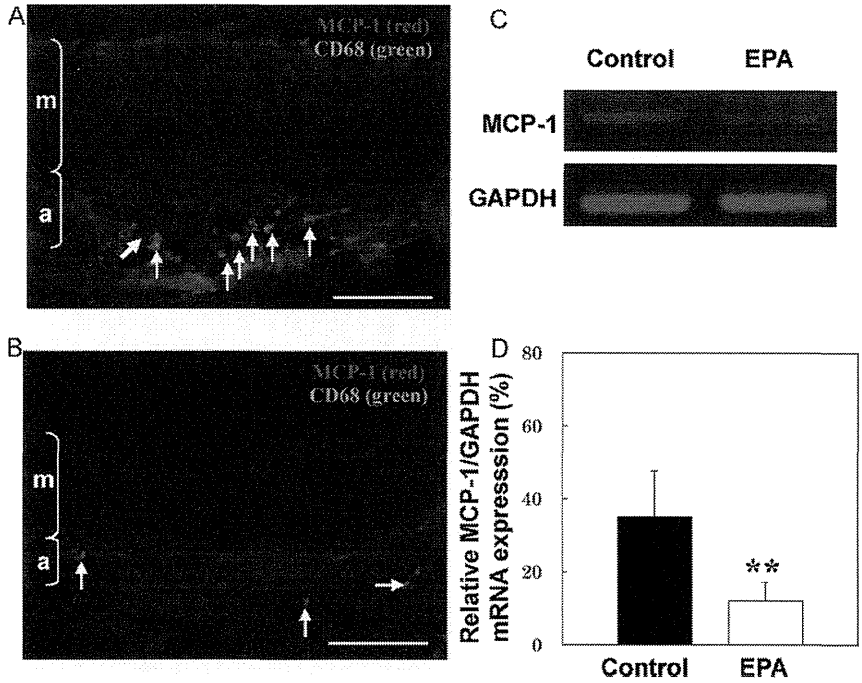

Fig. 5. MCP-1 expression in the calcified aorta. (A and B) Representative immunostaining for MCP-1 (red) and CD68 (green) of common iliac artery of rats in the control group (A) and EPA group (B). Macrophage is shown by an arrow (↑). Scale bar, 100 μm. (C) Representative MCP-1 mRNA expression assessed by RT-PCR. (D) MCP-1 mRNA expressions evaluated densitometrically and normalized to GAPDH ($n=6$ per group). m, media; a, adventitia. $^{**}p<0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In conclusion, we showed that EPA reduces AMC in warfarin-treated rats. Multiple effects of EPA may be beneficial for AMC caused by various mechanisms.

Conflict of interest

The authors report no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.12.001.

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Clinical Investigations

Risk of Sudden Death in End-Stage Hypertrophic Cardiomyopathy

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ABSTRACT

Background: It remains unclear whether end-stage hypertrophic cardiomyopathy (HCM) is associated with as high a rate of sudden death as occurs among HCM patients with preserved left ventricular (LV) systolic function. The purpose of this study was to evaluate the incidence of sudden death among patients with end-stage HCM and to identify high-risk end-stage patients.

Methods and Results: A total of 490 consecutive patients with HCM, who were diagnosed and followed-up at our hospital, were analyzed retrospectively. End-stage HCM was defined by an LV ejection fraction <50% on echocardiography during follow-up. Among the 490 HCM patients, 43 patients (8.8%) were diagnosed as having end-stage HCM during a mean follow-up period of 12 ± 7 years after the initial diagnosis. During a mean follow-up period of 5 ± 3 years after progression to end-stage HCM, sudden death occurred in 21 of 43 patients (47%). Cox proportional hazards analysis identified syncope as an independent predictor of sudden death (hazard ratio = 6.15; 95% confidence interval, 2.40-15.75; $P < .001$).

Conclusions: This study demonstrated that patients with end-stage HCM have a high incidence of sudden death. Therefore, it is suggested that an aggressive therapeutic strategy to counter sudden death should be considered for patients with end-stage HCM. (*J Cardiac Fail 2011;17:459–464*)

Key Words: Heart failure, epidemiology, prognosis, syncope.

In most patients with hypertrophic cardiomyopathy (HCM), left ventricular (LV) systolic function is normal or supernormal, whereas abnormalities of LV relaxation and filling are identified in approximately 80%.[1] A distinctive terminal phase of this disease, resembling the morphological and functional features of dilated cardiomyopathy, is observed in 2.4 to 15% of patients with symptomatic HCM.[2–8] Recently, it was reported that a common feature of end-stage HCM is progressive systolic dysfunction, which is superimposed on pre-existing diastolic dysfunction and is accompanied by LV dilatation, ventricular wall thinning, or both in approximately 50% of patients.[9] Development of such LV systolic dysfunction in HCM patients has serious clinical implications because it is associated with high rates of heart failure death and sudden death.[9] Although sudden death has been recognized as a prominent and devastating consequence of HCM, it has been unclear whether sudden death is as common in end-stage disease as it is among HCM patients with preserved LV systolic function.[1,10–12] Furthermore, the risk factors for sudden death in HCM patients who progress to end-stage disease are not fully understood. Accordingly, the purpose of this study was to evaluate the incidence of sudden death among patients with end-stage HCM and to identify a high-risk subgroup of end-stage patients.

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See page 464 for disclosure information.

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Methods

Study Population and Diagnostic Criteria

A total of 490 consecutive patients with HCM (334 with nonobstructive HCM and 156 with obstructive HCM; the mean age at

diagnosis: 46 ± 15 years) were diagnosed and followed-up at Tokyo Women's Medical University Hospital (Tokyo, Japan), which is a referral center, between 1980 and 2005.[13] We retrospectively analyzed the incidence and clinical characteristics of end-stage HCM during follow-up after the initial diagnosis of HCM. We also analyzed cardiovascular mortality in patients with end-stage HCM during follow-up after the diagnosis of end-stage disease. A diagnosis of HCM was based on echocardiographic evidence of LV hypertrophy (wall thickness >15 mm) in the absence of any other cardiac or systemic disease capable of producing similar hypertrophy.[1,8] Exclusion criteria were a history of hypertension or coronary artery disease and severe congestive heart failure. We also excluded patients who were diagnosed as having end-stage HCM at their first evaluation because it is difficult to distinguish end-stage HCM from idiopathic dilated cardiomyopathy in such patients.[14] The present study was conducted in accordance with the Declaration of Helsinki.

Definitions

End-stage HCM was defined by the detection of LV cavity enlargement and an LV ejection fraction (LVEF) <50% on echocardiography without a history of surgical or ablative septal reduction therapy during follow-up of HCM.[4–7] LV outflow tract obstruction from systolic anterior motion of the mitral valve with septal contact was considered to be present when the peak instantaneous gradient was estimated to be at least 30 mm Hg by continuous-wave Doppler echocardiography under resting conditions.[1,8] The diagnostic criteria for apical hypertrophy included the detection of asymmetric LV hypertrophy (predominantly confined to the LV apex) and an apical wall thickness ≥15 mm on 2-dimensional echocardiography or cardiovascular magnetic resonance imaging.[15]

Cardiovascular morbidity was defined as stroke, heart failure, and syncope. Cardiovascular death was defined as sudden death, heart failure–related death, stroke-related death, and heart transplantation. Sudden death included both sudden cardiac death and nonfatal cardiac arrest, including appropriate implantable cardioverter-defibrillator (ICD) interventions, such as antitachycardia pacing therapy and shock therapy. Heart failure–related death included support by an LV assist device and heart transplantation.[16] Nonsustained ventricular tachycardia (VT) was defined as one or more runs of 3 or more consecutive ventricular extrasystoles at a rate of >120 minutes and lasting for <30 seconds.[8]

Echocardiography

Echocardiographic studies were performed using commercially available ultrasound equipment. Complete M-mode, 2-dimensional, and Doppler studies were performed, in the left lateral decubitus or supine position via the standard parasternal, apical, and subcostal approaches. The severity and distribution of LV hypertrophy was assessed in the short-axis view by dividing the LV wall into 4 segments (anterior septum, posterior septum, anterolateral wall, and posterior wall) at the level of the mitral valve and also at the papillary muscles. Maximal LV wall thickness was defined as the greatest thickness in any single segment. LV outflow tract obstruction, caused by systolic anterior motion of the mitral valve with septal contact, was considered to be present when the estimated peak instantaneous gradient was ≥30 mm Hg during based on continuous-wave Doppler echocardiography under basal (resting) conditions. The ejection fraction was calculated from 2-dimensional echocardiographic images by the modified Simpson's formula.

Statistical Analysis

Analyses were performed with SAS ver. 9.1 software (SAS Institute, Cary, NC). Data are presented as the mean ± SD or as frequencies. Student's *t*-test was employed to compare with respect to normally distributed continuous variables between 2 groups, whereas the chi-square test was used to compare nominal variables. Cumulative event-free curves were drawn by the Kaplan-Meier method, and differences between curves were determined with the log-rank test. Multivariate Cox proportional hazards analysis with stepwise selection of variables was applied to evaluate the influence of syncope at the time of diagnosing end-stage HCM on total death, heart failure death, and sudden death. The influence of profile, interaction, and collinearity in the models were examined by regression diagnostic analysis. Two-tailed *P* values <.05 were considered to indicate a statistically significant difference.

