization in critical limb ischemia and myocardial infarction in human studies [10, 11]. Similarly, many studies have demonstrated the therapeutic potential of MSC; however, the underlying mechanisms contributing to cardiovascular protection might be different between MSC and MNC. The gene expression profiles under normoxia and hypoxia are largely different between MSC and MNC [70]. MNC express a number of genes involved in inflammatory response and chemotaxis. On the other hand, MSC express a number of genes involved in development (e.g. transgelin, actin-γ1), morphogenesis (e.g. bone morphological protein-2, transforming growth factor-β3), cell adhesion (e.g. neural cell adhesion molecule-1, cadherin-11) and proliferation (e.g. connective tissue growth factor, platelet-derived growth factor-A) under normoxia. Furthermore, focusing on genes encoding secretory proteins in response to hypoxia, the upregulated genes in MSC include several molecules involved in cell proliferation and survival such as VEGF-D, placenta growth factor (PGF), pre-B cell colony-enhancing factor 1 (PBEF1), heparin-binding epidermal growth factor-like growth factor (HB-EGF) and matrix metalloproteinase-9 (MMP-9), while the upregulated genes in MNC under hypoxia include proinflammatory cytokines such as chemokine (CXC motif) ligand 2 (CXCL2) and interleukin-1α (IL-1α) [70]. These results suggest that transplanted MSC may act to promote cell proliferation, including angiogenesis, and cell survival in response to hypoxia, while MNC may induce an inflammatory response, followed by angiogenesis. In fact, implantation of MNC into ischemic limbs has been reported to lead to local inflammatory reactions [71].

It is postulated that not only exogenously administered MSC, but also endogenous MSC migrate and participate in wound repair. In healthy animals, intravenous administration of MSC preferentially engraft in the bone marrow cavity; however, when rats were subjected to ischemia/reperfusion, a significant number of xenogeneic MSC could be identified in the circulation, and subsequently in the infarcted region of the heart [72]. Taking these findings together, MSC are considered to exert cardiac repair by a variety of mechanisms.

MSC for Treatment of Heart Failure

Myocardial injection of MSC improved cardiac function in a rat model of dilated cardiomyopathy, a myocardial disease characterized by a loss of cardiomyocytes and an increase in fibroblasts [73, 74], possibly through induction of angiogenesis and myogenesis, as well as by inhibition of myocardial fibrosis [67]. In this study, MSC transplantation significantly increased capillary density and decreased the collagen volume fraction in the myocardium. In addition, intravenous administration of MSC improved inflammatory changes and cardiac function in rats with acute myocarditis, suggesting an anti-inflammatory effect of MSC [69]. Recently, we have demonstrated that adipose tissue-derived monolayered MSC, generated by cell sheet technology using temperature-responsive culture dishes, repair scarred myocardium after myocardial infarction in rats [75]. Interestingly, the engrafted MSC sheet gradually grew after transplantation to form a thick stratum that included undifferentiated MSC as well as newly formed vessels, which were composed of graft-derived cells, host-derived cells or both. Unlike a fibroblast cell sheet, the monolayered MSC reversed wall thinning in the scar area, contributing to a reduction in left ventricle wall stress and improvement of cardiac function. Therefore, this new technology may overcome problems associated with needle injection of bone marrow cells into the scar area, i.e. difficulty to reconstruct sufficient cardiac mass.

There are few reports describing the results of clinical studies on the treatment of heart failure, but several studies are on going. It has been reported that intracoronary administration of autologous bone marrow-derived MSC improved cardiac function in patients with acute myocardial infarction [76] and chronic ischemic cardiomyopathy [77]. Katritsis and co-workers reported that intracoronary transplantation of bone marrow-derived MSC may contribute to regional regeneration of myocardial tissue following myocardial infarction [78], and intracoronary transplantation of these cells did not appear to be arrhythmogenic [79]. The National Institute of Health in the United States provides information on current clinical trials using MSC (www.clinicaltrials.gov). In December 2005, the Rigshospitalet in Denmark started a phase

I/II safety and efficacy study (NCT00260338) to evaluate the clinical effect of autologous bone marrow-derived MSC therapy in patients with severe chronic myocardial ischemia. In October 2006, Helsinki University started a prospective double-blind trial (NCT00418418) of intraoperative transmyocardial autologous bone marrow-derived MSC transplantation versus placebo in patients with heart failure scheduled to undergo coronary bypass operation. In October 2006, the National Heart, Lung, and Blood Institute in the United States started a phase II study (NCT00383630) to evaluate the effect of injected autologous bone marrow cells to improve heart function in individuals with a left ventricular assist device (LVAD) awaiting heart transplantation. This study is enrolling individuals undergoing surgery to receive an LVAD, and patients are randomly assigned to one of the following three groups: group 1 patients receive injected MSC while undergoing LVAD implantation; group 2 patients receive injected immunoselected CD34-positive hematopoietic stem cells while undergoing LVAD implantation; group 3 patients undergo LVAD implantation. We have also started a pilot study of intramyocardial injection of autologous bone marrow-derived MSC in patients with end-stage heart failure. In this study, 20 ml of bone marrow cells are aspirated from the ilium, and MSC are cultured and expanded with medium containing autologous serum for 3 weeks. A large number of MSC are directly injected into the myocardium at 40 sites, using a catheter.

Conclusions

MSC have emerged as a new therapeutic tool for cell therapy in heart failure, and exert their effects by differentiating into specific cell types as well as through paracrine actions such as angiogenic, cytoprotective, anti-inflammatory and anti-fibrotic effects. Whether autologous MSC have significant value in the treatment of heart failure is currently being explored in clinical trials.

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Sex Hormone and Gender Difference—Role of Testosterone on Male Predominance in Brugada Syndrome

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Testosterone in Brugada Syndrome. ***Introduction:*** **The clinical phenotype is 8 to 10 times more prevalent in males than in females in patients with Brugada syndrome. Brugada syndrome has been reported to be thinner than asymptomatic normal controls. We tested the hypothesis that higher testosterone level associated with lower visceral fat may relate to Brugada phenotype and male predominance.**

Methods and Results: **We measured body-mass index (BMI), body fat percentage (BF%), and several hormonal levels, including testosterone, in 48 Brugada males and compared with those in 96 age-matched control males. Brugada males had significantly higher testosterone (631 ± 176 vs 537 ± 158 ng/dL; P = 0.002), serum sodium, potassium, and chloride levels than those in control males by univariate analysis, and even after adjusting for age, exercise, stress, smoking, and medication of hypertension, diabetes, and hyperlipidemia, whereas there were no significant differences in other sex and thyroid hormonal levels. Brugada males had significantly lower BMI (22.1 ± 2.9 vs 24.6 ± 2.6 kg/m^2; P < 0.001) and BF% (19.6 ± 4.9 vs 23.1 ± 4.7%; P < 0.001) than control males. Testosterone level was inversely correlated with BMI and BF% in both groups, even after adjusting for the confounding variables. Conditional logistic regression models analysis showed significant positive and inverse association between Brugada syndrome and hypertestosteronemia (OR:3.11, 95%CI:1.22–7.93, P = 0.017) and BMI (OR:0.72, 95%CI:0.61–0.85, P < 0.001), respectively.**

Conclusions: **Higher testosterone level associated with lower visceral fat may have a significant role in the Brugada phenotype and male predominance in Brugada syndrome.** *(J Cardiovasc Electrophysiol, Vol. 18, pp. 415-421, April 2007)*

Brugada syndrome, gender, sex hormones, testosterone, body mass index

Introduction

Brugada syndrome is characterized by coved-type ST-segment elevation in the right precordial electrocardiographic (ECG) leads (V1–V3) and an episode of ventricular fibrillation (VF) in the absence of structural heart disease.[1-5] The prevalence of the disease is estimated to be up to 5 per 10,000 inhabitants and is one of the important causes of sudden cardiac death of middle-aged males, particularly in Asian countries including Japan.[4]

More than eight dozen distinct mutations in *SCN5A*, the gene encoding the α subunit of the sodium channel, have been so far identified in patients with Brugada syndrome and all mutations display an autosomal-dominant mode of transmission.[6,7] Therefore, males and females are expected to inherit the defective gene equally. However, more than 80% of patients in Western countries and more than 90% of patients in Asian countries affected with Brugada syndrome are males.[8] Recent experimental studies have unveiled the cellular mechanism of Brugada phenotype. The male predominance in the Brugada syndrome is suggested to be due, at least in part, to intrinsic differences in ventricular action potential (AP) between males and females.[9]

A male hormone, testosterone is reported to increase net outward currents[10-12] and is expected to accentuate Brugada phenotype, such as ST-segment elevation and subsequent episodes of VF in patients with Brugada syndrome. Testosterone is also known to decrease visceral fat.[13-15] Since patients with Brugada syndrome have been reported to be

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thinner than asymptomatic normal controls by Matsuo et al.,[16] we speculated that higher testosterone level associated with lower visceral fat may modulate Brugada phenotype and may relate to male predominance in patients with Brugada syndrome.

