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Adiponectin is a better predictor of endothelial function of the coronary artery than HOMA-R, body mass index, immunoreactive insulin, or triglycerides

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Abstract

Background: Previous studies have demonstrated that decreased levels of circulating adiponectin correlate with endothelial dysfunction in peripheral arteries. However, the relationship between adiponectin levels and endothelial function in coronary arteries remains unclear. The goal of the present study was to determine whether circulating adiponectin concentrations are a useful predictor of coronary endothelial function.

Methods: Thirty-six consecutive non-diabetic patients with normal or mildly diseased coronary arteries were enrolled in this study. Coronary endothelial function was evaluated by coronary vascular response to acetylcholine (Ach). The relationship between coronary vasoreactivity and adiponectin or other biochemical or anthropometric parameters was investigated. The predictive value of adiponectin level for assessment of coronary endothelial dysfunction was assessed at the best cut-off point.

Results: In a simple regression analysis, log-transformed adiponectin concentrations positively correlated with the percent change in coronary blood flow (CBF) and coronary artery diameter (CAD) induced by Ach ($r=0.62$, $p<0.0001$; $r=0.63$, $p<0.0001$, respectively). Insulin resistance index (HOMA-R), body mass index, immunoreactive insulin, and triglycerides concentrations also significantly correlated with the percent change in CBF and CAD. However, in a multiple regression analysis, log-transformed adiponectin concentration was the only independent predictor of the percent change in CBF and CAD ($p<0.0001$; $p<0.0001$, respectively). Furthermore, patients with adiponectin concentrations <6.3 mg/L demonstrated coronary endothelial dysfunction with high specificity both in terms of CBF and CAD response (85%; 88%, respectively).

Conclusions: Adiponectin is a better predictor of coronary endothelial function than other factors such as HOMA-R, body mass index, immunoreactive insulin, and triglycerides.

Keywords: Adiponectin; Endothelial dysfunction; Coronary artery

1. Introduction

Endothelial dysfunction is an early pivotal event in the development, progression, and manifestation of atherosclerosis [1,2]. Dysfunction of either the coronary or peripheral vascular endothelium is an independent predictor of

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cardiovascular events [3,4]. In addition to providing valuable prognostic information, the endothelium itself is an attractive primary target in the effort to optimize individualized therapeutic strategies to reduce cardiovascular morbidity and mortality [4,5].

Adiponectin is a collagen-like plasma protein identified in the human adipose tissue cDNA library [6] that is produced by the adipose tissue and that is abundant in the systemic circulation. Interestingly, plasma concentrations of adiponectin are reduced in the setting of obesity [7], type 2 diabetes mellitus [8], and coronary artery disease [9]. Adiponectin plays an important role in the regulation of insulin action [10,11], and several studies have demonstrated that adiponectin levels are negatively correlated with the degree of insulin resistance [12,13].

In addition to its effect on glucose metabolism, adiponectin also appears to modulate endothelial function. For example, adiponectin stimulates production of nitric oxide [14] and suppresses adhesion molecule expression [15] in vascular endothelial cells. Indeed, several clinical studies have demonstrated that hypoadiponectinemia correlated with endothelial dysfunction in peripheral arteries [16–18]. However, the relationship between adiponectin and endothelial function of coronary arteries remains unclear. If a correlation between circulating adiponectin levels and coronary endothelial function were confirmed, adiponectin would be useful as a marker of coronary artery endothelial function. Therefore, the goal of the present study was to investigate the impact of circulating adiponectin levels on coronary artery endothelial function and assess the utility of measuring adiponectin levels for the prediction of coronary endothelial function.

2. Methods

2.1. Study population

Seventy-eight consecutive patients who had been referred for cardiac catheterization to exclude coronary artery disease were considered for enrollment in this study. A total of 36 patients met the following inclusion criteria: 1) angiographically smooth arteries; 2) mild irregularities, <30% lumen diameter stenosis by visual assessment in any major conduit vessel; and 3) proximal coronary arteries >2.0 mm in diameter. Patients with a history of previous myocardial infarction, previous coronary revascularization, valvular heart disease, variant angina, cardiomyopathy, or myocarditis were excluded from this study [19].

Long-acting nitrates, calcium channel blocking agents, and β-adrenergic blockers were withheld for 48 h before the study to allow for the assessment of baseline coronary physiology. Smoker was defined as a current smoker. Upon admission to the hospital, subjects were instructed to refrain from smoking until completion of the study. Mean duration of abstention from smoking prior to blood sampling and cardiac catheterization was 4.4 days (range, 2–7 days). At baseline, patients with diabetes mellitus [fasting plasma glucose (FPG) ≥7.0 mmol/L at any two previous examinations, use of hypoglycemic drug therapy, or a current FPG ≥7.0 mmol/L or 2 h glucose level after 75 g oral glucose ≥11.1 mmol/L] or untreated endocrine diseases were excluded. All subjects reported that their body weight had been stable (±2 kg) for at least 3 months. Upon admission to the hospital, patients were instructed to refrain from intensive exercise until completion of the study. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Waist circumference was measured at the mid-level between the lower rib margin and the iliac crest. Hip circumference was measured as the widest measure over the buttocks and below the iliac crest. The waist-to-hip ratio was calculated as waist circumference in centimeters divided by hip circumference in centimeters.