Results

Incidence and Baseline Characteristics

Among 490 patients with HCM, 43 (8.8%) patients showed progression to end-stage disease during a mean follow-up period of 12 ± 7 years after the initial diagnosis of HCM. The annual incidence of end-stage HCM was 0.73%. Among these 43 patients, 28 patients (65%) had an LVEF <50% and left ventricular diastolic dimension (LVDD) ≥55 mm, whereas the remaining 15 patients (35%) only had an LVEF <50%. The patients with end-stage HCM were aged 44 ± 15 years (range, 6 to 68 years) at the initial diagnosis of HCM and 56 ± 12 years (range, 22 to 78 years) at the diagnosis of end-stage HCM (Table 1). Among these 43 patients, the incidence of a familial HCM and a family history of sudden death was 37% and 28%, respectively. The incidence of syncope at the diagnosis of end-stage HCM was 23% (10/43 patients). At the initial diagnosis of HCM, 25 patients (58%) had asymmetric septal hypertrophy, 11 (26%) had concentric hypertrophy, and 7 (16%) had apical hypertrophy. Interestingly, no patient with obstructive hypertrophy showed progression to end-stage HCM during the present study. At the diagnosis of end-stage HCM, mean LVDD and LVEF were 59 ± 6 mm and 36 ± 10%, respectively. Among the 43 patients with end-stage HCM, 30 patients (70%) were being treated with β-blockers, 35 patients (81%) with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, and 26 patients (60%) with spironolactone. Also, amiodarone was administered to 21 patients (49%) during follow-up. During a mean follow-up of 5 ± 3 years after the diagnosis of end-stage HCM, 13 (30%) patients developed persistent atrial fibrillation, and 25 patients (58%) had nonsustained VT. At the time of diagnosing end-stage HCM, an ICD had been implanted in 13 patients (30%), with the indication for implantation being primary prevention in 8 patients and secondary prevention in 5 patients. Also, after progression to end-stage HCM, 3 patients received ICDs for the primary prevention of sudden death.

Cardiovascular Mortality

During a mean follow-up period of 5 ± 3 years after progression to end-stage HCM, overall-related death occurred

Table 1. Baseline Characteristics of End-stage HCM Patients

Variables	End-stage HCM (n = 43)
Age at initial diagnosis of HCM, y	44 ± 15
Age at diagnosis of end-stage HCM, y	56 ± 12
Male	34 (79%)
Family history of HCM	16 (37%)
Family history of sudden death	12 (28%)
HCM-related morbidity at diagnosis of end-stage HCM	
Stroke	11 (26%)
Heart failure	10 (23%)
Syncope	10 (23%)
Chronic atrial fibrillation at diagnosis of end-stage HCM	13 (30%)
Nonsustained VT at diagnosis of end-stage HCM	25 (58%)
VF at diagnosis of end-stage HCM	1 (3%)
Echocardiographic findings at initial diagnosis of HCM	
LVDD, mm	46 ± 5
LVEF, %	61 ± 8
End-systolic left atrial diameter, mm	38 ± 8
Maximum LV wall thickness, mm	20 ± 4
Intraventricular septal thickness, mm	19 ± 4
Posterior wall thickness, mm	13 ± 3
Echocardiographic findings at diagnosis of end-stage HCM	
LVDD, mm	59 ± 6
LVEF, %	36 ± 10
End-systolic left atrial diameter, mm	44 ± 9
Intraventricular septal thickness, mm	11 ± 3
Posterior wall thickness, mm	10 ± 2
Subtype at initial diagnosis of HCM	
Asymmetric septal hypertrophy	25 (58%)
Concentric	11 (26%)
Apical	7 (16%)
Outflow tract or mid-cavity obstruction	0 (0%)
Therapy at diagnosis of end-stage HCM	
β–blocker	30 (70%)
ACE-I or ARB	35 (81%)
Spironolactone	26 (60%)
Amiodarone	21 (49%)
ICD	13 (30%)

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; HCM, hypertrophic cardiomyopathy; ICD, implanted cardioverter defibrillator; LVDD, left ventricular diastolic dimension; LVEF, left ventricular ejection fraction; VF, ventricular fibrillation; VT, ventricular tachycardia.

Data are the mean ± SD or n (percentage).

in 30 (70%) of 43 patients (Fig. 1A). Among these 30 patients, 28 patients died of sudden death (n = 21) or progressive heart failure–related death (n = 7). As a result, the annual cardiovascular death rate, annual sudden death rate, and annual heart failure–related death rate was 13%, 10%, and 3%, respectively (Fig. 1B). Among the 21 patients who died suddenly, 1 patient had nonfatal cardiac arrest, 10 patients had sudden cardiac death, and 10 of the 11 patients receiving ICDs for primary prevention had appropriate ICD interventions. One of the 7 patients with heart failure–related death had undergone heart transplantation. After the diagnosis of end-stage HCM, 3 patients died of noncardiovascular death. The time interval between the diagnosis of end-stage HCM and the occurrence of sudden death or heart failure–related death was 3.6 ± 3.0 and 5.8 ± 3.7 years, respectively.

Clinical Characteristics According to Cardiovascular Mortality

To evaluate possible predictors of sudden death in patients with end-stage HCM, we divided the subjects into 3 clinical subgroups according to cardiovascular mortality, which were an alive group (n = 13), a heart failure death group (n = 7), and a sudden death group (n = 21) (Table 2). There was no significant difference in the incidence of familial HCM or a family history of sudden death among these 3 clinical subgroups. Also, there was no significant difference of nonsustained VT or the maximum LV wall thickness at the diagnosis of HCM among these subgroups. However, there was a significant difference in the incidence of syncope at the diagnosis of HCM among the three subgroups. In the present study, 9 of the 10 (90%) patients with syncope at the time of diagnosis of end-stage HCM died of sudden death during a mean follow-up period of 5 ± 3 years. Figure 2 shows the probability of sudden death for end-stage patients with or without syncope at the diagnosis of end-stage HCM. The presence of syncope was associated with a significantly increased risk of sudden death ($P < .001$). Investigation of the medications being used at the time of diagnosing end-stage HCM revealed no significant differences for angiotensin-converting enzyme inhibitors/angiotensin receptor blocker and spironolactone among the 3 clinical subgroups. However, use of β-blockers was significantly more common among surviving patients than patients with heart failure–related death (85% vs. 29%, $P = .012$). Also, the use of amiodarone by patients with sudden death was significantly more common than by surviving patients (67% vs. 31%, $P = .042$). On the other hand, there was no significant difference in the rate of ICD implantation among the 3 clinical subgroups. In 7 (54%) of the 13 patients with ICDs, use of the device was appropriate. The annual rate of ICD activation was 4.6%. At the diagnosis of end-stage HCM, echocardiographic parameters like LVDD and LVEF showed no significant differences among the 3 clinical subgroups (Table 2).

Predictors of Sudden Death in Patients with End-stage HCM

To evaluate possible predictors of sudden death for the HCM patients who progressed to end-stage HCM, we performed multivariate Cox proportional hazards analysis with stepwise selection of variables. In this multivariate model, established major primary prevention risk factors for sudden death were entered (positive family history, maximum LV wall thickness ≥30 mm at the initial diagnosis of HCM, nonsustained VT at the diagnosis of end-stage HCM, and unexplained syncope at the diagnosis of end-stage HCM). As a result, the presence of syncope at the diagnosis of end-stage disease was identified as an independent predictor of sudden death (hazard ratio = 6.15; 95% confidence interval, 2.40-15.75; $P < .001$).

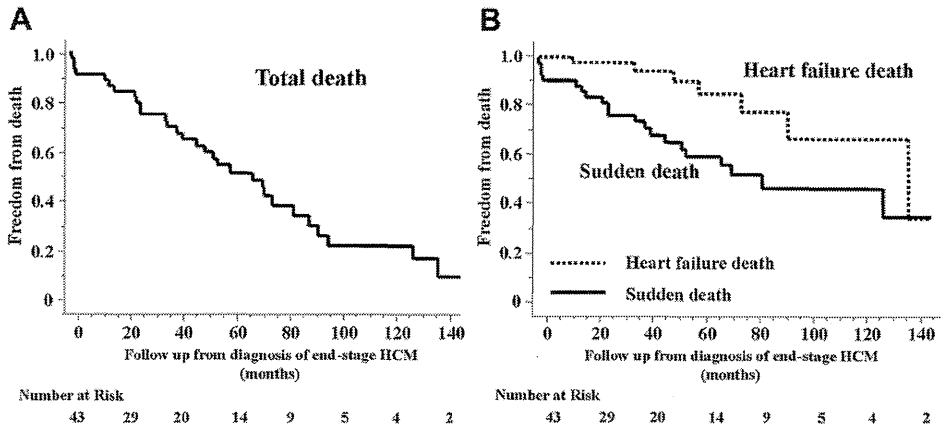

Fig. 1. Probability of cardiovascular mortality after the diagnosis of end-stage hypertrophic cardiomyopathy (HCM): (A) Total death, (B) Heart failure death and sudden death.

Discussion

This study demonstrated that 43 (8.8%) of 490 HCM patients progressed to end-stage disease during a mean follow-up period of 12 ± 7 years after the initial diagnosis of HCM, with the annual incidence of progression being 0.73%. The prevalence of cardiovascular mortality, especially sudden death, was also very high during a mean follow-up of 5 ± 3 years after the diagnosis of end-stage HCM. Furthermore, the presence of syncope at the time of diagnosing end-stage HCM diagnosis was an independent risk factor for sudden death among patients with end-stage HCM. Therefore, it is suggested that an aggressive therapeutic strategy for sudden death should be considered in patients with end-stage HCM, particularly those patients with syncope at the diagnosis of end-stage HCM.