Methods

Patient Population and Data Collection

The study population consisted of 48 males with Brugada syndrome who agreed to participate in this study and showed Type 1 "coved" ST-segment elevation in V1–V3 leads[17] ranging in age from 30 to 69 years with a mean age of 50 ± 11 years (mean ± SD). Brugada males who were less than 30 years old and more than 70 years old were excluded from this study to minimize the influence of age on the basal sex hormonal levels including testosterone. Forty of the forty-eight Brugada males have been included in our previous clinical studies.[18-20] In all patients, physical examination, chest roentgenogram, laboratory values, echocardiography with wall motion analysis, and Doppler screening excluded structural heart diseases. The clinical, electrocardiographic, and electrophysiologic characteristics of the 48 Brugada males are shown in Table 1. Average age of the 48 Brugada males at diagnosis was 47 ± 12 years old. Aborted cardiac arrest or VF was documented in 21 males (44%), syncope alone in 11 males (23%), and 16 males (33%) were asymptomatic. Family history of sudden cardiac death (SCD) was observed in eight males (17%). An *SCN5A* coding region mutation was identified in seven (17%) of 42 males in whom genetic screening was conducted. Implantable cardioverter defibrillator (ICD) was implanted in all 32 symptomatic males with documented VF and/or syncope. ICD was also implanted in nine of 16 asymptomatic males due to induction of VF during the electrophysiologic study. Type 1 ST-segment elevation was recorded spontaneously in 43 males (90%) and was induced by sodium channel blockers in five males (10%). Complete right bundle branch block was observed in three males (6%). Late potential was recorded by a signal-average ECG system in 27 (59%) of 46 males. During the electrophysiologic study, VF requiring direct cardioversion for termination was induced in 32 (73%) of 44 males. Average HV interval was 46 ± 11 msec.

TABLE 1
Clinical, Electrocardiographic, and Electrophysiologic Characteristics in the 48 Brugada Males

Clinical characteristics	
Age at diagnosis (years)	47 ± 12
Aborted cardiac arrest or VF (%)	21/48 (44%)
Syncope alone (%)	11/48 (23%)
Asymptomatic (%)	16/48 (33%)
Family history of SCD	8/48 (17%)
SCN5A mutation	7/42 (17%)
ICD implantation	41/48 (85%)
Follow-up period (month)	41 ± 2
Arrhythmic event (%)	9/48 (19%)
Electrocardiographic characteristics	
Spontaneous coved-type ST elevation	43/48 (90%)
CRBBB (%)	3/48 (6%)
RR (msec)	939 ± 113
PQ interval (II) (msec)	186 ± 34
QRS duration (V2) (msec)	104 ± 18
Corrected QT interval (V5) (msec)	394 ± 27
ST amplitude at J point (V2) (mV)	0.32 ± 0.16
Late potential (%)	27/46 (59%)
Electrophysiologic characteristics	
Induction of VF	32/44 (73%)
Mode (Triple/Double/Single)	16/15/1
HV interval (msec)	46 ± 11

CRBBB = complete right bundle branch block; ICD = implantable cardioverter defibrillator; SCD = sudden cardiac death; VF = ventricular fibrillation.

We first obtained data, such as the hormonal levels, visceral fat parameters, and ECG parameters in the 48 Brugada males prospectively between January and July in 2003, mainly at regular outpatient clinics for checking ICD. Only a Brugada male refused to participate during the recruitment of the case.

Thereafter, age-matched control males were randomly selected from the municipal population registry in Suita City. The hormonal and visceral fat data were collected sequentially between August and December in 2003. The municipal population registry in Suita City included 5,846 control subjects, among whom 1,052 males were age-matched to the 48 Brugada males. The 96 control males with a mean age of 50 ± 11 years were sequentially recruited from the age-matched 1,052 males. None of the recruited 96 control males refused to participate in this study. There were no significant differences in the clinical characteristics between the 96 control males and the remaining 956 age-matched males. Therefore, we had no way of knowing the body weight of the individuals who were selected to serve as controls from a very large database. Although K. Matsuo is a co-author of this study, none of the Brugada males and control males who appeared in the article by Matsuo[16] are included in the present study population.

All protocols were approved by the Ethical Review Committee in the National Cardiovascular Center. Written informed consent was obtained from all subjects.

Sex and Thyroid Hormonal Levels and Serum Electrolytes

Blood samples for analysis of basal hormone levels and serum electrolytes were obtained between 8:00 and 9:00 AM after an overnight fast. Plasma sex hormonal levels including testosterone, estradiol, DHEA-S, LH, and FSH were measured using commercially prepared immunoassay kits (testosterone, LH, and FSH: Chemiluminescent immunoassay [Bayer HealthCare, New York, NY, USA]; estradiol: Electrochemiluminescent immunoassay [Roche Diagnostics GmbH, Mannheim, Germany]; DHEA-S: Radioimmunoassay [Diagnostic Products Corporation, Los Angeles, CA, USA]). Thyroid hormonal levels including free T3, T4, and TSH, and serum electrolyte levels including sodium, potassium, and chloride were also measured.

Body Mass Index and Body Fat Percentage

Body weight (BW) was measured to the nearest 0.1 kg and height to the nearest cm. Body-mass index (BMI) was calculated as weight/height^2 (kg/m^2) as a parameter of visceral fat. We also measured body-fat percentage (BF%) by using body composition analyzer (Biospace Co., Ltd. Tokyo, Japan). These visceral fat parameters were measured just after blood sampling. In the 32 symptomatic Brugada males who had had documented VF and/or syncope, the BW and BMI were also measured within 48 hours after their clinical events during admission in our hospital or other emergent hospitals.

ECG Parameters

In the 48 males with Brugada syndrome, 12-lead ECG was recorded just before blood sampling, and ECG parameters were assessed by an investigator (WS) blinded to clinical information. The ECG parameters included RR interval, PQ interval measured in lead II, QRS interval measured in lead V2, QT interval, corrected QT (QTc) interval measured in leads V5, and ST amplitude at J point measured in lead V2.

Statistical Analysis

We first conducted univariate analysis by using unpaired *t*-test to compare each data between the Brugada males and the control males. Since several confounding variables, such as age, exercise (none, sometimes, regularly), stress (none, sometimes, regularly), current smoking (no, yes), and medication (no, yes) of hypertension, diabetes, and hyperlipidemia may affect the hormonal levels including testosterone level and the visceral fat parameters, analysis of covariance (ANCOVA) was used to compare least square mean values between the Brugada males and the control males adjusting for these confounding variables. Pearson's correlation coefficients were calculated between the testosterone level and the visceral fat parameters. Partial correlation coefficients were calculated between the testosterone level and the visceral fat parameters after adjusting for age, exercise, stress, current smoking, and medication. Moreover, conditional logistic regression models were used to calculate odds ratios and 95% confidence intervals adjusting for age, BMI, exercise, stress, current smoking, hypertension, diabetes, and hyperlipidemia. Hypertestosteronemia was defined as serum testosterone levels $\geq$700 ng/dL, which is 75 percentiles of testosterone levels among case and control combined groups. In the 32 Brugada males with documented VF and/or syncope, a paired *t*-test was used to compare the visceral fat parameters at the clinical cardiac events and at the measurement of hormonal and visceral fat data. A two-sided P value below 0.05 was considered to indicate significance. All statistical analyses were performed by using SAS software, Ver 8.2.