Hyperglycemia was defined as an FPG concentration >6.1 mmol/L. Dyslipidemia was defined as a low-density lipoprotein-cholesterol (LDL-C) concentration >3.33 mmol/L, and/or a triglyceride concentration >1.69 mmol/L, and/or a high-density lipoprotein-cholesterol (HDL-C) concentration <1.03 mmol/L, and/or having received treatment for dyslipidemia. Hypertension was defined as systolic blood pressure >140 mm Hg, and/or diastolic pressure >90 mm Hg, and/or having received treatment for hypertension.

Written informed consent was obtained from all patients after the purpose, nature, and potential risks of the study were explained to the subjects in accordance with guidelines established by the Committee for the Protection of Human Subjects in our institution.

2.2. Adiponectin measurements

Blood samples for measurement of fasting plasma adiponectin concentrations were obtained on the day of coronary flow study. Peripheral blood samples were drawn into tubes containing EDTA-sodium (1 mg/ml) and immediately placed on ice. All tubes were centrifuged at 4 °C for collection of plasma, which was stored at −80 °C until analysis. Plasma adiponectin level was determined using a validated sandwich enzyme-linked immunosorbent assay (ELISA) employing an adiponectin-specific monoclonal and polyclonal antibody supplied by a commercially available kit (Otsuka Pharmaceutical Co, Tokyo, Japan) [7].

2.3. Biochemical analysis

Blood glucose was measured by the glucose oxidase method (GAO3U, A&T Corp., Yokohama, Japan) [20]. Plasma levels of total cholesterol, triglyceride, HDL-C, uric acid and C-reactive protein were determined by an autoanalyzer (TBA-80-FR-Neo, Toshiba Co., Tokyo, Japan) using commercially available kits (total cholesterol, triglyceride, uric acid: Shino-Test Corp., Tokyo, Japan; HDL-C: Kyowa Medex Co. Ltd, Tokyo, Japan; C-reactive protein: Mitsubishi Kagaku

Iatron, Tokyo, Japan). LDL-C was estimated using Friedewald's formula [21]. Immunoreactive insulin (IRI) was determined by a specific enzyme immunoassay (EIA) (TOSOH Co. Ltd, Yamaguchi, Japan). Insulin resistance was calculated by the homeostasis model assessment method (HOMA-R), using FPG and insulin concentrations [22]. Assuming that normal weight subjects <35 years of age have an insulin resistance of 1, the value for insulin resistance was assessed by the formula: FPG (mmol/L)×IRI (mU/L)/22.5 [22,23].

2.4. Study protocol

Diagnostic coronary angiography was performed using a 6F Judkins catheter with a standard femoral percutaneous approach. Five thousand units of heparin was administered at the beginning of the procedure. Non-ionic contrast material was used for all patients. No nitroglycerin was given prior to the diagnostic procedure.

Coronary blood flow response to papaverine, acetylcholine (Ach), and nitroglycerin was studied according to previous reports [24,25]. After control coronary angiograms, interventions were performed as follows: 1) a 0.014-inch Doppler guidewire (Cardiometrics, Santa Anna, CA) was introduced into the left anterior descending coronary artery; 2) after obtaining a stable Doppler signal, a 5 ml bolus of papaverine at 2.5 g/L [an endothelium-independent vasodilator in resistance coronary arteries] was injected through a catheter; 3) infusion of Ach [an endothelium-dependent vasodilator in resistance and conduit coronary arteries] (0.5 mL/min) at a dose of 3 μg/min for 2 min was performed via the catheter [26,27]; and 4) a 5 ml bolus of nitroglycerin at 40 mg/L [an endothelium-independent vasodilator in conduit coronary arteries] was administered. Drugs were infused at least 5 min apart. Coronary arteriography was performed before and 2 min after each dose of Ach and after administration of nitroglycerin. Phasic coronary blood flow velocities, arterial blood pressure, and heart rate were monitored continuously and recorded. Measurements obtained during steady state conditions were used as control values for later analysis.

2.5. Quantitative coronary angiographic images

Technically suitable single-plane angiograms were selected for computer analysis. Quantitative coronary angiographic images (DBAC-1000; MID Corporation, Fukuoka, Japan) were recorded using validated densitometric analysis, as previously reported [28]. An end-diastolic still frame at each infusion (baseline, Ach, nitroglycerin) was selected from the angiographic sequence. Endothelium-dependent and -independent vasodilation of the conduit coronary artery was estimated by measuring the luminal diameter at the tip of the Doppler guidewire positioning at the proximal site of left anterior descending coronary artery. Experienced observers who were unaware of the coronary vascular reactivity tests or adiponectin levels made these measurements.

2.6. Assessment of coronary vasoreactivity

Doppler flow velocity spectra were analyzed on-line to determine time-averaged peak velocity. Volumetric coronary blood flow (CBF) was determined using the formula: CBF = cross-sectional area × average peak velocity × 0.5 [29]. Coronary flow reserve to papaverine was calculated as the ratio of maximal CBF induced by papaverine to basal CBF, which reflects the endothelium-independent function of the resistance coronary artery. Endothelium-dependent function was calculated as the percent increase of CBF or coronary artery diameter (CAD) in response to Ach. Endothelium-independent vasodilation of the conduit coronary artery was assessed by the percent increase of CAD in response to nitroglycerin [24,25]. This analysis was performed by experienced observers unaware of adiponectin levels.

