Clinical studies have shown that β-blockers could reduce incidence of cardiovascular events, as well as the mortality of patients with chronic heart failure. Carvedilol is a non-selective AR antagonist that blocks β1- and β2-ARs, as well as α1-AR. Carvedilol suppresses SNS activities, and decreases heart rate (HR) and contractility. This action is beneficial to patients with heart failure whose SNS is activated. Carvedilol also causes vasodilation and decreases peripheral vascular resistance without reflex tachycardia by concomitant blockade of α1- and β1-ARs.

Developments of coronary angioplasty and bypass operation for patients with myocardial infarction (MI) have decreased their mortality and morbidity. However, these treatments are still insufficient to prevent progression of cardiac remodeling and heart failure after MI. One of the reasons for this burden is activation of the sympathetic nervous system (SNS) after MI, which deteriorates cardiac functions. Activation of β-adrenergic receptors (β-ARs) predisposes to apoptosis of cardiac myocytes, suggesting the effectiveness of β-blockers.

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Cell-based regenerative therapy improved blood supply to the damaged heart, and minimized the area of infarction.\(^{11-13}\) These effects are mediated, in part, by cytokines such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) secreted by transplanted cells.\(^{14,16}\) Direct injection of cells into the heart has been problematic because of a significant loss of live cells and pro-arrhythmic effects.\(^{17,18}\) Tissue engineering using cell sheets has been developed to overcome these disadvantages; preparing cell sheets could improve viability of cells\(^{19,20}\) and prolong secretion of cytokines.\(^{21}\)

Adipose-derived stem cells (ASCs), belonging to the family of mesenchymal stem cells (MSCs), secrete cytokines such as VEGF, HGF and fibroblast growth factor (FGF), and prevent cardiac dysfunction and remodeling after MI.\(^{22,23}\) by inducing neovascularization.\(^{24,25}\) We have previously reported that ASC sheets decrease interstitial fibrosis, induce neovascularization, and prevent remodeling in rat MI hearts.

Catecholamines control mobilization and angiogenesis of bone marrow-derived endothelial progenitor cells.\(^{28}\) Recently, norepinephrine has been reported to activate MSC chemotaxis by increasing stroma cell-derived factor-1 (SDF-1).\(^{29}\) It has also been reported that activation of \(\beta\)-ARs is required to elevate \(\alpha\)-AR expression and signaling and to regulate secretion of cytokines by MSCs.\(^{30}\) Thus, carvedilol may influence the effects of ASC sheets on MI hearts via blocking actions of catecholamines and regulating secretion of cytokines such as VEGF.

In the present study, we examined whether administration of carvedilol before and after MI could influence ASC sheet effects on cardiac functions and remodeling after MI.

### Methods

#### In Vitro Study

**Engineering of ASC Sheets**

ASCs were isolated from the inguinal subcutaneous fat tissue of Lewis rats (200–250 g) and cultured as described previously.\(^{24}\) To prepare cell sheets, ASCs (1×10^6 cells; 3–4 passage) were cultured on 35-mm temperature-responsive culture dishes (UpCell, Cell Seed Inc., Tokyo, Japan) in an incubator at 37°C for 48 h. They were then maintained at 20°C for 1 h to be detached as an intact sheet.

**Real-Time Reverse Transcriptase-Polymerase Chain Reaction Analysis**

ASCs were incubated at 37°C under normal (21% O\(_2\)) or hypoxic (<2% O\(_2\)) conditions for 48 h using an AnaeroPack system (MITSUBISHI GAS CHEMICAL Inc., Tokyo, Japan). Total RNA was extracted from ASCs using a RNeasy Mini kit (QIAGEN inc., Valencia, CA, USA). Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of VEGF-A, HGF, FGF, hypoxia inducible factor-1α (HIF-1α), SDF-1, and \(\beta\)-actin was performed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Their mRNA levels were expressed as ratios to those of \(\beta\)-actin. mRNA levels of \(\alpha\)-1, \(\beta\)-1 and \(\beta\)-2 ARs were determined by quantitative RT-PCR. The primers used are shown in Table.\(^{24}\) The mRNA levels were determined by the comparative Ct method, and expressed as 2\(^{-\Delta\Delta C_t}\).