Results

Hormonal Levels, Serum Electrolytes, and Visceral Fat

Table 2 illustrates univariate analysis for comparing sex and thyroid hormonal levels, serum electrolytes, and visceral fat parameters between the two groups. Testosterone level was significantly higher in the Brugada males than in the control males, whereas there were no significant differences in other sex hormonal levels; estradiol, DHEA-S, LH, FSH, and thyroid hormonal levels; T3, T4, and TSH. Serum sodium, potassium, and chloride levels were all significantly higher in the Brugada males than in the control males. BMI, BF%, and BW were all significantly lower in the Brugada males than in the control males. All variables followed normal distribution, both in the 48 Brugada and 96 control males.

The comparison of the confounding variables that may affect the hormonal levels and the visceral fat parameters between the 48 Brugada males and the 96 control males was shown in Table 3. Even after adjusting for age, exercise, stress, current smoking, and medication (hypertension, diabetes, and hyperlipidemia), the testosterone level, serum sodium, potassium, and chloride levels were all significantly higher, and the visceral fat parameters were significantly lower in the 48 Brugada males than in the 96 control males (Table 4). There were also significant differences in these parameters between the 24 definite Brugada males with documented VF and/or *SCN5A* mutations and the 96 control males after adjusting for the confounding variables (Table 4).

Correlation between Testosterone, Visceral Fat, and Serum Electrolytes

Testosterone level was inversely correlated with all visceral fat parameters, BMI, BF%, or BW in both the Brugada males and the control males, even after adjusting for age,

TABLE 2

Sex and Thyroid Hormonal Levels, Serum Electrolytes, and Visceral Fat Parameters in the 48 Brugada Males and the 96 Age-Matched Control Males

	Brugada Males (n = 48)	Control Males (n = 96)	P Value
Sex hormones			
Testosterone (ng/dL)	631 ± 176	537 ± 158	0.002
Estradiol (pg/mL)	28.9 ± 7.6	31.1 ± 12.6	0.263
DHEA-S (ng/mL)	1,901 ± 850	1,966 ± 861	0.668
LH (mIU/mL)	4.6 ± 2.6	3.9 ± 2.0	0.073
FSH (mIU/mL)	6.2 ± 4.9	5.0 ± 2.9	0.066
Thyroid hormones			
Free T3 (pg/mL)	3.3 ± 0.4	3.4 ± 0.3	0.360
Free T4 (ng/dL)	1.3 ± 0.1	1.3 ± 0.2	0.089
TSH (μIU/mL)	1.9 ± 1.4	1.7 ± 1.4	0.619
Serum electrolytes			
Sodium (mEq/L)	143.7 ± 2.0	142.6 ± 2.0	0.003
Potassium (mEq/L)	4.6 ± 0.3	4.3 ± 0.3	<0.001
Chloride (mEq/L)	105.1 ± 2.1	103.6 ± 2.1	<0.001
Viceral fat			
BMI (kg/m^2)	22.1 ± 2.9	24.6 ± 2.6	<0.001
BF% (%)	19.6 ± 4.9	23.1 ± 4.7	<0.001
BW (kg)	62.9 ± 9.7	70.0 ± 8.6	<0.001

Values are mean ± SD where indicated.
BMI = body-mass index; BF% = body-fat percentage; BW = body weight.

TABLE 3

Comparison of the Confounding Variables Between the 48 Brugada Males and the 96 Age-Matched Control Males

	Brugada Males (n = 48)	Control Males (n = 96)	P Value
Exercise			
None (%)	39.6	44.8	
Sometimes (%)	41.6	43.8	
Regularly (%)	18.8	11.5	0.482
Stress			
None (%)	27.1	21.9	
Sometimes (%)	54.2	54.2	
Regularly (%)	18.8	24.0	0.684
Current smoking (%)	25.0	27.1	0.789
Medication			
Hypertension (%)	20.8	19.8	0.883
Diabetes (%)	2.1	13.5	0.028
Hyperlipidemia (%)	10.4	5.2	0.246

TABLE 4

Testosterone, Serum Electrolytes, and Visceral Fat Parameters in the Brugada Males and the 96 Age-Matched Control Males after Adjusting for Confounding Variables

	Brugada Males	Control Males (n = 96)	P Value
ALL Case (n = 48)			
Testosterone (ng/dL)	631 ± 44	538 ± 40	0.003
Sodium (mEq/L)	144.2 ± 0.5	143.2 ± 0.5	0.007
Potassium (mEq/L)	4.6 ± 0.1	4.3 ± 0.1	<0.001
Chloride (mEq/L)	105.5 ± 0.5	103.9 ± 0.5	<0.001
BMI (kg/m^2)	22.3 ± 0.7	24.9 ± 0.7	<0.001
BF% (%)	20.0 ± 1.3	23.9 ± 1.1	<0.001
BW (kg)	63.4 ± 2.4	70.1 ± 2.1	0.001
Definite Brugada case with VF and/or *SCN5A* (n = 24)			
Testosterone (ng/dL)	656 ± 59	550 ± 48	0.009
Sodium (mEq/L)	143.9 ± 0.7	142.9 ± 0.6	0.042
Potassium (mEq/L)	4.7 ± 0.1	4.4 ± 0.1	<0.001
Chloride (mEq/L)	105.2 ± 0.7	103.9 ± 0.6	0.006
BMI (kg/m^2)	21.5 ± 1.0	24.5 ± 0.8	<0.001
BF% (%)	19.9 ± 1.7	24.1 ± 1.4	<0.001
BW (kg)	60.5 ± 3.1	69.2 ± 2.5	0.001

Values are mean ± SE adjusted for age, exercise, stress, current smoking, and medication of hypertension, diabetes and hyperlipidemia. BMI = body-mass index; BF% = body-fat percentage; BW = body weight; VF = ventricular fibrillation.

exercise, stress, current smoking, and medication (Brugada: BMI, r = –0.394, P = 0.011; BF%, r = –0.390, P = 0.012; BW, r = –0.335, P = 0.032; Control: BMI, r = –0.333, p = 0.002; BF%, r = –0.333, P = 0.001; BW, r = –0.305, P = 0.004), suggesting that Brugada males had higher testosterone level associated with lower visceral fat compared with control males (Fig. 1). No significant correlations were observed between other serum electrolytes and testosterone level or visceral fat parameters. Testosterone level was not correlated with age, even after adjusting for exercise, stress, current smoking, and medication (r = 0.007, P = 0.947).

Conditional Logistic Regression Models Analysis

Conditional logistic regression models analysis showed significant positive and inverse association between Brugada syndrome, hypertestosteronemia (Odd Ratio (OR): 3.11, 95%CI: 1.22–7.93, P = 0.017), and BMI (OR: 0.72, 95%CI: 0.61–0.85, P < 0.001), respectively (Table 5). Other variables did not significantly increase or decrease risks of Brugada syndrome (Table 5).

Visceral Fat at Clinical Cardiac Events in Brugada Males

In the 32 symptomatic Brugada males with documented VF and/or syncope, the time-span between the clinical cardiac events and the measurement of hormonal and the visceral fat data was 42 ± 32 months (mean ± SD, 1–99 months). The BMI and BW at the clinical cardiac events (VF or syncope) were significantly lower than those at the measurement of hormonal and visceral fat data (BMI, 21.0 ± 2.6 vs 22.1 ± 2.9 kg/m^2; BW, 60.0 ± 8.9 vs 62.9 ± 9.7 kg: P < 0.001, respectively).

Testosterone versus ECG Parameters, Symptoms or SCN5A Mutation in Brugada Males

Baseline electrocardiographic data of the 48 Brugada males are shown in Table 1. No significant correlations were observed between testosterone level and ECG parameters, including ST amplitude (r = –0.123, P = 0.406) and QTc interval (r = –0206, P = 0.160), in the 48 Brugada males. There was no significant difference in testosterone level between 32 symptomatic and 16 asymptomatic Brugada males (649 ± 185 vs 593 ± 157 ng/dL: P = 0.298). No significant difference was observed in testosterone level between 43 Brugada males with spontaneous Type 1 ST-segment elevation and five Brugada males with sodium channel blocker-induced Type 1 ST-segment elevation (624 ± 171 vs 688 ± 230 ng/dL: P = 0.448). Testosterone level was also no different between seven Brugada males with *SCN5A* mutation and 41 Brugada males without *SCN5A* mutation (700 ± 198 vs 619 ± 172 ng/dL: P = 0.261).