2.7. Statistical analysis

Values are expressed as the mean ± SD or as the median (interquartile range) for skewed variables. Statistical significance was accepted when the p value was <0.05. Before statistical analysis, normal distribution and homogeneity of the variances were tested. Parameters that did not fulfill these tests (adiponectin and HOMA-R) were log-transformed. All assessed variables (e.g. age, sex, waist circumference, waist-to-hip ratio, log adiponectin, log HOMA-R, triglycerides, HDL-cholesterol, LDL-cholesterol, immunoreactive insulin, fasting plasma glucose, C-reactive protein, uric acid, mean blood pressure, and smoking) were entered as parameters for coronary vasoreactivity into both a linear regression and a multiple stepwise regression model. Log adiponectin values were entered the ROC-analyses to assess the best cut-off

Table 1
Patients characteristics

Age (years)	60 ± 12
Female	12 (33%)
Premenopausal	3 (8%)
Postmenopausal	9 (25%)
BMI (kg/m^2)	24.7 ± 3.8
Waist circumference (cm)	80.7 ± 9.6
Waist-to-hip ratio	0.85 ± 0.07
Mean BP (mm Hg)	93 ± 19
Smoking	8 (12%)
Hyperglycemia	6 (17%)
Dyslipidemia	23 (64%)
Hypertension	19 (53%)
Medications	
β-Adrenergic blockers	7 (19%)
Calcium channel blockers	10 (28%)
Long-acting nitrates	12 (33%)
ACE inhibitors	6 (17%)
Angiotensin II receptor blockers	3 (8%)
Statins	5 (14%)
Fibrates	0 (0%)

Values are given as mean ± SD or no. (%). BMI indicates body mass index; BP, blood pressure; ACE inhibitors, angiotensin-converting enzyme inhibitors.

Table 2
Biochemical data

Adiponectin (mg/L)	8.0 (6.3–10.0)
Log adiponectin	0.91±0.22
HOMA-R	1.87 (1.5–3.72)
Log HOMA-R	0.28±0.34
Triglycerides (mmol/L)	1.66±0.73
HDL-C (mmol/L)	1.33±0.26
LDL-C (mmol/L)	3.43±0.79
IRI (mU/L)	9.8±6.6
FPG (mmol/L)	5.49±0.64
C-reactive protein (mg/L)	2.34±4.34
Uric acid (μmol/L)	394.7±125.6

Values are given as mean±SD for normally distributed data and median (interquartile range) for non-parametric data. HOMA-R indicates homeostasis model assessment of insulin resistance index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; IRI, immunoreactive insulin; FPG, fasting plasma glucose.

point for detecting coronary endothelial dysfunction. The sensitivity, specificity, and predictive accuracy were calculated from 2×2 tables and were expressed as a percentage. The best cut-off point was chosen at the level that yielded the highest sensitivity, specificity, and predictive accuracy. Coronary endothelial dysfunction was defined as follows: Ach induced %change in CBF ≤0%; Ach induced %change in CAD ≤0% [24].

3. Results

3.1. Plasma adiponectin levels and patient characteristics

A total of 36 patients (24 men and 12 women) were enrolled in the study. Patient characteristics are summarized in Table 1. The mean age was 60±12 years. Characteristics of biochemical measurement data are shown in Table 2. The median (interquartile range) plasma adiponectin concentration was 8.00 (6.25–10.00) mg/L.

As shown in Fig. 1, log-transformed adiponectin concentration was inversely associated with log-transformed HOMA-R ($r=-0.58$, $p<0.001$), immunoreactive insulin (IRI) ($r=-0.58$, $p<0.001$), BMI ($r=-0.60$, $p<0.001$), and triglycerides levels ($r=-0.67$, $p<0.001$).

3.2. Changes in coronary blood flow induced by Ach

Relationships between percent change in CBF induced by Ach and clinical parameters including log-transformed adiponectin are shown in Table 3. Log-transformed adiponectin, BMI, log-transformed HOMA-R, IRI, and

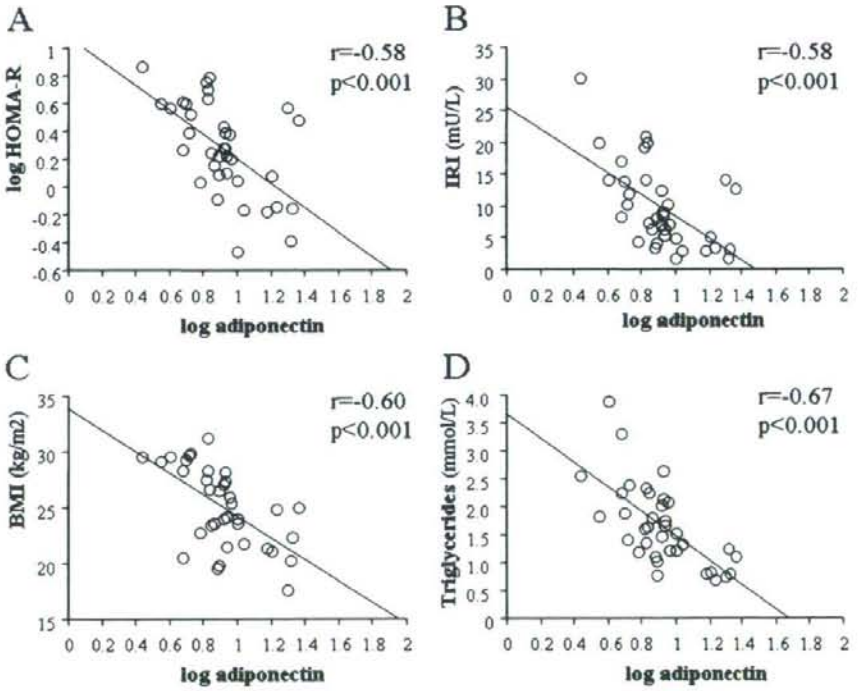

Fig. 1. Relationships between adiponectin and HOMA-R, IRI, BMI, and triglycerides. These graphs show the correlation between log adiponectin and log HOMA-R (A), IRI (B), BMI (C), and triglycerides concentrations (D).