**Western Blotting**

ASCs were harvested and lysed by sonication in PBS supplemented with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and a protease mixture (Rosche Diagnostics, Tokyo, Japan). Proteins were separated on SDS-PAGE and electrotransferred to PVDF membranes. The membranes were immunoblotted with a rabbit polyclonal antibody against \(\alpha\)-1-AR or \(\beta\)-1-AR.
protocols were approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Tottori University. Rats were randomly assigned to 4 groups: (1) no treatment (Control MI group, n=7); (2) ASC sheet transplantation alone (ST group, n=9); (3) carvedilol treatment alone (Car group, n=12); and (4) both ASC sheet transplantation and carvedilol treatment (ST+Car group, n=10). For all the groups, acute MI was created by ligation of the left anterior descending artery, as described previously.

Carvedilol at 5 or 20 mg · kg⁻¹ · day⁻¹ was orally administered to the Car and ST+Car group rats for 6 weeks (from 7 days prior to the creation of MI to 5 weeks after MI). ASC sheet transplantation was performed for the ST and ST+Car group rats 7 days after the creation of MI. We confirmed that transplanted ASC sheets existed on the surface of hearts by luminescent signals of ASCs derived from transgenic rats harboring the luciferase gene (data not shown). Details of the experimental protocol are shown in Supplementary Figure 1.

Measurements of Blood Pressure and HR
Systolic and diastolic blood pressure (SBP and DBP) and HR were measured by a tail-cuff system (BP-98A; Softron, Tokyo, Japan) a week before, just before, and 1, 3 and 5 weeks after MI (2 and 1 week before, just before, and 2 and 4 weeks after ASC sheet transplantation).

Echocardiography
Cardiac function was evaluated by echocardiography just before and 1, 3 and 5 weeks after MI (7 days before, just before, and 2 and 4 weeks after ASC sheet transplantation) using a 12-MHz transducer (LOGIQ P5J and 12L; GE Healthcare, Fairfield, CT, USA).

Figure 1. mRNA levels of vascular endothelial growth factor (VEGF)-A, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), hypoxia inducible factor (HIF)-1α and stroma cell-derived factor (SDF)-1 in adipose-derived stem cells (ASCs) under normoxia or hypoxia. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed to determine mRNA expression levels. They were normalized to β-actin mRNA levels and expressed as ratios to the control values under normoxia (n=5). **P<0.01 (vs. Normoxia).
Carvedilol Abolishes ASC Sheet Effects on MI Heart

Whether carvedilol affects the action of ASC sheets on MI heart functions was also determined using the Langendorff heart isolated from male Lewis rats (300–350 g, 12 weeks old). Animals were handled in accordance with the Tottori University Guide for the Care and Use of Laboratory Animals. Isolation and coronary perfusion of hearts were executed with a Langendorff perfusion system (model 7523-40; Masterflex, Barrington, IL, USA), as described previously.

Coronary arteries were perfused with a modified Tyrode’s solution [NaCl 144, KCl 5, CaCl2 1.5, MgCl2 0.9, HEPES 6, and glucose 5 (in mmol/L); pH 7.4 with NaOH] equilibrated with 100% oxygen.

Ventricular functions were assessed by measuring the left ventricular pressure (LVP) with a fluid-filled latex balloon inserted into the LV and inflated to give a LV end-diastolic pressure (LVEDP) of 5–15 mmHg. The transducer was connected to a PowerLab/8SP (AD Instruments, Castle Hill, NSW, Australia) to measure LVP and determine the maximum of LVP (LVPmax), LVEDP, LV developing pressure (LVDP), and maximum rates of LVP rise (dP/dtmax) and fall (dP/dtmin).

Data Analysis
All data are expressed as mean±SEM. A Student’s t-test and a Bonferroni multiple comparison test were used for comparisons.
Comparisons of 2 groups and multiple (≥3) groups, respectively. A probability value of <0.05 was considered significant.

Results

Effects of Hypoxia on Expressions of Angiogenic Factor mRNAs in ASCs

We quantified mRNAs of angiogenic factors in ASCs after exposure to normoxia or hypoxia for 48 h (Figure 1). Levels of VEGF-A mRNA were significantly elevated under hypoxia. Levels of FGF mRNA tended to increase under hypoxia, whereas those of HGF, HIF-1α and SDF-1 mRNA significantly decreased.