Follow-Up

Arrhythmic events occurred in nine (19%) of 48 Brugada males during average follow-up periods of 41 ± 2 months after blood sampling for the present study (Table 1). In more detail, arrhythmic events appeared in eight (38%) of 21 Brugada males with a history of aborted cardiac arrest or VF, in one (9%) of 11 Brugada males with syncope alone, but did not appear in any (0%) of 16 asymptomatic Brugada males.

Discussion

The major findings of the present study were: (1) Brugada males had significantly higher testosterone level, serum sodium, potassium, and chloride level, and significantly lower BMI, BF%, and BW than those in control males by univariate analysis, even after adjusting for age, exercise, stress, current smoking, and medications related to hypertension, diabetes and hyperlipidemia. (2) Testosterone level was inversely correlated with the BMI, BF%, and BW in both Brugada males and control males, even after adjusting for the confounding variables. (3) Conditional logistic regression models analysis showed strong positive association between Brugada syndrome and higher testosterone level (hypertestosteronemia) and strong inverse association between Brugada syndrome and BMI.

Testosterone in Brugada Phenotype and Male Predominance

For the past decade, numerous clinical, experimental, and molecular genetic studies have elucidated Brugada syndrome as a distinct clinical entity.[1-5,17] However, several problems remain unresolved, such as genetic heterogeneity, ethnic difference, and gender difference.[7] Di Diego and Antzelevitch recently suggested the cellular basis for male predominance in Brugada syndrome by using arterially perfused canine right ventricular wedge preparations.[9] Transient outward current (I_{to})-mediated phase 1 AP notch was larger in male dogs than in female dogs in the right ventricular epicardium, but not in the left ventricular epicardium, responsible for the male predominance in the Brugada phenotype. Recent clinical studies suggested that male hormone testosterone might be attributable to gender difference of the prevalence in this

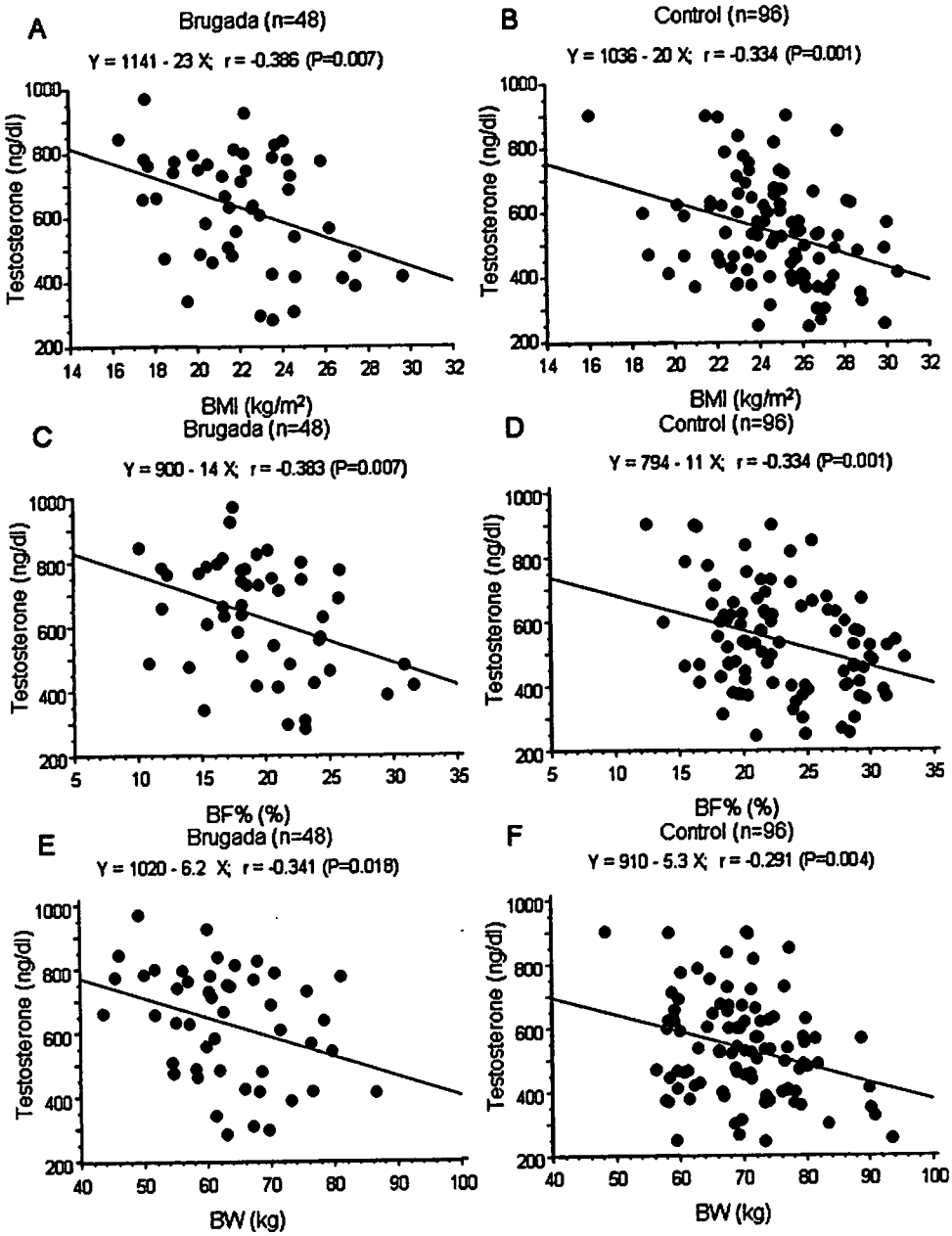

Figure 1. *Correlation between testosterone level and visceral fat parameters; body mass index (BMI) (A and B), body fat percentage (BF%) (C and D), and body weight (BW) (E and F) in the 48 Brugada males and the 96 age-matched control males. Testosterone level was inversely correlated with the BMI, BF%, or BW in both Brugada males and control males.*

syndrome. Matsuo et al. reported two cases of asymptomatic Brugada syndrome in whom typical coved ST-segment elevation disappeared following orchiectomy as therapy for prostate cancer,[21] indicating that testosterone may contribute to the Brugada phenotype in these two cases. Several experimental studies reported that testosterone increased outward potassium currents, such as the rapidly activating component (I_{Kr})[10,11] and the slowly activating component (I_{Ks})[12] of the delayed rectifier potassium current, and the inward rectifier potassium current (I_{K1}),[11] or decreased inward L-type calcium current (I_{Ca-L}).[12] Since the maintenance of the AP dome is determined by the fine balance of currents active at the end of phase 1 of the AP (principally I_{to} and I_{Ca-L}),[22,23] any agents that increase outward currents or decrease inward currents can increase the magnitude of the AP notch, leading to loss of the AP dome (all-or-none repolarization) in the epicardium, but not in the endocardium, contributing to a significant voltage gradient across the ventricular wall during ventricular activation, thus augmenting ST-segment elevation, the Brugada phenotype.[24] Therefore, testosterone would be expected to accentuate the Brugada phenotype. In the present study, males with Brugada syndrome had significantly higher testosterone level than age-matched control males, even after adjusting for age, exercise, stress, current smoking, and medication (hypertension, diabetes, and hyperlipidemia), which may affect the testosterone level. Moreover, conditional logistic regression models analysis showed strong positive association between Brugada syndrome and higher testosterone level (OR: 3.11). Our data suggest a significant role of testosterone, male hormone, in the Brugada phenotype. The

TABLE 5
Odds Ratios of Presence of Hypertestosteronemia and Confounding Risk Factors for Brugada Syndrome in Males

Variable	Odd Ratio	95% Confidence Interval	P Value
Hypertestosteronemia	3.11	1.22–7.93	0.017
Age	0.99	0.95–1.03	0.637
BMI	0.72	0.61–0.85	<0.001
Exercise	1.57	0.87–2.83	0.135
Stress	0.69	0.35–1.35	0.277
Current smoking	0.71	0.26–1.90	0.493
Hypertension	3.12	0.85–11.45	0.087
Diabetes	0.13	0.01–1.27	0.079
Hyperlipidemia	2.14	0.44–10.49	0.348

Hypertestosteronemia was defined as serum testosterone levels ≥ 700 ng/dL.

data also indicate that the male predominance in the Brugada phenotype is at least in part due to testosterone, which is present only in males.