Table 3
Simple and multiple regression analysis between %change in CBF and parameters

Parameter	Simple regression		Stepwise multiple regression		
	r	p	F value	p	Adjusted R^2
Age	−0.040	NS	0.457	NS	
Sex (female)	0.064	NS	0.014	NS	
Body mass index	−0.504	<0.01	1.445	NS	
Waist circumference	−0.311	NS	0.489	NS	
Waist-to-hip ratio	−0.258	NS	0.731	NS	
Log adiponectin	0.623	<0.0001	21.62	<0.0001	0.371
Log HOMA-R	−0.560	<0.001	3.531	NS	
Triglycerides	−0.414	<0.05	0.410	NS	
HDL-cholesterol	0.178	NS	0.035	NS	
LDL-cholesterol	−0.431	NS	2.740	NS	
Immunoreactive insulin	−0.501	<0.01	1.579	NS	
Fasting plasma glucose	−0.182	NS	0.016	NS	
C-reactive protein	−0.074	NS	0.007	NS	
Uric acid	−0.188	NS	0.177	NS	
Mean blood pressure	0.030	NS	1.341	NS	
Smoking	−0.076	NS	0.196	NS	

HDL-C indicates high-density lipoprotein cholesterol; LDL-C indicates low-density lipoprotein cholesterol; HOMA-R, homeostasis model assessment of insulin resistance index; NS, not significant ($p>0.05$).

Table 4
Simple and multiple regression analysis between %change in CAD and parameters

Parameter	Simple regression		Stepwise multiple regression		
	r	p	F value	p	Adjusted R^2
Age	−0.120	NS	0.587	NS	
Sex (female)	0.071	NS	0.028	NS	
Body mass index	−0.507	<0.01	1.453	NS	
Waist circumference	−0.306	NS	0.425	NS	
Waist-to-hip ratio	−0.277	NS	0.992	NS	
Log adiponectin	0.628	<0.0001	22.16	<0.0001	0.377
Log HOMA-R	−0.517	<0.01	2.014	NS	
Triglycerides	−0.449	<0.01	0.085	NS	
HDL-cholesterol	0.168	NS	0.279	NS	
LDL-cholesterol	−0.371	NS	1.990	NS	
Immunoreactive insulin	−0.536	<0.001	2.526	NS	
Fasting plasma glucose	−0.263	NS	0.549	NS	
C-reactive protein	−0.054	NS	0.373	NS	
Uric acid	−0.043	NS	0.374	NS	
Mean blood pressure	0.044	NS	0.151	NS	
Smoking	−0.040	NS	0.044	NS	

HDL-C indicates high-density lipoprotein cholesterol; LDL-C indicates low-density lipoprotein cholesterol; HOMA-R, homeostasis model assessment of insulin resistance index; NS, not significant ($p>0.05$).

triglycerides were significantly correlated with percent change in CBF induced by Ach in a simple regression analysis.

In a stepwise multiple regression analysis, log-transformed adiponectin was the only independent predictor of percent change in CBF induced by Ach ($p<0.0001$). The association between the percent change in CBF induced by Ach and log-transformed adiponectin is demonstrated in Fig. 2. Log-transformed adiponectin was positively correlated with percent change in CBF induced by Ach ($r=0.62$, $p<0.0001$), which suggests that lower levels of plasma adiponectin are associated with impaired endothelial function in resistance coronary arteries.

3.3. Changes in coronary artery diameter induced by Ach

Relationships between percent change in CAD induced by Ach and clinical parameters including log-transformed adiponectin are shown in Table 4. Log-transformed adiponectin, BMI, log-transformed HOMA-R, IRI, and triglycerides were significantly correlated with percent change in CAD induced by Ach in a simple regression analysis.

In a stepwise multiple regression analysis, log-transformed adiponectin was the only independent predictor of percent change in CAD induced by Ach ($p<0.0001$). The association between the percent change in CAD induced by Ach and log-transformed adiponectin is demonstrated in Fig. 3. Log-transformed adiponectin was positively

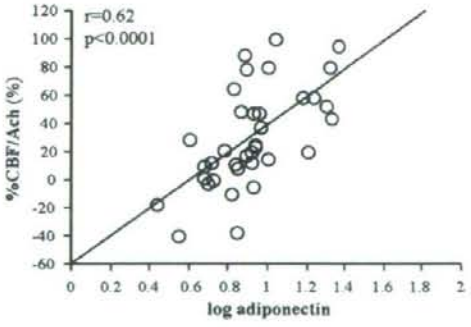

Fig. 2. Relationship between adiponectin and %change in CBF induced by Ach.