Previous studies have shown that the prevalence of end-stage HCM was approximately 2.4 to 15%, with an annual incidence of 0.5 to 1.0%.[2–8] This study demonstrated that 43 (8.8%) of 490 HCM patients progressed to end-stage disease during a mean follow-up of 12 years, with the annual incidence being 0.73%. Regarding the long-term prognosis of patients with end-stage HCM, the present study showed that cardiovascular death occurred in 28 (65%) of 43 patients during a mean follow-up period of 5 ± 3 years after the diagnosis of end-stage disease and the annual cardiovascular mortality rate was 13%, suggesting a very poor long-term prognosis. Harris et al reported that 66% of end-stage HCM patients died of either heart failure or sudden cardiac death or needed an ICD or heart transplantation, and the annual adverse event rate was 11%.[6] The adverse event rate in our study was similar to previous data.[4–6] In

Table 2. Clinical Characteristics According to the Outcome

	Alive	Heart Failure Death	Sudden Death	
	(n = 13)	(n = 7)	(n = 21)	*P* Value
Age at initial diagnosis of HCM, y	43 ± 16	40 ± 19	45 ± 12	.384
Age at diagnosis of end-stage HCM, y	56 ± 11	54 ± 18	57 ± 11	.253
Male	10 (77%)	4 (57%)	18 (86%)	.284
Family history of HCM	4 (31%)	2 (29%)	10 (48%)	.512
Family history of sudden death	3 (23%)	2 (29%)	7 (33%)	.815
HCM-related morbidity at diagnosis of end-stage HCM				
Stroke	2 (15%)	4 (57%)	4 (19%)	.084
Heart failure	3 (23%)	2 (29%)	5 (24%)	.959
Syncope	1 (8%)	0 (0%)	9 (43%)	.017
Chronic atrial fibrillation at diagnosis of end-stage HCM	5 (38%)	4 (57%)	4 (19%)	.141
Nonsustained VT at diagnosis of end-stage HCM	7 (54%)	2 (29%)	16 (76%)	.067
LVDD at diagnosis of end-stage HCM, mm	57 ± 5	59 ± 3	60 ± 7	.221
LVEF at diagnosis of end-stage HCM, %	38 ± 9	35 ± 11	34 ± 10	.318
Therapy at diagnosis of end-stage HCM				
β—blocker	11 (85%)	2 (29%)	16 (76%)	.023
ACE-I or ARB	11 (85%)	5 (71%)	18 (86%)	.672
Spironolactone	8 (62%)	3 (43%)	15 (71%)	.391
Amiodarone	4 (31%)	2 (29%)	14 (67%)	.063
ICD	4 (31%)	1 (14%)	11 (52%)	.154

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; HCM, hypertrophic cardiomyopathy; ICD, implanted cardioverter defibrillator; LVDD, left ventricular diastolic dimension; LVEF, left ventricular ejection fraction; VF, ventricular fibrillation; VT, ventricular tachycardia.
Data are the mean ± SD or n (percentage).

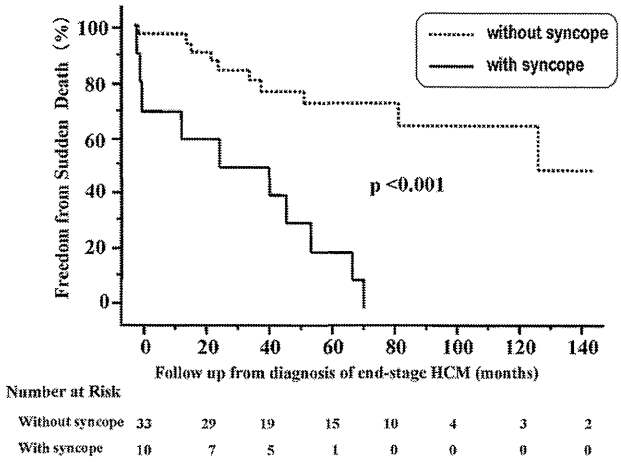

Fig. 2. Probability of sudden death in patients with end-stage hypertrophic cardiomyopathy (HCM) patients with or without syncope at the diagnosis of end-stage disease.

general, HCM is the most common cause of sudden death among young people, including competitive athletes, and sudden death is often the initial clinical manifestation of HCM.[1,8,17–20] Recently, Yacoub et al suggested that development of systolic dysfunction in HCM patients was associated with a high rate of heart failure–related mortality and sudden death.[9] Moreover, the onset of end-stage HCM represents a risk factor for sudden cardiac death and a potential indication for prophylactic ICD interventions.[9] However, it has been unclear whether sudden death was as common among HCM patients with end-stage disease as it is among HCM patients with preserved LV systolic function. In the present study, sudden death and heart failure–related death were observed in 78% and 22% of end-stage HCM patients, respectively, and the prevalence of sudden death was far higher than in previous reports.[4–6] The reason for this difference may be that the interval from diagnosis of end-stage HCM to heart transplantation was relatively short (less than 3 years) in previous reports, suggesting that early heart transplantation could prevent sudden death in patients with end-stage HCM from Western countries.[6] Another possible reason was that the use of β-blockers, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, and spironolactone showed obvious differences between previous reports and the present study. In particular, our data revealed higher administration rates of β-blockers and angiotensin-converting enzyme inhibitors/angiotensin receptor blockers among surviving patients compared with those who suffered from heart failure–related death. This is consistent with preliminary evidence that angiotensin-converting enzyme inhibitors may positively influence coronary microvascular dysfunction, which is associated with a higher incidence of end-stage HCM and a poor prognosis.[14] Moreover, the interval between the diagnosis of end-stage HCM diagnosis and sudden death or heart failure–related death was 3.6 ± 3.0 and 5.8 ± 3.7 years, respectively. This difference suggests that optimal medical therapy could prevent heart failure but not sudden death in patients who developed end-stage HCM. It has been reported that sudden death accounts for up to 50% of all deaths in patients with nonischemic dilated cardiomyopathy.[21] According to previous reports and our data, early detection of the end-stage phase could be regarded as another risk factor for sudden death in HCM patients, and prophylactic placement of an ICD in all end-stage HCM patients may be needed to prevent sudden death prior to heart transplantation.[6,17–19] However, further clinical research is needed to identify the optimal therapeutic strategy for end-stage HCM to prevent both heart failure death and sudden death in these patients.

Regarding risk factors for sudden death in end-stage HCM, our study showed that the presence of syncope at the diagnosis of end-stage disease was an independent predictor of sudden death by stepwise multivariate Cox proportional hazards analysis. Recently, it has been reported that syncope was a risk factor for sudden death in a large cohort of patients with HCM.[1,19,22,23] Therefore, end-stage HCM patients with syncope at the diagnosis of end-stage disease may have a substantially higher risk of sudden death than patients without syncope. However, further large-scale studies will be needed to confirm whether the presence of syncope is associated with sudden death in patients who progress to end-stage HCM.

The present study had several limitations. First, this study was retrospective nature and relatively small size. Second, the subjects of this investigation were limited to patients who were diagnosed as having HCM and followed-up at our hospital, which was the referral center, suggesting that center referral bias could influence our data on the clinical spectrum of end-stage HCM.[17,24] Third, in the present study, we defined sudden death as including appropriate ICD interventions. However, the definition of sudden death in patients with HCM remains unsettled.[25] Further clinical trials will be required to determine whether appropriate ICD intervention is a useful surrogate end point for sudden death in patients with HCM. Fourth, we could not evaluate the pathophysiological correlates of the progressive morphologic and functional changes identified in this study. Fifth, genetic analysis was not performed in our end-stage HCM patients, so we could not assess the correlations between genotype and phenotype.

In conclusion, this study demonstrated that patients with end-stage HCM have a high incidence of sudden death. Furthermore, end-stage HCM patients with syncope at the diagnosis of end-stage disease may have a substantially higher risk of sudden death than patients without syncope. Therefore, when HCM is evolving into the terminal phase, aggressive therapy for both heart failure death and sudden death should be considered. However, a further large-scale clinical study is needed to confirm whether the presence of syncope is associated with sudden death and to identify the optimal therapeutic strategy for HCM patients who progress to end-stage disease.

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Disclosures

None.

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Creation of mouse embryonic stem cell-derived cardiac cell sheets

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ABSTRACT

Research on heart tissue engineering is an exciting and promising area. Although we previously developed bioengineered myocardium using cell sheet-based tissue engineering technologies, the issue of appropriate cell sources remained unresolved. In the present study, we created cell sheets of mouse embryonic stem (ES) cell-derived cardiomyocytes after expansion in three-dimensional stirred suspension cultures. Serial treatment of the suspension cultures with noggin and granulocyte colony-stimulating factor significantly increased the number of cardiomyocytes by more than fourfold compared with untreated cultures. After drug selection for ES cells expressing the neomycin-resistance gene under the control of the α-myosin heavy chain promoter, almost all of the cells showed spontaneous beating and expressed several cardiac contractive proteins in a fine striated pattern. When ES-derived cardiomyocytes alone were seeded onto temperature-responsive culture dishes, cell sheets were not created, whereas cocultures with cardiac fibroblasts promoted cell sheet formation. The cardiomyocytes in the cell sheets beat spontaneously and synchronously, and expressed connexin 43 at the edge of adjacent cardiomyocytes. Furthermore, when the extracellular action potential was recorded, unidirectional action potential propagation was observed. The present findings suggest that stirred suspension cultures with appropriate growth factors are capable of producing cardiomyocytes effectively and easily, and that ES-derived cardiac cell sheets may be a promising tool for the development of bioengineered myocardium.

1. Introduction

Since heart failure is still a major cause of mortality in many developed countries, myocardial regeneration is considered to be a promising therapy for severe heart failure. Despite the principal concept of cardiac regeneration that transplanted stem cells differentiate into cardiomyocytes and replace the injured myocardium with newly cardiomyocytes, several recent studies have shown that paracrine effects mediated by growth factors secreted from the transplanted cells and the host tissue are the major mechanisms for adult stem cell transplantation-mediated improvement of cardiac function [1,2]. Since the adult human heart contains approximately 4×10^9 cardiomyocytes in the left ventricle [3], which suggests that it is an appropriate cell source for collecting huge amounts of cardiomyocytes, cell delivery methods and cell organization may be prerequisites for heart tissue reconstruction. Recently bioengineered myocardium has been proposed as one of the solutions for creating heart tissue [4]. We previously developed an original scaffold-free tissue engineering technology, designated "cell sheet-based tissue engineering", using temperature-responsive culture dishes covalently grafted to the temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) [5]. Lowering the culture temperature promotes a rapid surface transition from hydrophobic to hydrophilic, which enables us to collect a viable monolayer cell sheet with full preservation of the cell–cell contacts and extracellular matrices [6]. We also developed a cell sheet-based bioengineered myocardial tissue [7–9] using neonatal rat cardiomyocytes. However, the development of methods for the use of stem cell-based cardiomyocytes may be requisite for creating cell sheets and bioengineered heart tissue, since recent induced pluripotent stem (iPS) cell technologies have enabled the creation of autologous cardiomyocytes for clinical translation [10,11]. Furthermore, the following uncertainties

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remain for the cardiac cell sheets: (1) the ability to obtain a suitable number of stem cell-derived cardiomyocytes; (2) the necessity of using non-cardiomyocytes; and (3) the actual electrophysiological functions of cell sheets of stem cell-derived cardiomyocytes.