Lower Visceral Fat May Be a Predictor for Brugada Phenotype

Matsuo et al. recently reported in their epidemiologic study that cases with the Brugada-type ECG had significantly lower BMI than that in control subjects.[16] Similarly, in the present study, males with Brugada syndrome had significantly lower visceral fat parameters, BMI, BF%, and BW than those in age-matched control males, even after adjusting for several confounding variables. Moreover, conditional logistic regression models analysis showed strong inverse association between Brugada syndrome and BMI (OR: 0.72). All of the visceral fat parameters were inversely correlated with testosterone level in both Brugada and control males, even after adjusting for the confounding variables. It has been well demonstrated that testosterone level in obese males is decreased compared to normal males of similar age.[13] Tsai et al. reported that lower baseline total testosterone level independently predicted an increase in visceral fat in the Japanese-American male cohort for 7.5 years.[15] Reversely, Marin et al. reported that testosterone treatment of middle-aged abdominally obese males was followed by a decrease of visceral fat mass measured by computerized tomography.[14] These data suggest that primarily higher level of testosterone in Brugada males compared to that in control males may result in lower visceral fat in Brugada males, which would be an "innocent bystander" sign of Brugada phenotype. In reverse, if primary lower visceral fat (body weight loss) would result in higher testosterone level, the weight loss could be a trigger for Brugada phenotype, just like fever is.[25] It is noteworthy that the visceral fat parameters at the clinical cardiac events (VF or syncope) in the 32 symptomatic Brugada males were significantly lower than those at the time of blood sampling for this study. This indicates that testosterone level is expected to be additively higher at the clinical cardiac events, which may contribute to spontaneous episodes of VF or syncope.

Other Hormonal Levels and Serum Electrolytes

Estradiol, female hormone, is reported to reduce the expression of Kv4.3 channels, which are important molecular components of I_{to} currents.[26] However, in contrast to testosterone, other sex hormonal levels including estradiol were not different between the Brugada males and the control males in the present study. Although thyroid hormones are also demonstrated to alter membrane currents, such as I_{to} and I_{Ca-L},[27,28] no significant differences were observed in the thyroid hormonal levels between the two groups in the present study.

On the other hand, serum sodium, potassium, and chloride levels were all significantly higher in the Brugada males than in the control males, even after adjusting for several confounding variables. Recently, many agents and conditions that cause an outward shift in current activity at the end of phase 1 AP have been known to unmask ST-segment elevation, as found in the Brugada syndrome, leading to the acquired form of this disorder.[4,29] Electrolyte abnormalities, such as hyperkalemia, are reported to amplify ST-segment elevation like that in Brugada syndrome.[30] The lower visceral fat found in the Brugada males is expected to decrease serum level of insulin, leptine, a novel adipocyte-derived hormone, or ghrelin, a novel growth hormone-releasing peptide, suppressing β-adrenergic receptor or plasma norepinephrine level, resulting in an increase of serum potassium level.[31,32] Further studies including measurement of levels of insulin, leptine, and ghrelin will be required to elucidate the precise mechanism.

Study Limitations

Although the testosterone level was significantly higher in the Brugada males than in the control males, no statistically significant correlations were observed between the testosterone level and the ST amplitude in the Brugada males. The degree of the ST-segment elevation is variable between Brugada patients because it is influenced by several factors other than sex hormonal levels or electrolytes levels, such as basal autonomic tone, presence of *SCN5A* mutation, or probably intrinsic current density of I_{to}, etc., in the right ventricular epicardial cells. The threshold of ST-segment elevation for spontaneous induction of VF also varies between Brugada patients. Therefore, the Brugada phenotype, such as ST-segment elevation or spontaneous induction of VF, may correlate with the testosterone level day to day individually (intra-personally) in each Brugada male, but may not correlate among the pooled data obtained from many Brugada males, probably due to inter-person difference of the ST-segment elevation.

There were no significant differences in testosterone level between symptomatic and asymptomatic Brugada males, between Brugada males with spontaneous ST elevation and those with sodium channel blocker-induced ST elevation, or between Brugada males with and without *SCN5A* mutation, all of which are probably due to a relatively small number of Brugada males in the present study. Further evaluation with increasing number of Brugada males will be required.

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CNP infusion attenuates cardiac dysfunction and inflammation in myocarditis

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Abstract

Myocarditis is an acute inflammatory disease of the myocardium for which there is currently no specific therapy. We investigated the therapeutic potential of C-type natriuretic peptide (CNP) in acute experimental autoimmune myocarditis. One week after injection of porcine myosin into male Lewis rats, CNP (0.05 μg/kg/min) was continuously administered for 2 weeks. CNP infusion significantly increased maximum dP/dt, decreased left ventricular end-diastolic pressure, and improved fractional shortening compared with vehicle administration. In vehicle-treated hearts, severe necrosis and marked infiltration of CD68-positive inflammatory cells were observed. Myocardial and serum levels of monocyte chemoattractant protein-1 were elevated in myocarditis. However, these changes were attenuated by CNP infusion. In addition, treatment with CNP significantly increased myocardial capillary density. Guanylyl cyclase-B, a receptor for CNP, was expressed in myocarditic heart, and cyclic guanosine monophosphate was elevated by CNP infusion. In conclusion, CNP infusion attenuated cardiac function in acute myocarditis through anti-inflammatory and angiogenic effects.

Keywords: Angiogenesis; Inflammation; MCP-1; Myocarditis; Natriuretic peptides

Acute myocarditis is a non-ischemic heart disease characterized by myocardial inflammation. Acute myocarditis is associated with rapidly progressive heart failure, arrhythmias, and sudden death [1]. Immunomodulatory therapies such as immunoglobulin and interferon are regarded as promising for myocarditis [2,3]; however, the efficacy of those treatments still remains controversial [3,4]. Other treatment options are restricted to supportive care for heart failure or arrhythmias. The lack of specific treatment and the potential severity of the illness underlie the importance of novel and effective therapeutic strategies for myocarditis.

There are three main natriuretic peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), all of which signal through natriuretic receptors and cyclic guanosine monophosphate (cGMP) signaling pathways. ANP and BNP are predominantly secreted from cardiac myocytes. They have anti-hypertrophic effects on cardiac myocytes in an autocrine manner and also have inhibitory effects on collagen synthesis of cardiac fibroblasts in a paracrine manner, and thus have suppressive effects on cardiac remodeling. Cardioprotective effects of ANP and BNP have already been demonstrated, and they are used clinically for the treatment of heart failure. On the other hand, CNP, originally identified in the porcine brain [5], is predominantly

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expressed in vascular endothelial cells and plays a role in the local regulation of vascular tone and remodeling [6]. However, the potential therapeutic effects of CNP in heart disease are not well understood.

Recently, it has been shown that CNP is also synthesized in cardiac fibroblasts and inhibits collagen synthesis of cardiac fibroblasts more potently than ANP and BNP [7]. In addition, CNP proved to have more potent anti-hypertrophic effects than ANP in cultured cardiac myocytes [8]. More recently, infusion of CNP has been shown to improve cardiac function after myocardial infarction through anti-fibrotic and anti-hypertrophic effects [9]. These findings indicate the therapeutic potential of CNP in heart disease. However, it remains unknown whether CNP infusion improves acute myocarditis leading to severe heart failure. In the present study, cardiac myosin purified from pig hearts was injected into rats, and autoimmune myocarditis was induced [10].

Thus, the purposes of this study were (1) to investigate whether infusion of CNP improves cardiac function in a rat model of acute myocarditis, and (2) to investigate the mechanisms responsible for the effect of CNP on the myocarditic heart.

Materials and methods

Model of acute myocarditis. We produced a rat model of acute myocarditis by injecting pig cardiac myosin. In brief, purified myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [11]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl, mixed with an equal volume of complete Freund's adjuvant containing 11 mg/ml of Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg) and 0.2 ml of the antigen-adjuvant emulsion was injected into the foot pads.

CNP preparation and treatment. CNP was diluted in PBS with 5% glucose and administered via an ALZET mini-osmotic pump (DURECT Corporation, Cupertino, CA, USA) inserted subcutaneously, which discharged CNP at a rate of 0.05 μg/kg/min for the duration of 14 days beginning 1 week after myosin injection.