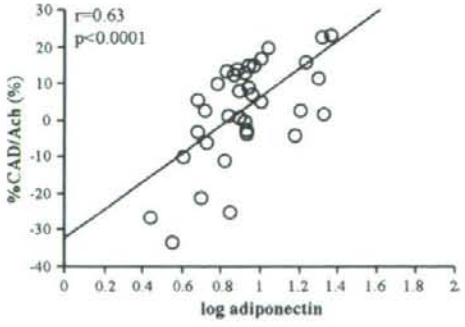

Fig. 3. Relationship between adiponectin and %change in CAD induced by Ach.

correlated with percent change in CAD induced by Ach ($r=0.63$, $p<0.0001$), which suggests that lower levels of plasma adiponectin are associated with impaired endothelial function in conduit coronary arteries.

3.4. Relationship between adiponectin levels and endothelium-independent vasodilation

There was no correlation between log-transformed adiponectin and percent change in CBF induced by papaverine ($r=0.001$, $p=0.996$) or percent change in CAD induced by nitroglycerin ($r=0.161$, $p=0.551$), which suggests that plasma adiponectin levels have no association with endothelium-independent vasodilating response in resistance and conduit coronary arteries.

3.5. Predictive value of adiponectin levels for coronary endothelial function

The best cut-off point for the prediction of coronary endothelial dysfunction was log adiponectin <0.8 (plasma adiponectin concentration <6.3 mg/L). The sensitivity of this criterion for prediction of endothelial dysfunction of resistance coronary artery was 75%, while the specificity was 85%, and the predictive accuracy was 83%. The sensitivity of this criterion for prediction of endothelial dysfunction of conduit coronary artery was 70%, the specificity was 88%, and the predictive accuracy was 83%.

4. Discussion

The present study demonstrated that adiponectin levels positively correlated with changes in coronary blood flow and coronary artery diameter induced by acetylcholine, suggesting that decreased adiponectin levels are associated with coronary endothelial dysfunction in conduit and resistance coronary arteries. Furthermore, body mass index (BMI), log-transformed HOMA-R, immunoreactive insulin (IRI), and triglycerides levels were also significantly and negatively correlated with changes of both coronary blood flow and coronary artery diameter induced by acetylcholine. However, among these parameters, adiponectin level was the only independent factor that correlated with coronary endothelial function.

4.1. Association of hypoadiponectinemia with coronary endothelial dysfunction

Several clinical studies have described an association between hypoadiponectinemia and endothelial dysfunction. For example, Ouchi et al. [18], demonstrated that plasma adiponectin level strongly correlated with the vasodilator response to reactive hyperemia in hypertensive patients as determined by the analysis of forearm blood flow by strain-gauge plethysmography, which is an established non-invasive method for the assessment of endothelial function. Although a previous study suggested that endothelial dysfunction detected in the arm correlated with coronary endothelial dysfunction [30], these data did not indicate whether the correlation between adiponectin and endothelial function was similar when comparing coronary and peripheral arteries. Further, although the brachial artery does develop atherosclerosis [31], it is clinically different from the obstructive disease that affects the coronary circulation. For these reasons, the present study was designed to investigate the more specific association between adiponectin and Ach-induced vasodilation in the coronary artery. Data from the present study suggest that assessment of plasma adiponectin levels is a non-invasive method of predicting endothelial dysfunction, even in the coronary circulation.

In contrast to our results, Singhal et al. [32] demonstrated no significant correlation between adiponectin concentration and brachial artery flow-mediated endothelial-dependent dilatation in a large study of young and healthy adolescents (age 13–16 years). Based on these observations, they speculated that longer time periods of low adiponectin concentration may be required to impair endothelial function or that other risk factors found in adults are required for the adverse effect of adiponectin on vascular physiology, which is consistent with observations from other studies [16–18].

4.2. Coronary endothelial function does not correlate with waist circumference

Despite prior reports of the relationship between adiponectin concentrations and intra-abdominal fat [33,34], our data unexpectedly failed to show any significant correlation between endothelium-dependent vasodilatation and waist circumference or waist-to-hip ratio. Williams et al. [34] demonstrated that brachial artery flow-mediated vasodilatation was associated with waist circumference and waist-to-hip ratio in healthy, relatively young subjects. Similarly, Arcaro et al. [35] reported that body fat distribution predicts the degree of endothelial dysfunction. To remove the potential confounding element of gender on data analysis, the relationship between waist circumference or waist-to-hip ratio and coronary endothelial function was analyzed in a gender-specific manner. In 24 male patients, waist circumference was significantly associated with both the percent change in coronary blood flow (CBF) and coronary artery diameter (CAD) in a simple regression analysis ($r=-0.44$, $p<0.05$; $r=-0.43$, $p<0.05$, respectively). However, multiple regression analysis of male patients demonstrated that waist circumference was a non-independent predictor of the coronary endothelial dysfunction. It is possible that inclusion of subjects with confounding risk factors for endothelial dysfunction (e.g., hypertension, increased insulin resistance, dyslipidemia) may account for these findings, as previous studies [34,35] excluded patients with risk factors from their analysis. Indeed, a recent study reported that waist circumference was not an independent

predictor of endothelial dysfunction after adding insulin resistance to the multivariable regression analysis [36]. Analysis of 12 females in our study population failed to identify any significant correlation between waist circumference and CBF or CAD response to acetylcholine. Future studies should include a larger population of females and perform subgroup analysis based on menopausal status, which may influence the endothelial function [37].