Many studies have reported that embryonic stem (ES) cells can differentiate into cardiomyocytes through embryoid body (EB) formation [12,13], and these studies have usually used hanging-drop methods. However, such methods may not be suitable for large-scale cultures, and three-dimensional stirred suspension cultures have therefore been used to create EBs and induce cardiac differentiation of ES cells [14,15]. Although suspension cultures are useful in terms of scaling up, it may be difficult to control the EB size, which may hinder cardiac differentiation [16]. On the other hand, robust studies have reported that several growth factors are related to heart development [17] and that certain growth factors such as noggin and granulocyte colony-stimulating factor (GCSF) promote cardiac differentiation of ES cells [18] and induce cardiomyocyte proliferation [19]. Accordingly, suspension cultures with the use of appropriate growth factors may promote cardiac differentiation, thereby enabling the collection of more cardiomyocytes.

The aims of this study were to establish easy and effective methods for collecting cardiomyocytes from mouse ES cells and creating cardiac cell sheets, and to elucidate the electrophysiological functions of ES-derived cardiac cell sheets.

2. Materials and methods

2.1. Animals and reagents

Wild-type C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University. The following antibodies were used for immunocytochemistry: mouse monoclonal anti-sarcomeric α-actinin (Sigma-Aldrich, St. Louis, MO); mouse monoclonal anti-β-myosin heavy chain and mouse monoclonal anti-NG2 (Millipore, Temecula, CA); mouse monoclonal anti-cardiac troponin T (cTnT) (Thermo Scientific, Rockford, IL); guinea pig monoclonal anti-vimentin (Progen, Heidelberg, Germany); rabbit polyclonal anti-connexin 43 (Zymed Laboratories, South San Francisco, CA); and rabbit polyclonal anti-von Willebrand factor (Dako, Tokyo, Japan). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Unless otherwise specified, all reagents were purchased from Sigma-Aldrich.

2.2. Mouse ES cell cultures

R1 ES cells ubiquitously expressing EYFP and the neomycin phosphotransferase gene under the control of the α-myosin heavy chain promoter, and a phosphoglycerate kinase gene in front of a hygromycin-resistance gene were maintained as described previously [20].

For cardiac differentiation, trypsinized ES cells were seeded at 5×10^4 cells/mL (total, 125 mL/flask) into spinner flasks (Integra Biosciences, Zizers, Switzerland) and cultured as described previously [20] with a few modifications. Briefly, ES cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) without leukemia inhibitory factor until day 10. On day 6, the volume of medium was scaled up to 250 mL/flask. On day 10, 400 μg/mL G418 was added until day 18. The differentiation and selection media were changed every day. For noggin treatment, the cells were cultured with noggin (150 ng/mL) from 3 days before to 1 day after starting the suspension culture. For GCSF treatment, the cells were cultured with GCSF (1 ng/mL) from day 6 to day 10 of the spinner flask culture. The cell number at each time point was measured after EB dissociation with 0.25% trypsin/EDTA.

2.3. Cell isolation

Cardiomyocytes and fibroblasts were obtained from the hearts of neonatal mice (1–2 days of age) as described previously [21]. Cardiac fibroblasts from passage 4 were used for the experiments. Immunocytochemical analyses revealed that >99% of the cardiac fibroblasts expressed vimentin, and did not express von Willebrand factor (endothelial cell marker; data not shown) or NG2 (pericyte marker; data not shown).

2.4. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and subjected to immunostaining as described previously [2]. Images of the stained samples were obtained by laser confocal microscopy (Carl Zeiss, Jena, Germany) or fluorescence microscopy (Nikon, Tokyo, Japan) with NIS-Elements software (Nikon).

2.5. Cell sheet preparation

Before seeding the cells, the surface of the temperature-responsive dishes (UpCell; CellSeed, Tokyo, Japan) was coated with FBS for 2 h. A mixed cell suspension of ES cell-derived cardiomyocytes and cardiac fibroblasts isolated from neonatal mouse hearts was plated onto each UpCell dish at 3.2×10^5 cells/cm^2, and the cells were cultured in DMEM supplemented with 15% FBS at 37 °C. After 4 days of culture, the cells were incubated at 20 °C. After 2 h, the cells spontaneously detached from the culture dish and floated in the medium as a monolayer cell sheet.

2.6. Electrophysiological analysis

The electrical activities of the cardiomyocyte sheets were obtained from the extracellular potentials measured by a multi-electrode array (MED) system (Alpha MED Sciences, Osaka, Japan) as described previously [9]. For monolayered cell sheet, 8 × 8 array prove (MED-515A: array size; 1.05 × 1.05 mm, distance between electrodes, 150 μm) and for partially overlaid cell sheets, cardiac sheet prove (MED-P5A15: electrode size; 50 μm, minimum distance between electrodes; 450 μm) were used.

2.7. Statistical analysis

Data are presented as means ± SD. The differences between groups were evaluated by analysis of variance followed by Bonferroni's correction. Values of $P < 0.05$ were considered to be statistically significant.

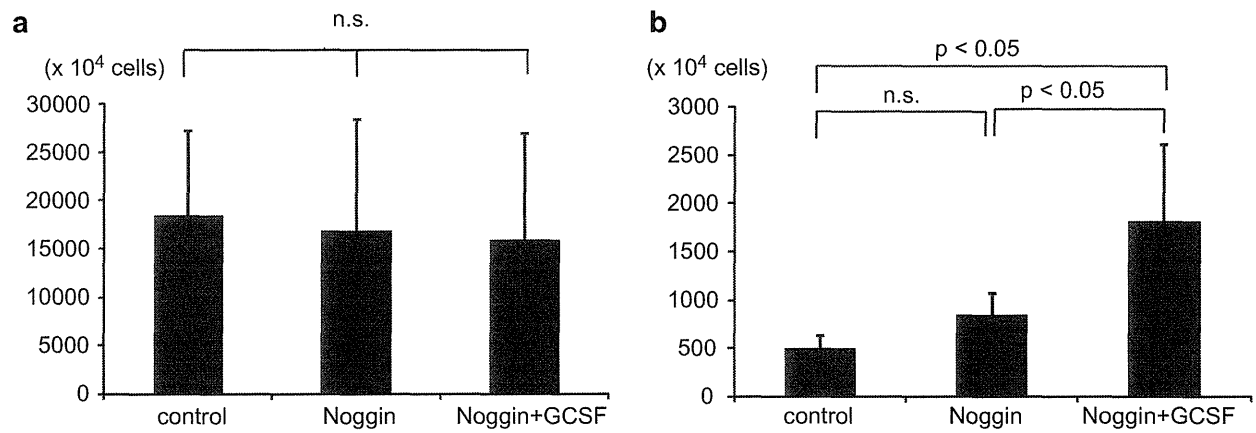

Fig. 1. Serial treatment with noggin and GCSF increases the number of cardiomyocytes in suspension cultures. The numbers of cells at 10 days (a) and 18 days (b) in suspension cultures ($n = 3$) are shown. The data are means ± SD.

Table 1
The total number of cells at day 0, 10 and 18 in each condition (n=3). The data are means ± SD.

	Day 0	Day 10	Day 18
Control (× 10^4 cells)	625	18350 ± 8888	491 ± 143
Noggin (× 10^4 cells)	625	16725 ± 11707	829 ± 243
Noggin + GCSF(× 10^4 cells)	625	15838 ± 11124	1800 ± 813

3. Results

3.1. Stirred suspension cultures for collecting ES-derived cardiomyocytes

Suspension cultures of mouse ES cells are considered to be promising methods for cell expansion and differentiation. When ES cells were seeded at 5 × 10^4 cells/mL in spinner flasks (6.25 × 10^6 cells/flask), we observed an approximately 30-fold increase in the cells forming EBs after 10 days in culture (Fig. 1a, Table 1). When the cells were treated with neomycin from day 10 to day 18, approximately 5 × 10^6 cells remained at day 18 (Fig. 1b,