Expressions of α/β-AR mRNAs and Proteins in ASCs Under Hypoxia

We quantified mRNAs and proteins of α1-, β1- and β2-ARs expressed in ASCs under hypoxia. ASCs expressed mRNAs of α1- and β1-ARs, but not that of β2-AR (Supplementary Figure 2A). Expressions of α1-AR and β1-AR proteins in ASCs were confirmed by Western blotting (Supplementary Figure 2B).

Effects of Norepinephrine on Expressions of Angiogenic Factor mRNAs in ASCs

Treatment with norepinephrine (10 μmol/L) significantly increased the mRNA level of VEGF-A under hypoxia, but failed to change mRNA levels of HGF, FGF, HIF-1α and SDF-1 (Supplementary Figure 1). Even in the presence of carvedilol (10 μmol/L), norepinephrine significantly increased VEGF-A mRNA. Carvedilol did not significantly alter expressions of mRNAs of any cytokines in the presence or absence of norepinephrine.

Carvedilol Abolished the Beneficial Effects of ASC Sheets on Cardiac Fibrosis and Neovascularization After MI

We studied effects of ASC sheets and carvedilol on cardiac functions and remodeling after MI in vivo. EF was significantly lower in the MI group than in the sham-operated, non-MI group (data not shown). Four weeks after ASC sheet transplantation, EF and FS in the ST group, but not in the Car group or the ST+Car group, were significantly higher than those in the control MI group, without any significant difference in LVESD and LVEDD (Figure 2). There was no significant difference in SBP, DBP or HR among the 4 groups (Supplementary Figure 4).

The plasma level of ANP measured 4 weeks after transplantation was slightly lower in the ST group than in the sham-operated, non-MI group (data not shown). Four weeks after ASC sheet transplantation, EF and FS in the ST group, but not in the Car group or the ST+Car group, were significantly higher than those in the control MI group, without any significant difference in LVESD and LVEDD (Figure 2). There was no significant difference in SBP, DBP or HR among the 4 groups (Supplementary Figure 4).

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Figure 3 shows LV functions of Langendorff-perfused hearts isolated from rats in the 4 groups. LV max, LVDP and dP/dt min were significantly improved in the ST group in comparison to those in the control MI group, but were comparable in the control MI and ST+Car groups. Thus, the ASC-induced changes in LV max, LVDP and dP/dt min were abolished by carvedilol treatment.

Carvedilol Abolished the Beneficial Effects of ASC Sheets on Cardiac Fibrosis and Neovascularization After MI

Cardiac fibrosis and neovascularization were evaluated by the histological analyses 4 weeks after transplantation.
Carvedilol Abolishes ASC Sheet Effects on MI Heart

(5 weeks after MI). In the control MI group, extensive fibrosis occurred around the infarcted region. This fibrosis after MI was reduced in the ST group, but not in the ST+Car group (Figure 4A). Figure 4B shows the summary data obtained from 7 to 11 experiments. The extent of cardiac fibrosis was significantly lower in the ST group than in the control MI group. The reduction of fibrosis did not occur either in the Car or ST+Car group. The capillary density was remarkably increased in the ST group, as compared with those in the control MI and Car groups, although there was no difference between the control MI and ST+Car groups (Figure 5A). As summarized in Figure 5B, the capillary density was significantly higher in the ST group than in the control MI group, while comparable in the control MI and Car groups; it was significantly lower in the ST+Car group than in the ST group. Thus, carvedilol prevented the reduction of fibrosis and enhancement of neovascularization by ASC sheets without affecting them in itself.

Effects of Carvedilol on VEGF-Induced Phosphorylation of VEGF Receptors and Tube Formation in HUVECs

To clarify how carvedilol prevented the enhancement of neovascularization by ASC sheets, we examined effects of carvedilol on VEGF-induced phosphorylation of the VEGFR2 (Y1175) and tube formation of HUVECs. In the presence of VEGF, carvedilol (10 μmol/L) significantly reduced the phospho-VEGFR2 (Y1175) without altering the total VEGFR2 protein level (Supplementary Figure 6).