4.3. Utility of circulating adiponectin levels for predicting coronary endothelial function

In the present study, coronary endothelial dysfunction was more strongly correlated with a low adiponectin level than with an increased level of HOMA-R, IRI, triglycerides, and BMI. Previous studies have shown links between adiponectin levels and coronary vascular disease (CVD). For example, in a prospective study, men with high plasma adiponectin levels without previous CVD were at lower risk of myocardial infarction. This association was independent of traditional cardiovascular risk factors [38]. In another study, an independent correlation between low adiponectin concentration and the development of acute coronary syndrome was demonstrated [39]. Further, a case–control study performed in Japan demonstrated that plasma adiponectin levels less than 4 mg/L were associated with an increased risk of coronary artery disease [40]. These results are comparable with observations from the present study that adiponectin was the only independent predictor of coronary endothelial function among all of the variables investigated. Additionally, the present study demonstrated that a cut-off level of log adiponectin <0.8 (plasma adiponectin concentration <6.3 mg/L) had high specificity for the detection of coronary endothelial dysfunction. However, the predictive cut-off value of adiponectin for coronary endothelial dysfunction may be suboptimal. Future study of larger populations that include separate analysis of men and women and subgroup analysis of menopausal status could improve the predictive value of these data, as plasma adiponectin concentrations are modulated by gender [41] and menopausal status [42].

Adiponectin circulates in plasma in three forms: a trimer, a hexamer, and a larger multimeric high-molecular weight (HMW) form. Although biological activities of these isoforms are a matter of controversy, there is some evidence that HMW adiponectin may be the active form of the protein. Kobayashi et al. demonstrated that HMW adiponectin suppressed apoptosis in cultured cells, suggesting a vasculoprotective property of HMW adiponectin [43]. The ELISA method used in the current study measured the monomeric form of adiponectin [7]. Thus, we could not evaluate the predictive value of HMW adiponectin levels for coronary endothelial dysfunction. However, the present data suggest an independent relationship between total adiponectin concentrations and coronary endothelium-dependent vasodilatation.

Several groups have investigated the short-term dynamics of plasma adiponectin concentrations. For example, Merl et al. reported that serum adiponectin concentrations were stable over 72 h of fasting in normal and over-weight subjects, and adiponectin levels were similar before and after fasting [44]. Based on these data, they concluded that adiponectin reflected long-term changes in body weight rather than short-term regulatory influences. Hotta et al. demonstrated no daily change in adiponectin levels in both the non-diabetic and diabetic subjects who received breakfast, lunch and dinner [8]. By contrast, Gavrila et al. reported that serum adiponectin declined at night, reaching a nadir in the early morning in normal weight healthy men [45], and recent studies reported that adiponectin levels decreased after acute smoking [46] or rowing [47]. Characterization of the day-to-day or intra-day variability of adiponectin concentration may improve its utility as a predictor of endothelial dysfunction and various disease states.

4.4. Clinical implications

The present study characterized an independent relationship between adiponectin and coronary endothelial dysfunction. Therefore, plasma adiponectin may be a useful non-invasive predictor of coronary endothelial dysfunction, even in patients without overt coronary atherosclerotic lesions or diabetes mellitus. If a causal relationship between adiponectin and coronary endothelial dysfunction is established, strategies to increase adiponectin levels, such as through reduction of visceral fat, caloric restriction, and moderate physical exercise [48,49], may be of use in reducing morbidity and mortality from cardiovascular disease. However, there is no current therapy that specifically raises adiponectin without changing other variables such as insulin sensitivity or lipid levels.

4.5. Limitations

This study possesses several limitations. First, our data was based on a single measurement of adiponectin concentrations, and the time course relationship with vascular events cannot be predicted from the current study. Additionally, the methodology of coronary angiography and selective intra-coronary infusion used in this study is not amenable to establishing causality through repeat exams. Second, the present study is a cross-sectional study, and its findings warrant confirmation through a prospective study. Third, because of relatively small numbers of participants in our study population, further investigation involving a large number of patients with adjustments for confounding factors (e.g., gender, menopausal status) is required to confirm the value of adiponectin as a marker of coronary endothelial function and its clinical implications. Finally, the study population included only non-diabetic patients with normal or mildly diseased coronary arteries. Thus, the present findings may not be applicable to patients with diabetes or advanced coronary artery disease.

5. Conclusions

This in vivo study demonstrated that circulating adiponectin level correlated with coronary artery endothelial function. This study supports a role for adiponectin as a better predictor of coronary endothelial function when compared with other known predictors.

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Relationship between hyperglycemia and coronary vascular resistance in non-diabetic patients

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Abstract

Background: Hyperglycemia upon hospital admission in patients with acute myocardial infarction is associated with the no-reflow phenomenon after successful reperfusion, and increased mortality. However, the mechanism underlying this phenomenon remains unclear. Therefore, the aim of this study was to characterize coronary hemodynamics in a homogenous group of non-diabetic patients without coronary artery disease.

Methods and Results: A total of 104 consecutive non-diabetic patients (mean age, 62±14 years) without coronary artery disease underwent Doppler flow study of the left anterior descending coronary artery. Vascular reactivity was examined by intra-coronary administration of papaverine, acetylcholine (Ach), and nitroglycerin using a Doppler guidewire. Coronary vascular resistance (CVR) was calculated as the mean arterial pressure divided by coronary blood flow (CBF). Baseline CVR was shown as CVR at control and minimal CVR was shown as CVR with papaverine administration. Fasting plasma glucose (FPG) level had a significant, positive correlation with baseline CVR and minimal CVR ($r=0.24$, $p<0.02$ and $r=0.21$, $p<0.05$, respectively). Hemoglobin A1c (HbA1c) also had a significant, positive correlation with baseline CVR and minimal CVR ($r=0.31$, $p<0.01$ and $r=0.32$, $p<0.01$, respectively). The percent change in CBF induced by Ach was inversely correlated with HbA1c but not with FPG ($r=0.22$, $p<0.05$ and $r=0.06$, $p=0.57$, respectively). By contrast, neither FPG nor HbA1c had significant correlation with coronary flow reserve to papaverine.