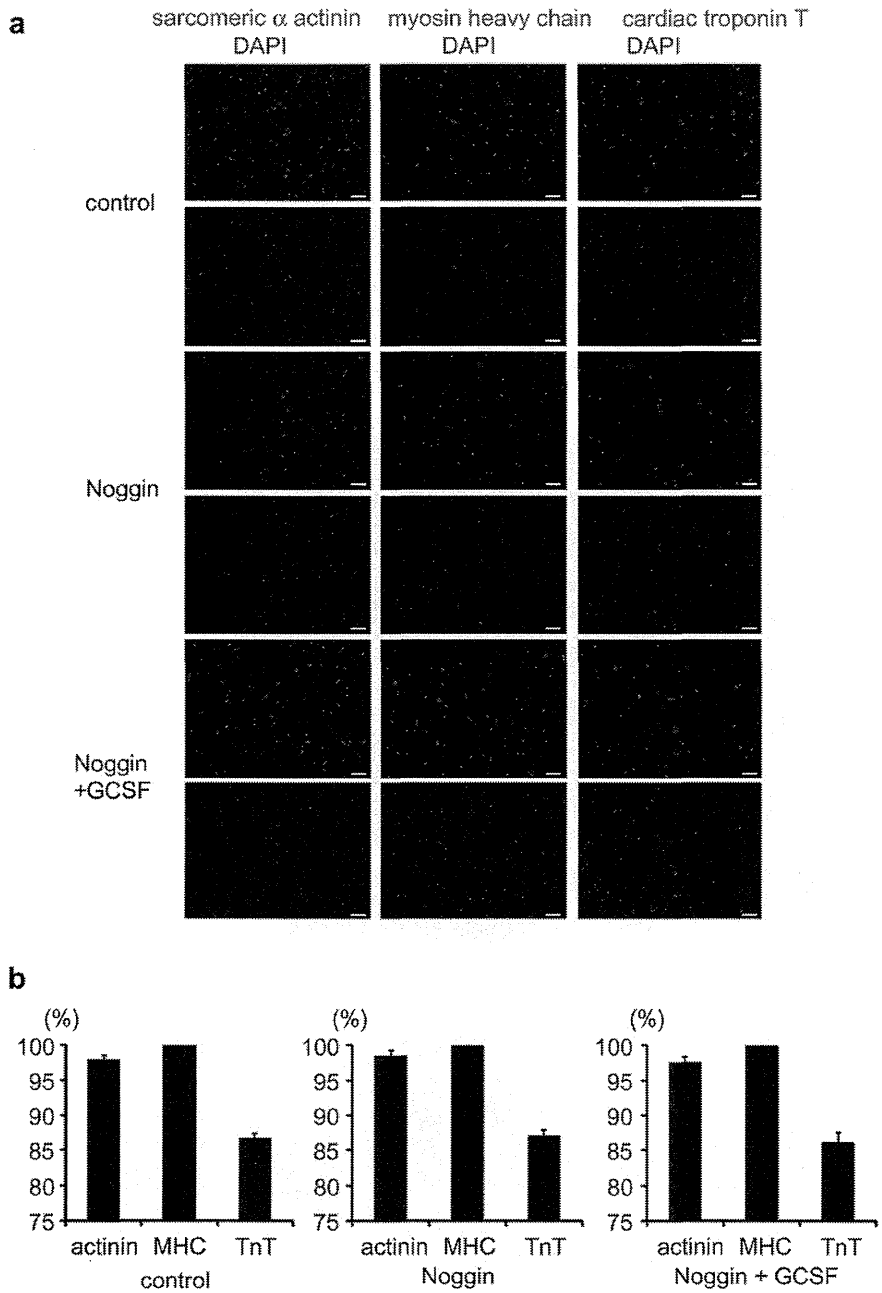

Fig. 2. Purity of cardiomyocytes after neomycin selection. (a) At 18 days after starting the culture in spinner flasks (at 8 days of culture with neomycin), the cells were dissociated and seeded onto 1% gelatin-coated 3.5-cm dishes. At 12 h after seeding, the cells were fixed and immunostained for sarcomeric-α actinin (left), myosin heavy chain (middle) and cTnT (right). The upper panels show images of the contractive proteins and the lower panels show images of DAPI nuclear staining. Scale bars, 100 μm. (b) The percentages of α-actinin (+), myosin heavy chain (+) and cTnT (+) cells were calculated and are shown in a graph (n = 3). The data are means ± SD.

Table 1). After dissociation with trypsin/EDTA, the cells were seeded onto gelatin-coated dishes. At 12 h after seeding, spontaneous beating was observed in almost all of the attached cells (Supplementary video 1). The purity of the cardiomyocytes in the remaining cells was examined by immunocytochemistry. As shown in Fig. 2, more than 99% of the cells were positive for myosin heavy chain, about 98% of the cells were positive for sarcomeric α-actinin and about 85% of the cells were positive for cTnT. These findings indicate that almost all of the remaining cells after neomycin selection in the suspension cultures might be cardiomyocytes, although the percentage of each cardiac contractive protein expression may vary probably according to the levels of maturation. Confocal microscopy observations revealed that the differentiated cardiac cells showed a fine striated pattern and that many cells had a single nucleus while some cells had double and triple nuclei (Fig. 3). These findings suggest that an 18-day culture period may promote not only cardiomyocyte differentiation but also cardiomyocyte maturation.

Supplementary video related to this article can be found at doi: 10.1016/j.biomaterials.2011.05.042.

Although we were able to collect many cardiomyocytes to a certain extent using the suspension culture system, better culture conditions should further increase the number of cardiomyocytes. Therefore, we tried to increase the number of cardiomyocytes in our suspension cultures by treatment with noggin [18] and/or GCSF [19]. The ES cells were treated with noggin from 3 days before to 1 day after starting the suspension cultures, and with GCSF from day 6 to day 10 in the suspension cultures. Even when ES cell cultures were treated with noggin and/or GCSF, the number of cells at day 10 in the suspension cultures did not differ compared with untreated cell cultures (Fig. 1a). However, at 8 days after neomycin selection (18 days after starting the culture), a significant increase in the number of remaining cells was observed in the cultures treated with noggin and GCSF (Fig. 1b, Table 1). Moreover, the remaining cells showed a similar expression pattern of cardiac contractive proteins to the untreated cells (Fig. 2), indicating that noggin and GCSF treatment may increase the number of cardiomyocytes. Conversely, noggin (Fig. 1b, Table 1) or GCSF (data not shown) alone did not significantly increase the number of cells after neomycin selection. Since it has been reported that transient early treatment with noggin (from 3 days before to 1 day after EB formation) induces the mesoendoderm [18] and that GCSF induces the proliferation of fetal cardiomyocytes and ES-derived cardiomyocytes [19], the sequential treatment with noggin and GCSF may strongly increase the number of cardiomyocytes in large-scale suspension cultures.

3.2. Cell sheet creation using ES cell-derived cardiomyocytes and cardiac fibroblasts

Next, we tried to create cardiac cell sheets using ES-derived cardiomyocytes and UpCell temperature-responsive culture dishes. According to our previous cell sheet experiments using neonatal rat cardiomyocytes [22], ES-derived cardiomyocytes were

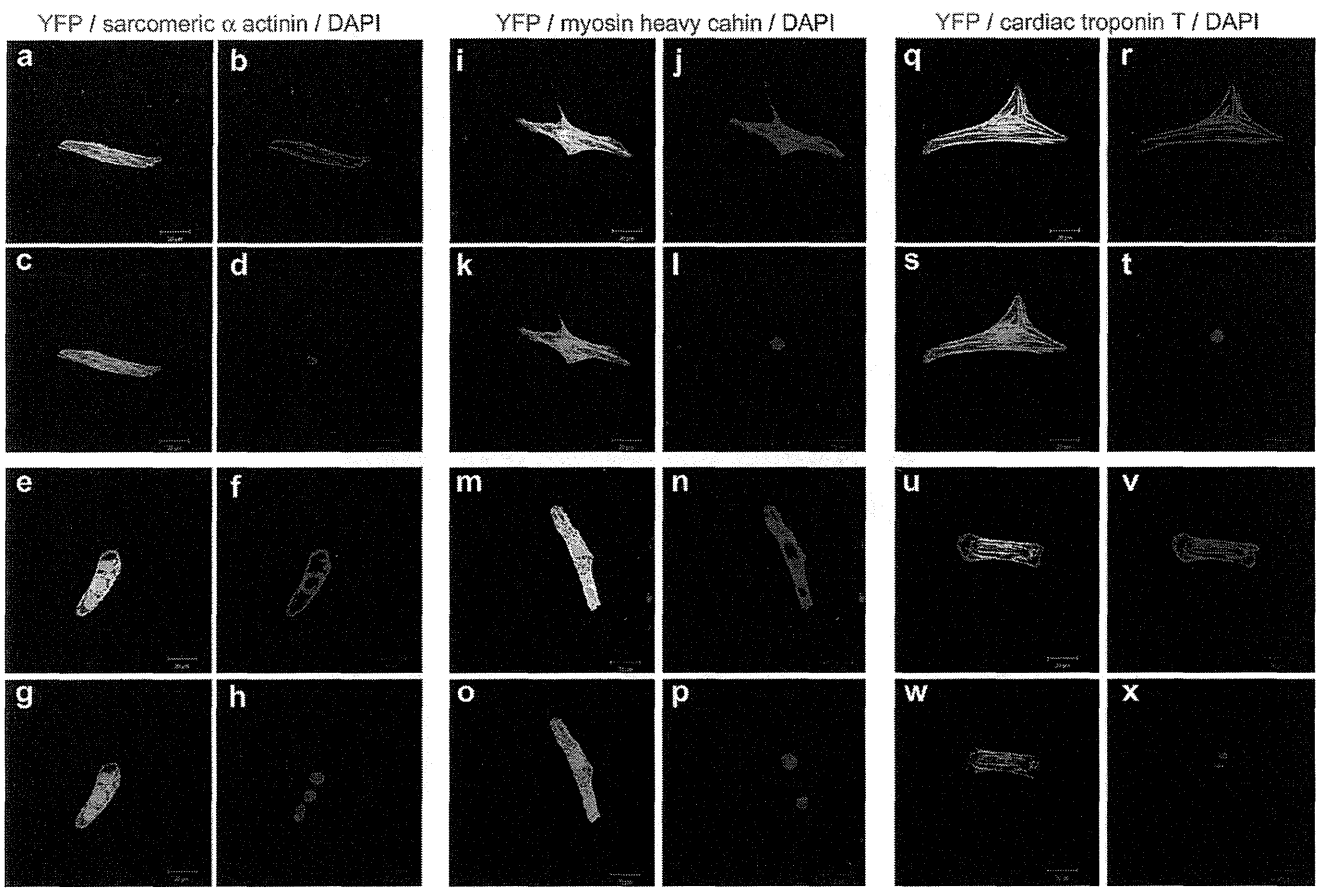

Fig. 3. Confocal microscopy images of the expression of cardiac contractive proteins in ES-derived cardiomyocytes after neomycin selection. Left panels: sarcomeric α-actinin; middle panels: myosin heavy chain; right panels: cTnT. Panels a–d, e–h, i–l, m–p, q–t and u–x show the same images, respectively. Panels a, e, i, q, m and u show merged images. Panels b, f, j, n, r and v show the expression of the contractive proteins (red). Panels c, g, k, o, s and w show YFP expression (green). Nuclei were stained with DAPI (blue in panels d, h, l, p, t and x). Scale bars, 25 μm.