Experimental groups. Rats with sham operation or those with acute myocarditis were treated with vehicle or CNP. Fifty-four male 10-week-old Lewis rats (Japan SLC, Hamamatsu, Japan) were randomly placed into four groups and received the following treatments: (1) sham rats given vehicle ($n = 12$), (2) sham rats given CNP ($n = 12$), (3) myocarditis rats given vehicle ($n = 15$), and (4) myocarditis rats given CNP ($n = 15$).

Echocardiography. Echocardiography was performed at day 21 post-myosin injection. Rats were anesthetized with sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). Left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), anterior wall thickness (AWT), and posterior wall thickness (PWT) were measured, and taken as an average of three beats. Fractional shortening (%FS) was calculated as follows;

$$\%FS = (LVDd - LVDs)/LVDd \times 100$$

Hemodynamic study. Hemodynamic measurements were taken at day 21 post-myosin injection. A 1.5F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery (Millar Instruments, Houston, TX, USA). Heart rate was also monitored with electrocardiogram. As indexes of hemodynamics, heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum dP/dt, and minimum dP/dt were used. Anesthesia was maintained with sodium pentobarbital, and the above mentioned indexes were recorded simultaneously during spontaneous ventilation after an equilibration period of a minimum of 20 min.

Histopathology. The heart was excised above the origin of the great vessels, and the heart and body weights were recorded. A midventricular portion of the heart was fixed with formalin and embedded in paraffin, and 4-μm sections were cut and stained with either hematoxylin and eosin (H&E) or Masson's trichrome stain, or subjected to immunohistochemical staining. H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) for the characterization of myocardial injury and inflammation, without knowledge of the experimental groups, on the following scale: (0) no or questionable presence, (1) limited focal distribution, (2 and 3) intermediate severity, and (4) coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue.

Immunohistochemistry. Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Sections were incubated with DakoCytomation protein block, then with anti-von Willebrand factor (vWF) (DakoCytomation, Glostrup, Denmark), CD68 (DakoCytomation), or monocyte chemoattractant protein-1 (MCP-1) (BD Biosciences, San Jose, CA, USA) antibodies, followed by sequential incubations with HRP-linked rabbit anti-mouse IgG (DakoCytomation). The reaction products were visualized using 0.5% diaminobenzidine and 0.03% hydrogen peroxide. Sections were counterstained with hematoxylin. The numbers of vWF-stained capillaries and CD68-stained cells were determined in ten randomly selected fields (vWF; 400×, CD68; 200×).

Enzyme-linked immunosorbent assay (ELISA). Serum MCP-1 level on day 21 post-myosin injection was measured using a Rat MCP-1 ELISA Kit (Biosource International, Camarillo, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of guanylyl cyclase-B (GC-B) mRNA, a receptor for CNP, was examined by RT-PCR. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and myocarditis rats given vehicle ($n = 5$ in each group). Total RNA was extracted from heart with RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed (PCR Amplification Kit, Takara, Shiga, Japan). The complementary DNA was amplified by the PCR using specific primers for GC-B or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers for GC-B were as follows [12]: sense primer 5′-AACGGGCG CATTGTGTATATCTGCGGC-3′ and antisense primer 5′-TTATCA CAGGATGGGTCGTCCAAGTCA-3′. For GAPDH, the primers were as follows: sense primer 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and antisense primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

Radioimmunoassay. To investigate whether subcutaneous administration of CNP has a biological activity in heart, we measured myocardial level of cGMP. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and those given CNP ($n = 10$ in each groups). Myocardial level of cGMP was measured with a radioimmunoassay kit (cGMP assay kit; YAMASA Co., Chiba, Japan).

Statistical analysis. Data were presented as means ± SEM. Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Differences were considered significant at $P < 0.05$.

Results

Improvement in cardiac function by CNP treatment

Myocarditis rats given vehicle had two deaths 19 and 21 days after myosin injection, respectively, whereas those treated with CNP showed no mortality. At 3 weeks post-myosin injection, Myocarditis rats given vehicle showed decreased maximum dP/dt and minimum dP/dt, and

increased LVEDP compared with the sham rats (Fig. 1A–C), indicating the presence of acute heart failure in this model. Such parameters subsequently returned to baseline with CNP treatment. On echocardiography, rats with myocarditis showed an increase in LVDd and a significant reduction in %FS (Fig. 1D–F). CNP infusion significantly improved %FS in myocarditis rats. Myocarditic hearts showed significantly increased heart weight to body weight ratio, which was reduced by CNP treatment (Table 1). MAP was significantly decreased in myocarditis rats, and the decrease was significantly attenuated by CNP treatment. CNP did not significantly influence cardiac function in sham rats.

Attenuation of inflammatory cell infiltration by CNP treatment

Histological examination showed that myocardial necrosis and tissue granulation as well as inflammation and edema were markedly increased in our model of acute myocarditis (Fig. 2A and B). CNP administration significantly attenuated necrotic changes observed in myocarditis rats. CNP-treated hearts exhibited a consistent tendency for a reduction of tissue granulation, inflammation and edema, on blinded histological grading by a cardiovascular pathologist (H.I-U.) as compared to vehicle-treated hearts. Although, CNP is known to have potent anti-fibrotic activ-