Discussion

In this study, we found that: (1) hypoxia increased VEGF-A mRNA in ASCs; (2) ASC sheet transplantation improved contractile functions of MI hearts, suppressed fibrosis, and enhanced neovascularization; (3) carvedilol treatment before and after MI abolished these beneficial actions of ASC sheets; and (4) carvedilol abolished promoting effects of VEGF on VEGFR2 (Y1175) phosphorylation and tube formation of HUVECs.

ASCs have been reported to improve cardiac functions after MI by inducing neovascularization and suppressing fibrosis. These actions of ASCs may be attributable to the release of cytokines and/or their trans-differentiation into cardiomyocytes. We reported that ASC sheets survived on infarcted areas as layers for at least 2 weeks after transplantation, and that vWF-positive capillaries were created under the sheet. ASCs secrete VEGF, FGF and HGF in vitro and in vivo, which may contribute to the increment of capillaries. In this study, the mRNA level of VEGF-A was significantly increased in ASCs under hypoxia. ASCs expressed mRNAs of α1- and β1-ARs, and norepinephrine further increased VEGF-A mRNA in ASCs under hypoxia. It has been reported that increased expression of α-ARs and enhancement of their downstream signaling play an
important role in the secretion of cytokines by MSCs. Thus, ASCs may exert beneficial effects on MI hearts via the norepinephrine-induced enhancement of VEGF secretion. In this study, the expression of HIF-1α mRNA was significantly suppressed under hypoxia, which was very similar to our previous data. There was a difference in mRNA level of HIF-1α between our study and a previous study, which may be attributable to the difference in the timing of measurements of HIF-1α mRNA.

Carvedilol is a third-generation vasodilating β-blocker that has been shown to reduce morbidity and mortality of patients with heart failure. Inhibition of β-ARs on cardiomyocytes prevents their responses to the SNS, leading to decreased HR and contractility. This action of the agent is beneficial for patients with heart failure whose SNS is activated. Carvedilol has a relatively weak inhibitory action on α1-ARs, which leads to decreased peripheral vascular resistance and an antihypertensive effect; there happens no reflex tachycardia due to simultaneous blockade of β1-ARs in the heart. In this study, carvedilol did not affect norepinephrine-induced elevation of VEGF-A mRNA in ASCs, suggesting that the norepinephrine effect was not mediated by β- or α1-ARs. Further experiments are necessary to identify the signaling pathway responsible for the norepinephrine-induced elevation of VEGF-A mRNA.

The most prominent finding in the present study was that carvedilol abolished beneficial effects of ASC sheets on contractile functions, neovasculization and fibrosis of MI hearts. Although several reports indicate that administration of carvedilol after MI exerts cardioprotective actions, there have been no reports to clarify whether treatment with carvedilol prior to MI could improve contractile functions of MI hearts. Following our previous report showing the effects of pretreatment with an angiotensin II receptor blocker, irbesartan, on cardiac functions, we examined the effects of oral administration of carvedilol at 5 and 20 mg · kg⁻¹ · day⁻¹ before and after MI on the contractile function of MI hearts. As shown in Figure 2 and Supplementary Figure 7, carvedilol at 5 mg · kg⁻¹ · day⁻¹ did not influence contractile functions of MI hearts, whereas carvedilol at the higher dose of 20 mg · kg⁻¹ · day⁻¹ significantly improved the MI heart function 1 week after MI (just before ASC sheet transplantation). In contrast, treatment with carvedilol at 5 mg · kg⁻¹ · day⁻¹ abolished the beneficial effects of ASC sheets on contractile functions, neovasculization and fibrosis of MI hearts. To confirm the present study results from the in vivo measurement of LV contractile function, we performed the measurement 5 weeks after MI (just before ASC sheet transplantation). As shown in Figure 5, carvedilol at 5 mg · kg⁻¹ · day⁻¹ did not influence contractile functions of MI hearts, whereas carvedilol at the higher dose of 20 mg · kg⁻¹ · day⁻¹ significantly improved the MI heart function 4 weeks after ASC sheet transplantation.
Carvedilol Abolishes ASC Sheet Effects on MI Heart

functions, we performed the Langendorff experiment to measure LV functions of isolated MI hearts 5 weeks after MI (4 weeks after transplantation). Although ASC sheets improved systolic and diastolic functions of isolated MI hearts, carvedilol abolished the beneficial effects of ASC sheets (Figure 3), which is consistent with the results from the in vivo experiment (Figure 2).