Conclusion: These data demonstrate that elevated glucose levels are associated with increases in baseline and minimal coronary vascular resistance. These changes may contribute to unfavorable coronary hemodynamics in non-diabetic patients without coronary heart disease.

Keywords: Coronary vascular resistance; FBS; HbA1; Hyperglycemia

1. Introduction

Hyperglycemia upon hospital admission in patients with acute myocardial infarction (AMI) is associated with the no-reflow phenomenon after successful reperfusion [1], resulting in larger infarct size and worse functional recovery. Further, hyperglycemia in patients with ST-segment elevation acute myocardial infarction is an important predictor of impaired epicardial flow before reperfusion therapy [2], and hyperglycemia in patients with AMI is associated with increased mortality [3–7]. However, the mechanisms underlying these adverse effects of hyperglycemia remain unknown. Therefore, the aim of this study was to characterize coronary hemodynamics in a

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Table 1
Clinical characteristics of the study patients

Age (years)	62±14 (17–85)
Male gender	69 (66%)
Risk factors	
Hypertension	57 (55%)
Hyperlipidemia	35 (34%)
Smoker	27 (26%)
Laboratory data	
FPG (mg/dl)	94±8 (74–120)
HbAlc (%)	5.2±0.4 (4.1–6.3)
Total-cholesterol (mg/dl)	197±37 (114–314)
Triglycerides (mg/dl)	122±64 (40–368)
HDL–cholesterol (mg/dl)	57±15 (28–98)
LDL–cholesterol (mg/dl)	113±28 (53–211)

HDL: high density lipoprotein; LDL: low density lipoprotein; Values are mean±SD.

homogenous group of non-diabetic patients without coronary artery disease.

2. Methods

2.1. Study population

A total of 187 consecutive non-diabetic patients who had been referred for cardiac catheterization to exclude coronary artery disease were considered for enrollment in this study. Of these, 104 patients met the following inclusion criteria: 1) angiographically smooth arteries; 2) mild irregularities, <30% lumen diameter stenosis by visual assessment in any major conduit vessel; 3) proximal coronary arteries >2.0 mm in diameter; and 4) lacking a history of previous myocardial infarction, previous coronary revascularization, valvular heart disease, variant angina, cardiomyopathy, or myocarditis.

Patients meeting the following criteria were considered to have obvious diabetes and excluded: 1) previous diagnosis of diabetes, 2) current treatment by oral hypoglycemic agents or insulin, or 3) concentration of fasting plasma glucose (FPG) >126 mg/dl or hemoglobin A1c (HbA1c) >6.5% at admission [8].

Informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

2.2. Study protocol

Diagnostic coronary angiography was performed using a 6F Judkins catheter with a standard femoral percutaneous approach. Heparin (5000 units) was administered at the beginning of the procedure. Non-ionic contrast material was used for all patients. No nitroglycerin was given prior to the diagnostic procedure. Coronary blood flow (CBF) response to papaverine, acetylcholine (Ach), and nitroglycerin was studied according to previous reports [9,10]. After control coronary angiograms, interventions were performed as follows: 1) a 0.014-inch Doppler guidewire (Cardiometrics, Santa Anna, CA, USA) was introduced into the left anterior descending coronary artery; 2) after obtaining a stable Doppler signal, a bolus of papaverine (an endothelium-independent vasodilator in resistance coronary arteries) (12.5 mg/5 ml) was injected through a catheter; 3) infusion of Ach (an endothelium-dependent vasodilator in resistance and conduit coronary arteries) (0.5 ml/min) at a dose of 3 μg/min for 2 min was performed via the catheter [11,12,4] a bolus of nitroglycerin (an endothelium-independent vasodilator in conduit coronary arteries) (200 μg/5 ml) was administered. Drugs were infused with a minimum 5-min interval. Coronary arteriography was performed before and 2 min after each dose of Ach and after administration of nitroglycerin. Phasic CBF velocities, arterial blood pressure, and heart rate were monitored continuously and recorded. Measurements obtained during steady state conditions were used as control values for later analysis.

Doppler flow velocity spectra were analyzed on-line to determine time-averaged peak velocity. Volumetric CBF was determined from the formula: CBF=cross-sectional area×average peak velocity×0.5 [13]. Coronary flow reserve to papaverine was calculated as the ratio of maximal CBF induced by papaverine to basal CBF, which was equivalent to

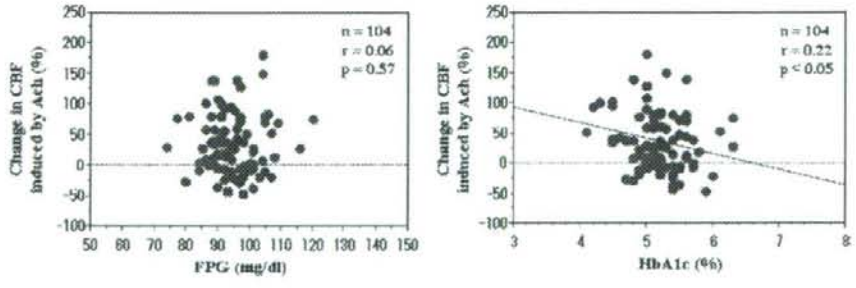

Fig. 1. Scattergram illustrating the correlation between percent change in CBF induced by Ach and FPG (left panel) and HbA1c (right panel).