seeded onto FBS-coated UpCell dishes at 3.2×10^5 cells/cm^2. When we checked the cell distribution on the dishes by fluorescence microscopy after 4 days in culture, many spontaneously beating YFP(+) cell aggregates were observed (Supplementary video 2). However, synchronous beating among the aggregates was not observed. When the cells were cultured in a 20 °C incubator, the aggregates detached from the culture dishes and no cell sheet formation was observed (Fig. 4a). Furthermore, even when the number of seeded cells was increased to 14.8×10^5 cells/cm^2 (four times the initial number of seeded cells) and the dishes were precoated with gelatin, fibronectin or laminin, the cell aggregates still detached from the culture dishes (data not shown), suggesting that the cell–cell interactions between cardiomyocytes may be insufficient to create cell sheets. To examine the cell components in the cell sheets, we created cell sheets using neonatal mouse cardiomyocytes seeded at 3.2×10^5 cells/cm^2. At 4 days after cell seeding, cell sheets were created upon culture in a 20 °C incubator. Immunocytochemical analyses showed the coexistence of cardiomyocytes and vimentin-positive cells in the cell sheets (Fig. 4b), suggesting that a certain level of non-cardiomyocytes that produce extracellular matrix may be necessary to create cell sheets. Therefore, we tried to create cell sheets by coculturing ES-derived cardiomyocytes and cardiac fibroblasts isolated from neonatal mouse hearts. To examine the appropriate ratio for the cell numbers of cardiomyocytes and fibroblasts, we cocultured ES-derived cardiomyocytes and fibroblasts at ratios of 9:1, 8:2, 7:3, 6:4 and 5:5. When cardiomyocytes and fibroblasts were cocultured at the ratio of 9:1, cell aggregates that may have been larger than those formed by cardiomyocytes alone were detached from the culture dishes after culture at 20 °C (Fig. 4a). On the other hand, when cardiomyocytes were cocultured with fibroblasts at the ratio of 8:2, YFP(+) cells were evenly distributed and beat synchronously with one another (Supplementary video 3), and cell sheets were created at 4 days after culture at 20 °C (Fig. 4a). Furthermore, even when the ratio of fibroblasts was increased up to 50%, cell sheets were created (Fig. 4a), suggesting that the existence of fibroblasts was important for creating cardiac cell sheets using ES-derived cardiomyocytes.

Supplementary video related to this article can be found at doi: 10.1016/j.biomaterials.2011.05.042.

3.3. Electrophysiological evaluation of ES-derived cardiac cell sheets

Finally, we examined the electrophysiological functions of the ES-derived cardiac cell sheets created by the mixture of 80% cardiomyocytes and 20% fibroblasts. Confocal microscopy observations revealed vimentin(+) cells surrounding cTnT(+) cells in horizontal and vertical directions in the cell sheets. Consistent with the observation that the cardiomyocytes in the cell sheets beat spontaneously and synchronously, connexin 43 was expressed at the edge of adjacent cardiomyocytes (Fig. 5a). To confirm the electrophysiological connections among the cardiomyocytes in the cell sheets, the extracellular action potentials were measured using the

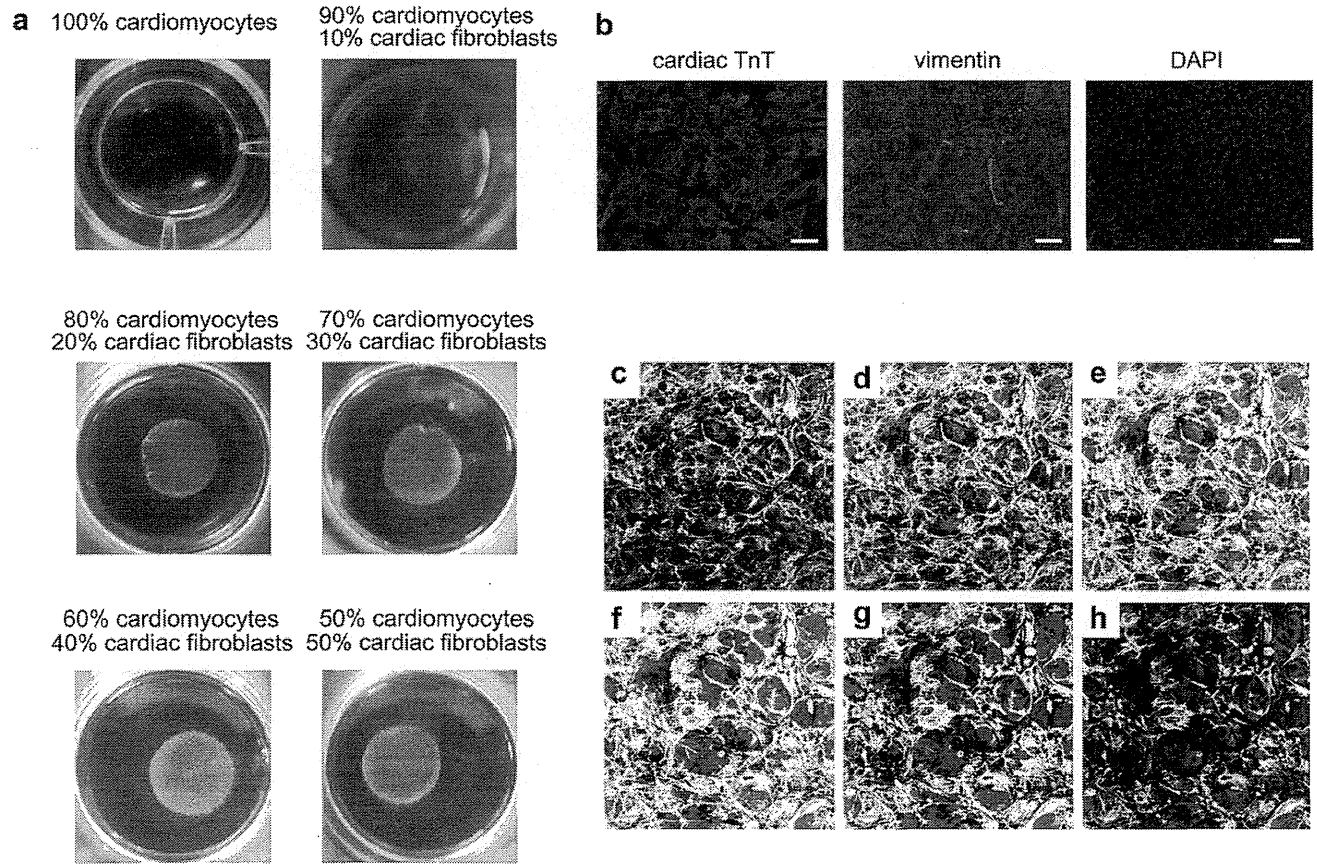

Fig. 4. Cell sheet creation using ES-derived cardiomyocytes and cardiac fibroblasts. (a) Representative images of cell aggregates and cell sheets in the well of a 24-well plate after 2 h of incubation at 20 °C. (b) Distribution of cardiomyocytes and fibroblasts in a neonatal mouse cardiac cell sheet. Left: cTnT (red); middle: vimentin (green); right, DAPI (blue). Scale bars, 50 μm (c–h) Distribution of cardiomyocytes and fibroblasts in ES-derived cardiac cell sheets. The cell sheets were stained for cTnT (red) and vimentin (white). Images were taken at 1-μm intervals in the vertical direction. Scale bar, 50 μm.

MED system [9]. As shown in Fig. 5b, unidirectional action potential propagation was observed. To clarify the electrophysiological connections between cell sheets, a cell sheet was seeded onto one side of the MED system. After 30 min, another cell sheet was seeded onto the other side of the MED system. Under the conditions used, the two cell sheets were partially overlaid and synchronous beating in the two cell sheets was observed (Fig. 5c). These findings indicate that electrical communication was established between the two cardiac cell sheets.

4. Discussion

Many reports have suggested that ES cells differentiate into cardiomyocytes via EB formation [12,13]. Since the classical methods to create EBs using hanging-drop procedure might not be useful for obtaining enough amounts of cardiomyocytes for fabricating bioengineered myocardium, various methods to overcome its limitation have been developed including multi-well plates [16,23], microwell substrates [24,25] and stirred suspension culture [14]. Consistent with previous studies [14,15], many EBs were created and cardiac differentiation was observed in the present study. Stirred suspension cultures have been thought to be unsuitable for differentiation because it is difficult to control the size of the EBs. In the present study, the number of cardiomyocytes was around 4.9×10^6 cells after neomycin selection (~78.1% of the initial ES cell number and ~2.7% of the cell number at day 10). It has been reported that ~1.5% of cells were cardiomyocytes on day 10 when mouse ES cells were cultured on a size-controlled cell pattern surface [26], suggesting that size control of EBs may not affect cardiac differentiation as obviously as previously thought. Furthermore, we applied serial treatment with noggin and GCSF to the stirred suspension cultures. The serial treatment significantly increased the number of cardiomyocytes to 1.8×10^7 cells (3.7-fold the number of untreated cells), whereas noggin treatment alone tended to increase the number of cardiomyocytes. Zandstra et al. reported that around 9.0×10^6 cardiomyocytes were obtained when mES cells were cultured in stirred suspension culture after neomycin selection, and retinoic acid treatment enhanced cardiomyocytes production doubly [14]. Although the number of cardiomyocytes without growth factor treatments in our study was smaller than that in their report, time-dependent growth factor stimulation with noggin and GCSF might produced more cardiomyocytes effectively with synergistic promotions of cardiac differentiation and proliferation in stirred suspension cultures.