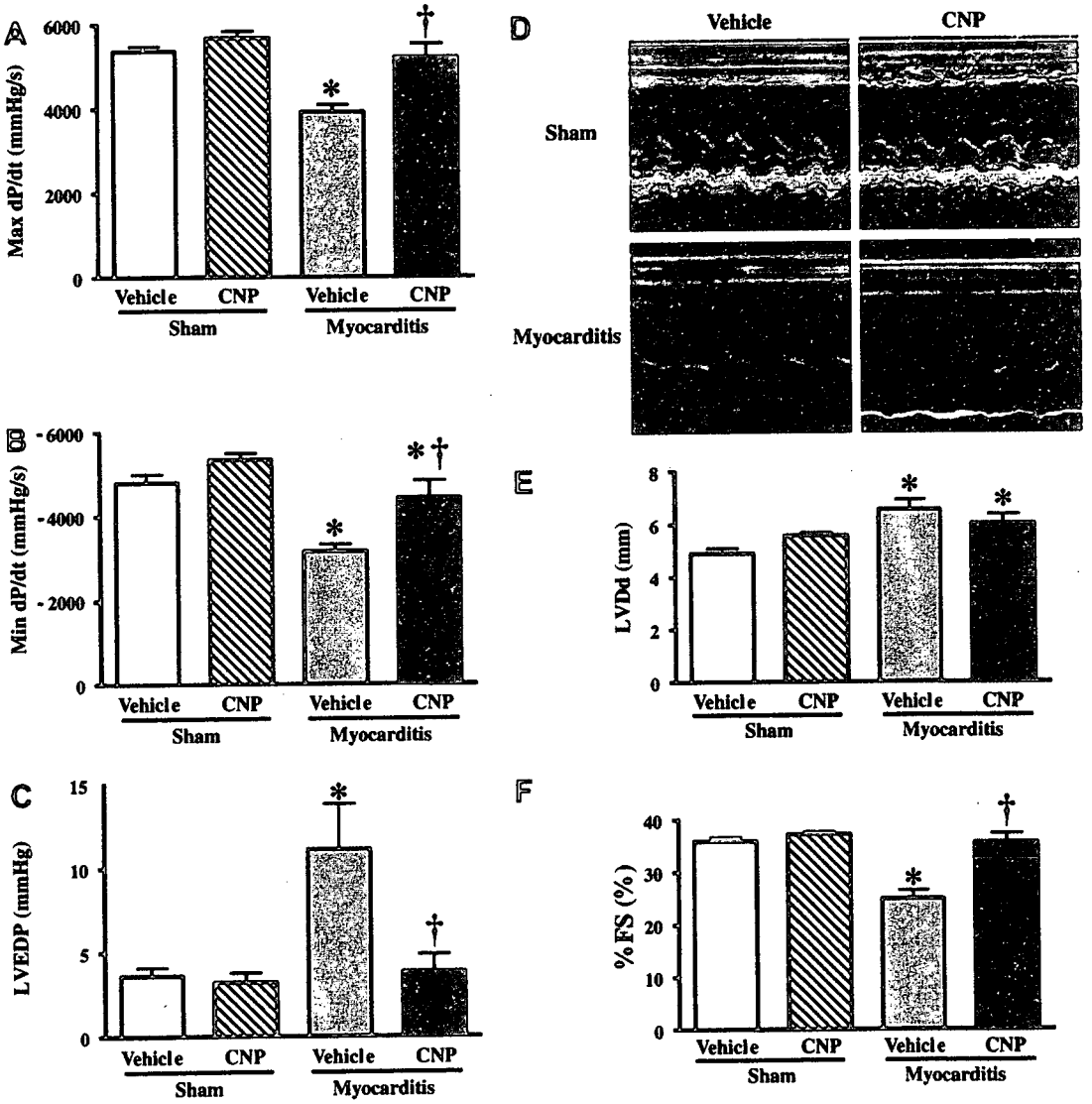

Fig. 1. Effects of CNP administration on hemodynamic parameters in acute myocarditis. (A) Maximum dP/dt (Max dP/dt), (B) minimum dP/dt (Min dP/dt), and (C) left ventricular end-diastolic pressure (LVEDP) were measured in sham rats given vehicle, sham rats given CNP, myocarditis rats given vehicle, and myocarditis rats given CNP. (D) Representative echocardiographic images showing wall thickening and poor myocardial movement in rats with myocarditis and improved cardiac contractility in those treated with CNP. (E,F) CNP administration in myocarditis tended to attenuate the increase in left ventricular diastolic dimension (LVDd) and significantly improved fractional shortening (%FS). Values are means ± SEM. $^{*}P < 0.05$ *vs.* Sham-Vehicle, $^{\dagger}P < 0.05$ *vs.* Myocarditis-Vehicle.

Table 1
Physiological and catheter-based parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 12$)	CNP ($n = 13$)
BW (g)	282 ± 2	282 ± 3	208 ± 4*	224 ± 3*,†
HW/BW (g/kg)	2.86 ± 0.04	2.81 ± 0.03	6.33 ± 0.25*	5.29 ± 0.20*,†
HR (bpm)	428 ± 7	422 ± 5	367 ± 13*	431 ± 13*,†
MAP (mmHg)	111 ± 4	103 ± 4	87 ± 3*	105 ± 5†
LVSP (mm Hg)	124 ± 5	125 ± 4	104 ± 4*	123 ± 6†

BW, body weight; HW/BW, heart weight to body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure. Data are means ± SEM.

* $P < 0.05$ *vs.* Sham-Vehicle.

† $P < 0.05$ *vs.* Myocarditis-Vehicle.

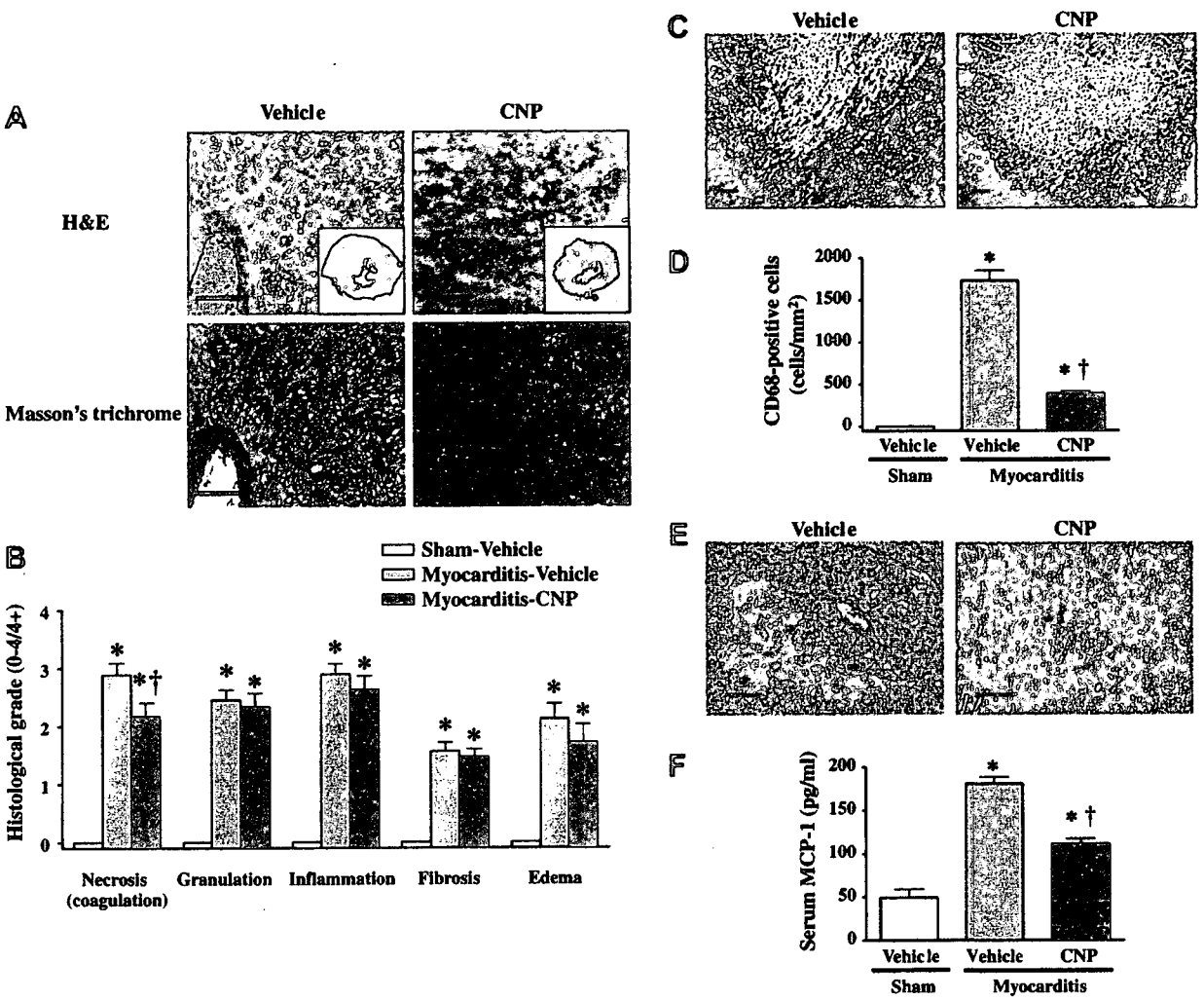

Fig. 2. Histological analysis of the myocardium. (A) Representative myocardial sections showed markedly decreased inflammation and tissue necrosis (H&E) and a comparable degree of early fibrosis (Masson's trichrome) in CNP-treated hearts as compared to myocarditic hearts. Insets are transverse section of the left ventricular section (H&E). (B) Semiquantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were lower in myocarditis rats treated with CNP as compared to untreated rats. Sham rats exhibited no measurable pathological change. Scale bar is 100 μm. (C) Representative myocardial sections immunohistochemically-stained for CD68 demonstrated a marked decrease in CD68-positive cells, including giant cells, in CNP-treated hearts as compared to vehicle-treated hearts. Scale bar is 100 μm. (D) Semi-quantitative counts of CD68-positive cells demonstrate a significant reduction in CNP-treated hearts. (E) Representative MCP-1-stained myocardial sections from rats with acute myocarditis. Scale bar is 100 μm. (F) Serum level of MCP-1 measured by ELISA. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

ity [9], myocardial fibrosis was not significantly attenuated by CNP infusion (Fig. 2B), probably due to the acute nature of this experiment (Table 2).

Notably, marked histiocytic infiltration was demonstrated by the presence of CD68-positive cells, including multinucleated giant cells, in rats with myocarditis, and this

Table 2
Echocardiographic parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 9$)	CNP ($n = 11$)
LVDd (mm)	5.6 ± 0.1	5.6 ± 0.1	6.5 ± 0.4*	6.0 ± 0.3
LVDs (mm)	3.6 ± 0.1	3.5 ± 0.1	4.9 ± 0.4*	3.9 ± 0.2†
%FS (%)	36 ± 1	37 ± 1	25 ± 2*	36 ± 2†
AWT diastole (mm)	1.9 ± 0.1	1.9 ± 0.1	3.1 ± 0.2*	2.8 ± 0.2*
PWT diastole (mm)	1.9 ± 0.1	1.8 ± 0.1	3.5 ± 0.3*	3.6 ± 0.4*

LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; %FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. Data are means ± SEM.
* $P < 0.05$ *vs.* Sham-Vehicle.
† $P < 0.05$ *vs.* Myocarditis-Vehicle.

was significantly attenuated by CNP treatment (Fig. 2C and D). In myocarditis, there was an increase in MCP-1 expression localized to the vascular endothelium and also in myocytes surrounding and adjacent to areas of inflammatory infiltration (Fig. 2E). The hearts in myocarditis rats treated with CNP showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in myocarditis rats, whereas it was significantly decreased in those treated with CNP (Fig. 2F).