Carvedilol did not reduce the mRNA level of VEGF-A in the absence or presence of norepinephrine, indicating that preventive effects of carvedilol on ASC sheet-induced cardioprotection are not ascribable to its inhibition of VEGF-A transcription or reductions in the norepinephrine action. VEGF promoted phosphorylation of VEGFR2 (Tyr1175) and tube formation of HUVECs, which were abolished by carvedilol. Consistent with our finding, β-blockers such as carvedilol and labetalol have been reported to exert anti-angiogenic effects on neuroblastoma cells; this action was potentiated by propranolol, nebivolol, atenolol or metoprolol.35 Besides, carvedilol inhibited angiogenesis through the VEGF-Src-ERK signaling pathway in cirrhosis livers.36 Ding et al36 demonstrated that carvedilol inhibited tube formation of HUVECs induced by VEGF-A through inhibition of phosphorylation of VEGFR2, ERK and Src. This finding was partly confirmed by the fact that carvedilol inhibited the VEGF- or ASC-conditioned medium-induced enhancement of the tube formation by HUVECs (Figure 6). This pathway may be involved in carvedilol inhibition of the ASC sheet effects observed in the present study.

Although the precise mechanism that underlies the carvedilol effects on ASC sheet-induced cardioprotection is unknown, a clinical implication of this study may be clear. ASC sheets would be applicable to drug-refractory patients who wait for heart transplantation. Given the observed carvedilol effects, we have to pay attention to the period and timing of carvedilol administration before and after ASC sheet transplantation. We cannot apply the present study results to clinical settings directly, because effects of carvedilol on MI hearts and ASC sheet therapy may depend on the method of its administration; β-blockers, including carvedilol, improved prognosis of MI patients when administered with a time lag of more than 24 h after MI, but failed to do so when administered immediately after MI.31 These results suggest that carvedilol should not be administered to patients before or immediately after MI. We need further investigations on whether carvedilol administered 24 h or later after MI (post-treatment with the agent) inhibits cardioprotective actions of ASC sheets. Carvedilol at 25 or 50 mg/day have been reported to be prescribed to patients with chronic heart failure,37,38 and

Figure 6. Inhibitory effects of carvedilol on vascular endothelial growth factor (VEGF)- or adipose-derived stem cell (ASC) conditioned medium-induced enhancement of tube formation by human umbilical vein endothelial cells (HUVECs). (A) Effects on the VEGF-induced enhancement of tube formation. Representative images of tube formation (top) and averaged luminal areas of tubular structures (bottom) are shown. HUVECs were placed in Matrigel with or without VEGF (50 ng/mL) in the presence or absence of 10 μmol/L carvedilol (Car). After 8 h, tubular structures were imaged (magnification: ×100). Averaged lumen areas were determined using ImageJ software (n=6). **P<0.01. (B) Effects on ASC-conditioned medium-induced enhancement of tube formation. Representative images are shown. HUVECs were placed in Matrigel in the conditioned medium of ASCs (0.5×10^6 cells) in the presence or absence of 10 μmol/L carvedilol. After 8-h incubation, tubular structures were imaged (magnification: ×100).
Watanabe et al.\textsuperscript{29} reported that carvedilol doses of 25 and 50 mg for humans correspond to those of 2 and 5 mg/kg, respectively, for rats, when corrected by body surface areas. Therefore, our experimental condition of the carvedilol dose (5 mg · kg$^{-1}$ · day$^{-1}$) may be applicable to clinical settings. Nevertheless, differences in carvedilol effects between the groups, with its treatment only after MI and both before and after MI, and the effects of carvedilol at a broader range of doses should be determined in future studies.

There is a limitation in the present study. MI damage just before ASC sheet transplantation (1 week after MI) should be the same in the 4 groups to assess the ameliorating effects of ASC sheets on dysfunctions and remodeling of MI hearts properly. In the present study, however, it was impossible to confirm that the MI damage was comparable among the 4 groups because we aimed to evaluate the effects of oral administration of carvedilol before MI (before ASC sheet transplantation); pretreatment with carvedilol may affect myocardial damage caused by MI.

**Funding**

None.

**Conflicts of Interest**

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**References**


**Supplementary Files**

Please find supplementary file(s):