Cell sheet technologies have been used as promising cell delivery tools for regenerative medicine in both animal experiments and clinical settings [2,27–29]. Although transplantation of cardiac cell sheets formed from neonatal rat cardiomyocytes was reported to improve the cardiac function in a rat infarcted heart model [30] and serial subcutaneous transplantation of cardiac sheets enabled the creation of thickened heart-like tissue of >1 mm [7], methods for creating cell sheets and the function of cell sheets formed from ES

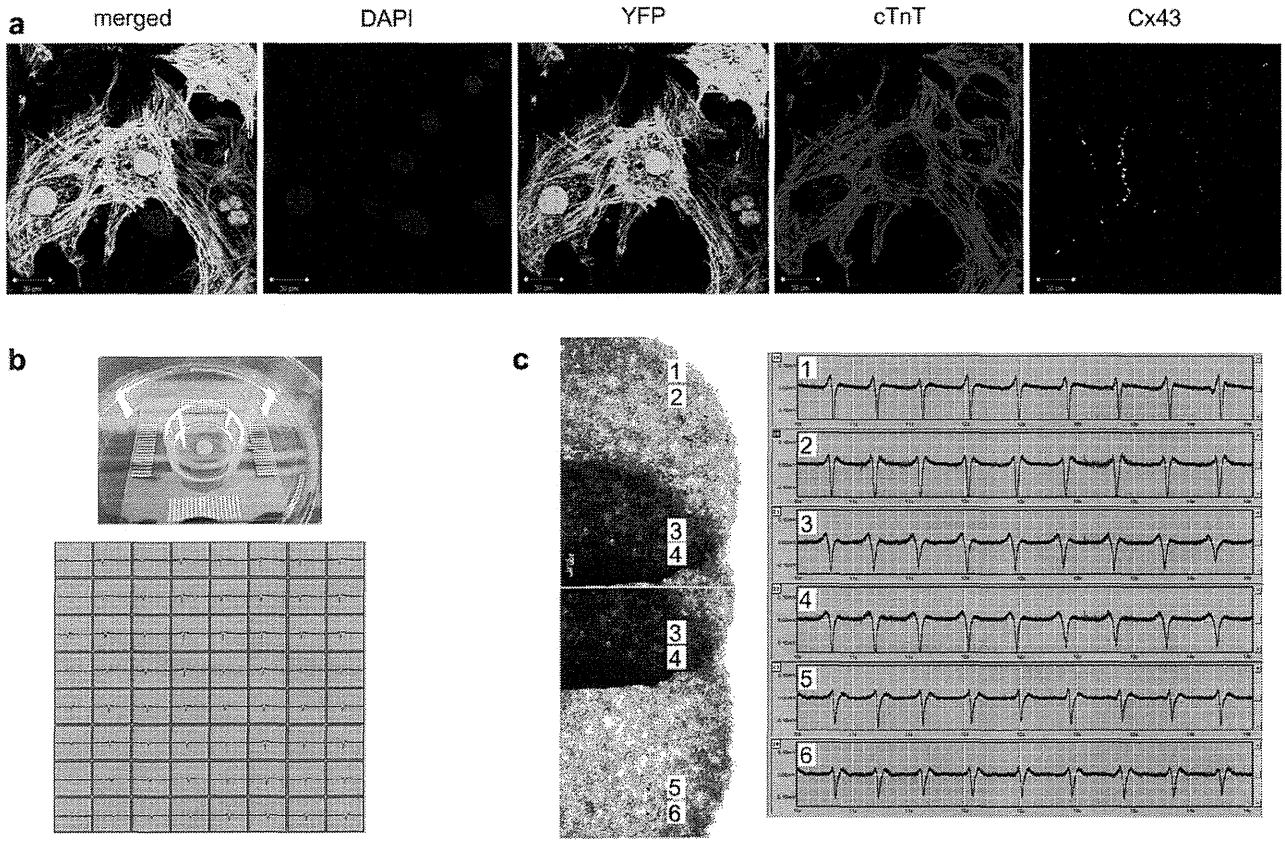

Fig. 5. Electrophysiological functions of ES-derived cardiac cell sheets. (a) Confocal microscopic images of cell sheets. Connexin 43 (white) is expressed at the edge of adjacent cardiomyocytes (YFP, green; cTnT, red). Nuclei were stained with DAPI (blue). Scale bars, 20 μm. (b) Extracellular action potential evaluations using an MED system. The upper panel shows a macroscopic view of a cell sheet on the MED system. Unidirectional action potential propagation is observed in the monolayer cardiac cell sheet (lower). (c) Electrophysiological connection between two cell sheets. The two cell sheets were partially overlaid on the MED system. The left panels show microscopic view of the electrode positions in cell sheets. Right panel shows the extracellular action potential at each electrode position. The number in each action potential shows the position of electrode under the cell sheets and No. 3 and 4 are under the overlaid area. Scale bars, 200 μm.

cell-derived cardiomyocytes have remained elusive. In the present study, ES-derived cardiomyocytes seeded onto temperature-responsive culture dishes did not form cell sheets, even when the number of seeded cells was increased. On the other hand, when we cocultured ES-derived cardiomyocytes with cardiac fibroblasts on the temperature-responsive culture dishes, cell sheets were created. These findings indicate that the existence of non-cardiomyocytes potentially expressing extracellular matrix components may be important for forming cardiac cell sheets. We further found that the cardiomyocytes in these cell sheets expressed connexin 43 at the edge of adjacent cardiomyocytes and beat spontaneously and synergistically with neighboring cardiomyocytes. Furthermore, the MED system revealed that unidirectional action potential propagation was present in the cell sheets, suggesting that ES-derived cardiac cells sheets might have the electrical functional gap junctions. Consistent with previous report indicating that the excitation of a cardiac cell sheet of neonatal rat cardiomyocytes was conducted and spread to other cell sheet via newly formed gap junction [9], the action potential of a ES-derived cardiac cell sheet could be propagated to another cell sheet. These findings suggest that ES-derived cardiac cell sheet might have functions as bioengineered myocardium in term of synchronous electrical coupling as similar to cardiac cell sheets of neonatal rat cardiomyocytes [9,22] and might provide us the helpful models for evaluating genetic disordered myocardium if patient-specific iPS cells are available [31,32]. Vascularization of cell sheets is considered to be important for fabricating thickened myocardium [7], recently we have developed the pre-vascularized cell sheets in vitro [33]. Since endothelial cells have been already reported to be isolated from ES-derived EBs [34], to create the multilayered ES-derived cardiac cell sheets with vascularization using ES-derived vascular endothelial cells is our next challenge.

5. Conclusions

In the present study, we have demonstrated the usefulness of combined treatment with noggin and GCSF of stirred suspension cultures for cardiac differentiation and the function of cardiac cell sheets using ES-derived cardiomyocytes. Recently, iPS cell technologies have enabled the creation of autologous cardiomyocytes [10]. Further understanding of the mechanisms of the expansion and differentiation in stirred suspension cultures may provide new insights for the development of human ES/iPS cell cardiomyocyte culture systems and human heart tissue engineering using cell sheet technologies.

Competing interests statement

T.S. is a consultant for CellSeed Inc. T.O. is an investor in CellSeed Inc.

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The Pleiotropic Effects of ARB in Vascular Endothelial Progenitor Cells

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Abstract: Angiotensin II regulates blood pressure and contributes to endothelial dysfunction and the progression of atherosclerosis. Bone marrow-derived endothelial progenitor cells (EPCs) in peripheral blood contribute to postnatal vessel repair and neovascularization. Impaired EPC function in patients with hypertension and diabetes inhibits the endogenous repair of vascular lesions and leads to the progression of atherosclerosis. The number of EPCs in peripheral blood is inversely correlated with mortality and the occurrence of cardiovascular events. Angiotensin II-mediated signaling is implicated in oxidative stress, inflammation and insulin resistance, factors that cause EPC dysfunction. Blockade of the angiotensin II type 1 receptor may therefore present a new therapeutic target for enhancing EPC function.

Keywords: EPC, angiotensin II, ARB, oxidative stress, PPARγ.

INTRODUCTION

The renin-angiotensin system (RAS) plays a major role in the physiological regulation of the cardiovascular system. Angiotensin II (AngII) is a pivotal molecule in the RAS. AngII causes vasoconstriction and increased blood pressure and is implicated in inflammation, endothelial dysfunction, atherosclerosis, hypertension, and congestive heart failure. Most of the pathophysiological actions of AngII in the cardiovascular system are mediated through the AngII type 1 (AT_1) receptor. Pharmacological inhibition of the RAS is one of the great success stories of cardiovascular medicine. Evidence accumulated over the past decade shows that RAS blockade with angiotensin converting enzyme (ACE) inhibitors and AngII typel receptor blockers (ARBs) prevents progression of cardiac hypertrophy and atherosclerosis and reduces morbidity and mortality in patients with heart failure [1]. Although RAS blockade is thought to reduce cardiovascular events by lowering blood pressure, evidence suggests that ARBs also protect the cardiovascular system by mechanisms independent of their antihypertensive effect, including anti-atherogenic, anti-diabetic, anti-platelet aggregating, anti-arrhythmic and hypouricemic actions [2].

Repairing injured vessels and promoting neovascularization are promising strategies for the treatment of ischemic heart disease. Angiogenesis, the proliferation and migration of preexisting endothelial cells, was thought to be the major mechanism of postnatal vessel repair and neovascularization. Recent evidence shows that bone marrow-derived endothelial progenitor cells (EPCs) in peripheral blood also contribute to these processes [3]. EPCs migrate to injured areas and differentiate into mature functional endothelial cells *in situ* [4]. Cardiovascular risk factors, such as hypertension, diabetes, dyslipidemia, smoking, and aging, influence EPC number and functions, including migration and colony-forming ability [5, 6]. In diseases of the vessel wall, such as atherosclerosis, EPCs show impaired function and a reduction in number of up to 40 % [5]. Vasa *et al.* demonstrated that EPCs from patients with coronary artery disease (CAD) have an impaired migratory function that is negatively correlated with the number of vascular risk factors [5]. In patients with CAD bone marrow-derived mononuclear cells (BM-MNCs), presumed to include EPCs, have a reduced capacity for neovascularization [7]. Hill *et al.* report that EPC numbers are inversely correlated with endothelial function [6]. These findings suggest that EPC number and function are surrogate markers for endothelial function. Impaired EPC function may limit the endogenous repair of vascular lesions and cause progression of atherosclerosis. As the number and colony-forming ability of EPCs predict cardiovascular events, a strategy for improving EPC function may present a novel therapeutic target for reducing vascular risk.

In this article, we review recent experimental and clinical data that support the benefits of ARB treatment on EPC function as a therapeutic target for cardiovascular disease. We focus particularly on hypertension and diabetes.