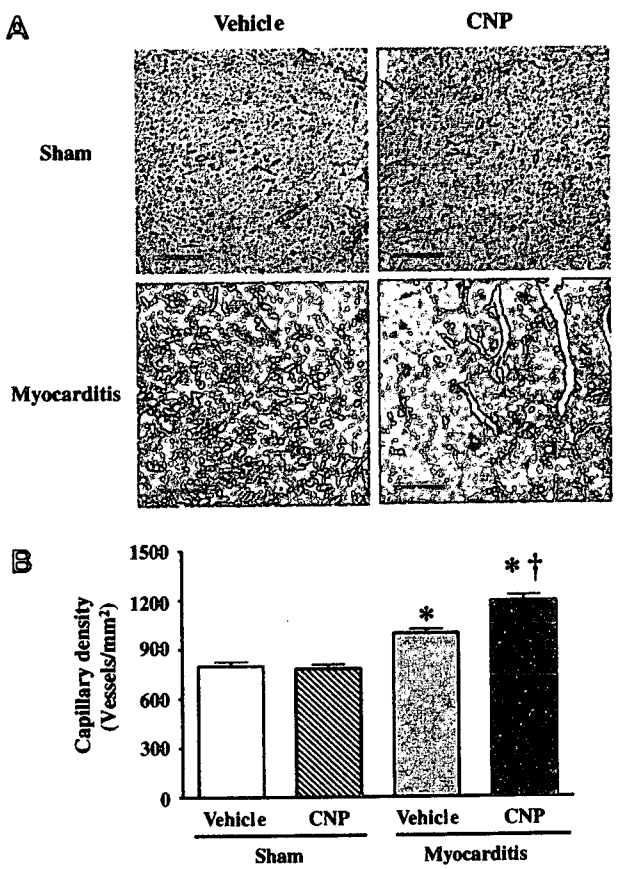

Fig. 3. Angiogenic potential of CNP in acute myocarditis. (A) Representative myocardial sections immunohistochemically-stained for vWF exhibit increased microvasculature in control myocarditic hearts, which was more marked in CNP-treated hearts. (B) Capillary density measured in 10 random representative high powered fields showed a significant increase in rats with acute myocarditis and a further increase in those treated with CNP. Scale bar is 100 μm. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

Effect of CNP on angiogenesis

To determine the angiogenic effect of CNP treatment in the myocardium, immunohistochemical analysis of vWF was performed. Capillary density in the heart was increased in myocarditis, particularly in areas directly adjacent to tissue necrosis (Fig. 3). Notably, capillary density was increased over that in acute myocarditis alone. The clustering of relatively small vessels seen in CNP-treated myocarditic hearts was indicative of recent endothelial regeneration or angiogenesis. On the other hand, CNP did not significantly influence the capillary density in the sham rats.

Expression of GC-B and cGMP in myocardium

RT-PCR demonstrated that GC-B mRNA was expressed in myocarditic heart (Fig. 4A). Myocardial level

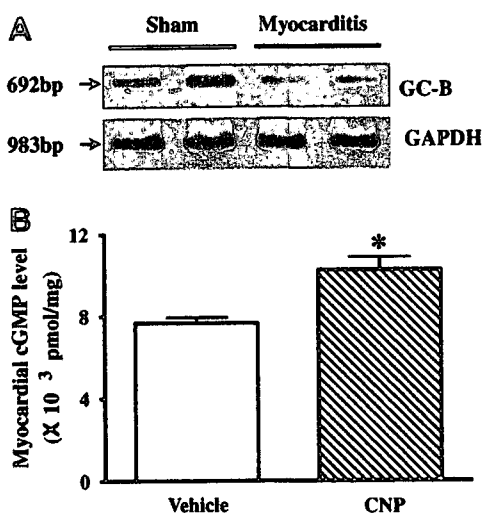

Fig. 4. Expression of GC-B and cGMP in the myocardium. (A) RT-PCR analysis of GC-B mRNA expression in myocarditic heart. (B) Myocardial level of cGMP measured by radioimmunoassay. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle.

of cGMP was significantly elevated by the subcutaneous infusion of CNP (Fig. 4B). These results suggest that subcutaneous infusion of CNP (0.05 μg/kg/min) has biological effects on myocaditic heart.

Discussion

In this study, we focused on the therapeutic potential of CNP in the acute phase of autoimmune myocarditis. We showed that CNP treatment 1 week following myosin injection but prior to the development of myocarditis (1) preserved cardiac function after acute myocarditis, (2) significantly decreased tissue necrosis, inflammatory cell infiltration and MCP-1 expression in the heart and serum, and led to a tendency for reduced overall inflammation, granulation and edema, and (3) stimulated angiogenesis in myocarditic hearts beyond the baseline increase seen in myocarditis.

The rat model of myosin-induced experimental autoimmune myocarditis closely resembles human giant cell myocarditis [11]. This disease model is triphasic, consisting of antigen priming phase from days 0–14, an autoimmune response phase from days 14–21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [13]. In our experiments, CNP was administered 1 week following myosin injection, corresponding to an early time point in the disease process. In the present study, CNP treatment significantly improved cardiac function as determined by increased maximum dP/dt and %FS as well as decreased LVEDP in rats with acute myocarditis. Importantly, earlier studies have shown that the vasodilator effect of CNP is much less potent than that of ANP [5,9,14,15]. ANP and BNP cause vasodilatation and hypotension, thus limiting their use as treatment for patients with severe heart failure. Because the effects of CNP on blood pressure and HR were very small, CNP treatment is considered as a safer alternative for the treatment of those patients [16]. Indeed, administration of CNP did not decrease arterial pressure, but sustained its biological activity.

Our data showed a significant decrease in inflammatory cell infiltration and a consistent tendency for decreased overall inflammation and edema by CNP treatment. In addition, CNP infusion decreased MCP-1 expression in the heart and serum. A previous study has demonstrated that CNP reduces macrophage infiltration by inhibition of MCP-1 expression [17]. These findings suggest that attenuation of inflammatory cell infiltration by CNP may be regulated, at least in part, by suppression of MCP-1 expression.

Recently, it was shown that CNP has anti-fibrotic properties in pulmonary fibrosis and myocardial infarction, through a cGMP-dependent pathway [9,18]. However, since the present experiments were carried out in the acute phase of myocarditis, the anti-fibrotic effect of CNP in the myocarditic heart was not clear. Further studies are necessary to examine the anti-fibrotic effects of CNP in the chronic phase of myocarditis.

We demonstrated that CNP induces endothelial regeneration beyond the increase seen in myocarditis. In rabbit balloon injury, infectious vein graft disease and hindlimb ischemia models, CNP overexpression stimulated reendothelialization via a cGMP-dependent pathway [19]. Endothelial dysfunction including microvascular constriction and microaneurysm formation has previously been reported in myocarditis [20], as well as chronic impairment of endothelial-dependent vasorelaxation of coronary resistance vessels in myocarditis [21]. Thus, the endothelial regenerative effects of CNP are likely to be beneficial in preventing myocardial injury and dysfunction in acute myocarditis. In this study, capillary density in normal heart was not increased by CNP infusion. In inflammatory tissue, it is speculated that CNP does not have an effect on initiation of angiogenesis, but promote angiogenesis at the phase of forming mature blood vessels. However, a further examination is necessary to elucidate the mechanisms of angiogenic effects.

Considering the importance of natriuretic peptides, such as ANP and BNP, in the diagnosis and treatment of cardiovascular diseases, there is currently much interest in the role of CNP. Since, CNP has marked cardioprotective effects including anti-inflammatory and angiogenic effects, and has less vasodilator effects, which enable the use of this peptide in patients with hypotension, this molecule may have great potential for the treatment of patients with acute myocarditis.

In summary, administration of CNP ameliorated cardiac dysfunction in a rat model of acute myocarditis. The beneficial effects may be due, at least in part, to anti-inflammatory and angiogenic effects. This work expands the beneficial effects of CNP to acute myocarditis, and increases our understanding of the role of natriuretic peptides in severe heart failure.

Acknowledgments

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