EPC IN HYPERTENSION

Increased arterial blood pressure is associated with microvascular dysfunction, increased peripheral vascular resistance, and impaired post ischemic neovascularization in clinical studies and animal models of hypertension [8, 9]. While low levels of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [10, 11], and defective endothelial function [12] contribute to impaired angiogenesis in hypertensive animals, EPC dysfunction may also contribute to the pathogenesis of hypertension. Vasa *et al.* report that the number and migratory capacity of EPCs are reduced in patients with hypertension [5], and Umemura *et al.* report that hypertension is an independent predictor of reduced EPC numbers [13]. Hypertension is associated with an increase in reactive oxygen species (ROS). ROS are thought to reduce nitric ox-

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ide (NO) bioavailability, which may lead to defective mobilization of EPCs from bone marrow [14]. Imanishi *et al.* report that ROS also affect the proliferation, senescence and apoptosis of EPCs [15, 16]. You *et al.* report that hypertension-induced increases in ROS inhibit the differentiation of BM-MNCs into cells with an endothelial phenotype *in vitro* [9], leading to a reduced therapeutic effect *in vivo*. These findings suggest ROS may be a major cause of impaired EPC function in hypertension. AngII increases oxidative stress, inflammation, and alters endothelial function *via* the AT_1 receptor. Kobayashi *et al.* report that an AngII infusion reduces the number and accelerates senescence of EPCs in rats [17]. AngII is also reported to accelerate EPC senescence by a gp91 phox–mediated increase in oxidative stress in humans [16]. Accordingly, ARBs decrease oxidative stress in endothelial cells [18]. It is thus possible that ARBs might improve EPC function by inhibiting AngII-mediated ROS. Valsartan, an ARB, inhibits the senescence of EPC caused by AngII-mediated oxidative stress *in vitro* [15]. ARBs such as losartan [9, 19] and candesartan [20] improve impaired EPC function in hypertensive animals by attenuating oxidative stress *via* the reduced expression of gp91-phox, p22-phox, and p47-phox. In a prospective study in normotensive and moderately hypertensive individuals, Bahlmann *et al.* found that olmesartan increases EPC numbers [21]. These findings support the important role of the RAS in the regulation of EPC bioactivity in hypertensive patients.

EPC IN DIABETES

In patients with diabetes, atherosclerosis progression is accelerated by direct endothelial damage and by the reduced availability and function of EPCs. EPC numbers are reduced in patients with type 1 and type 2 diabetes mellitus and EPCs from diabetic patients have an impaired capacity for adhesion, proliferation, and tubulization [22]. Uncontrolled plasma glucose levels, assessed by glycated hemoglobin and free plasma glucose levels, are inversely correlated with the number of EPCs. In contrast, improvement in glycemic control after treatment is associated with increased EPC numbers [23]. Chen *et al.* report that advanced glycation end products (AGE) impair the function of EPCs by affecting Akt and cyclooxygenase-2 [24]. Recent reports suggest that high glucose levels decrease the number of human EPCs *in vitro* through the reduced expression of SIRT1 [25]. SIRT1 down-regulates p53 activity and prolongs the lifespan of cells [26]. Hyperglycemia also impairs the proliferation and increases the apoptosis of EPCs through up-regulation of p16Ink-4a and p21Waf-1 [27]. Krankel *et al.* report that hyperglycemia causes reduced MMP-9 activity leading to a decreased capability of EPCs to invade a target tissue and incorporate into tubular structures [27]. Hyperglycemia also enhances protein phosphatase 2A activity in EPCs, causing a reduction in eNOS phosphorylation at Ser^{1177} and a decline in NO production [27]. In addition, hyperglycemia shifts the endothelial differentiation of EPCs to a pro-inflammatory phenotype [28]. The degree of impairment of EPC function is related to the severity of diabetic vasculopathies such as peripheral artery disease [29]. EPCs are thus thought to play an important role in the pathogenesis of diabetic vasculopathy.

AngII-mediated signaling is also important in the pathogenesis of the vascular complications of diabetes. As hyperglycemia-mediated endothelial dysfunction is largely attributed to oxidative stress *via* arachidonic acid metabolism, glucose oxidation, and AGE formation [30], blockade of RAS signaling is a promising potential therapeutic target for preventing diabetic complications. In clinical trials, inhibition of the RAS prevents the progression of diabetic nephropathy [31, 32]. Consistent with the evidence that ARB inhibition of oxidative stress improves EPC function in hypertension, olmesartan and irbesartan increase EPC numbers in diabetic patients 12 weeks after treatment [21].

Recent evidence suggests that endothelial dysfunction is already present in humans with insulin resistance and hyperinsulinemia before they become diabetic [33]. In insulin-resistant patients, the progression of atherosclerosis is associated with down-regulation of the phosphatidylinositol 3 kinase (PI3K)/Akt/eNOS pathway [34]. Inactivation of the PI3K/Akt/eNOS pathway is also reported to reduce mobilization of EPCs from bone marrow through a decrease in NO bioavailability [35]. Su *et al.* report that valsartan induces NO production in endothelial cells through Src/PI3K/Akt-dependent phosphorylation of eNOS [36]. As activation of the PI3K/Akt signal contributes to statin-induced EPC proliferation and inhibition of the senescence of EPCs [37, 38], a strategy to activate the PI3K/Akt signal by ARB treatment could present a target for preventing EPC dysfunction in patients with insulin resistance. AngII infusion decreases insulin sensitivity in diabetic and non-diabetic mice [39]. ARBs reduce insulin resistance by promoting the insulin-induced tyrosine phosphorylation of the insulin receptor substrate (IRS)-1, the association of IRS-1 with p85, and the translocation of GLUT4 [40]. Several clinical trials report that ARB treatment inhibits the new occurrence of diabetes in patients with hypertension [41, 42] and CAD [43]. Recently Lee *et al.* have reported that ARBs improve glucose tolerance in OLETF rats, an animal model of type 2 diabetes [44]. They also report that ARB treatment increases the number of small differentiated adipocytes that produce adiponectin. Adiponectin is the major adipokine that sensitizes the body to insulin [45] and it also promotes the migration of EPC through the PI3K/Cdc42/Rac1 pathway [46]. These findings suggest that ARBs may not only directly improve EPC function in diabetes by inhibiting oxidative stress, but also indirectly affect EPC function by improving insulin sensitivity and up-regulating adiponectin production.

THE PPARγ DEPENDENT EFFECTS OF ARB

Telmisartan has recently been identified as a partial agonist of peroxisome proliferator-activated receptor gamma (PPARγ) [47]. Other clinically approved ARBs have little or no effect on PPARγ activity with the exception of irbesartan and a metabolite of losartan, both of which are less potent activators of PPARγ than telmisartan [48, 49]. PPARs are transcription factors belonging to the nuclear receptor superfamily that heterodimerize with the retinoid X receptor and bind to PPAR-responsive elements in target gene promoters. The activation of PPARγ in adipose tissue promotes adipose differentiation and increases the number of small insulin-sensitive adipocytes [50]. Thiazolidinediones (TZD), full agonists of PPARγ, increase endothelium-derived NO pro-

duction [51] and reduce vascular inflammation [52], suggesting that PPARγ activation might be anti-atherosclerotic. Telmisartan is thought to functionally activate PPARγ and to induce adiponectin expression *via* PPARγ activation [53]. We recently reported that telmisartan increases the number of human peripheral blood-derived EPC *in vitro via* a PPARγ dependent pathway *in vitro* [54]. Our results are consistent with evidence that TZD increases EPC numbers [55, 56]. By contrast, valsartan treatment does not affect the EPC numbers [54], suggesting that different ARBs have differing effects on EPC proliferation. We also found that the telmisartan-mediated increase in EPCs is regulated by the PI3K/Akt pathway [54]. As down-regulation of the PI3K/Akt/eNOS pathway in patients with diabetes mellitus increases endothelial dysfunction and reduces mobilization of EPC from bone marrow, activation of the PI3K/Akt signal by telmisartan may be a novel therapeutic target for improving endothelial function. Pioglitazone, a TZD, attenuates AngII-induced cellular senescence and oxidative stress in endothelial cells *in vitro* [57]. Pioglitazone treatment also increases the number of circulating EPCs in type 2 diabetics and non-diabetic patients with CAD [58, 59]. As telmisartan causes AT_1 receptor blockade and PPARγ activation, it might be expected to improve vascular function and promote neovascularization *via* the proliferation of EPCs in ischemic tissue in the clinical setting.

CONCLUSIONS

Accumulating data suggest that oxidative stress and inflammation in patients with cardiovascular risk factors impair the proliferation, migration, and differentiation of EPCs. ARBs improve EPC function by reducing oxidative stress and inflammation, increasing insulin sensitivity and activating PPARγ. Impaired EPC bioactivity is thought to play the critical role in the progression of atherosclerosis and reduced EPC numbers and impaired EPC function are associated with increased mortality in patients with cardiovascular risk factors. However, treatments that improve EPC bioactivity have not yet been shown to prevent cardiovascular death or new myocardial infarction. Further basic and clinical research is thus required to elucidate the interaction between pharmacological interventions such as ARB treatment and the occurrence of cardiovascular events in terms of the effects on EPC function. Improving our understanding of EPC biology will help us develop new treatments for ischemic cardiovascular disease.

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NON-STANDARD ABBREVIATIONS

ACE = Angiotensin converting enzyme

AGE = Advanced glycation end products

AngII = Angiotensin II

ARBs = Angii typel receptor blockers

AT_1 = AngII type 1

BM-MNCs = Bone marrow-derived mononuclear cells

CAD = Coronary artery disease

EPCs = Endothelial progenitor cells

HGF = Hepatocyte growth factor

IRS = Insulin receptor substrate

NO = Nitric oxide

PI3K = Phosphatidylinositol 3 kinase

PPARγ = Peroxisome proliferator-activated receptor gamma

RAS = Renin-angiotensin system

ROS = Reactive oxygen species

TZD = Thiazolidinediones

VEGF = Vascular endothelial growth factor

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