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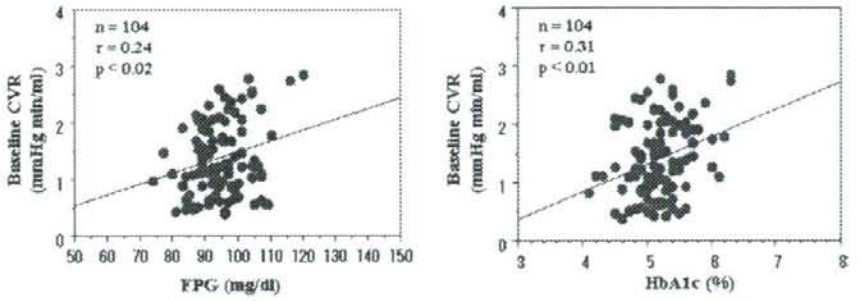

Fig. 2. Scattergram illustrating the correlation between baseline CVR and FPG (left panel) and HbA1c (right panel).

the endothelium-independent function of the resistance coronary artery. Endothelium-dependent function was calculated as the percent increase of CBF in response to Ach [9,14].

Coronary vascular resistance (CVR) was calculated by mean arterial pressure divided by CBF. Baseline CVR is shown as CVR at control, and minimal CVR is shown as CVR with papaverine administration.

2.3. Statistics

All data are expressed as the mean value±SD. One-way analysis of variance (ANOVA) was used for comparison of continuous variables, and significance of difference was calculated using the Scheffe F test. Differences were considered significant at $p<0.05$. Statistical analysis was performed with Statview, ver. 5.0 (SAS Institute Inc., Cary, NC).

3. Results

A total of 104 patients were evaluated. Patient characteristics are shown in Table 1. All patients enrolled in this study had concentrations of FPG<126 mg/dl and HbA1c<6.5%.

3.1. Changes in coronary blood flow

The relationship between FPG, HbA1c, and percent change in CBF induced by Ach are shown in Fig. 1. Percent change in CBF induced by Ach (i.e., namely endothelium-dependent vasodilatation in resistance arteries) was inversely correlated with HbA1c but not with FPG, which suggests that chronically higher levels of glucose concentration are associated with impaired endothelial function in resistance coronary arteries in non-diabetic patients. Coronary flow reserve to papaverine (i.e., endothelium-independent vasodilatation in resistance arteries) did not correlate with FPG or HbA1c ($r=0.08$, $p=0.42$; $r=0.02$, $p=0.85$, respectively) in non-diabetic patients.

3.2. Changes in coronary artery diameter

Neither FPG nor HbA1c was correlated with percent change in coronary artery diameter (CAD) induced by Ach (i.e., namely endothelium-dependent vasodilatation in epicardial arteries) ($r=0.01$, $p=0.97$ and $r=0.03$, $p=0.82$, respectively). Similarly, percent change in CAD induced by nitroglycerin (i.e., namely endothelium-independent vasodilatation in epicardial arteries) did not correlate with FPG or HbA1c ($r=0.01$, $p=0.94$ and $r=0.06$, $p=0.57$, respectively). Thus, neither FPG nor HbA1c were correlated with endothelium-dependent and -independent vasodilation of the epicardial coronary arteries in non-diabetic patients.

3.3. Coronary vascular resistance

The relationship between baseline CVR and minimal CVR to FPG and HbA1c are shown in Figs. 2 and 3, respectively.

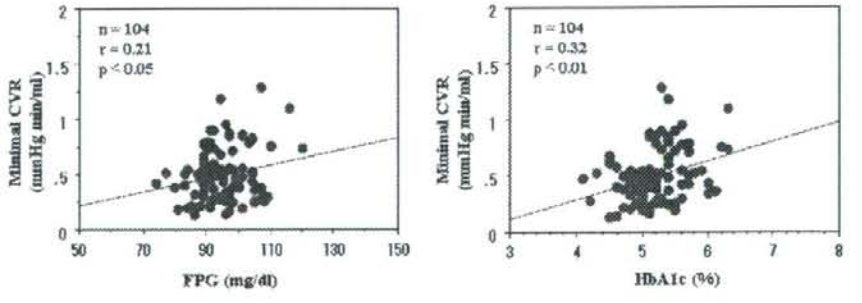

Fig. 3. Scattergram illustrating the correlation between minimal CVR and FPG (left panel) and HbA1c (right panel).

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FPG and HbA1 had a significant, positive correlation with baseline CVR and minimal CVR. These results suggest that acute and chronic high glucose concentrations are associated with increased vascular resistance in coronary arteries at baseline and at a hyperemic state in non-diabetic patients.

4. Discussion

The present study demonstrated that chronic exposure to high glucose concentrations is associated with coronary endothelial dysfunction and that baseline and minimal CVR are elevated in the context of acute and chronic hyperglycemia in non-diabetic patients without coronary artery disease. These data suggest that elevated blood glucose concentrations may contribute to impaired coronary flow in non-diabetic patients.

4.1. Hyperglycemia and coronary endothelial function

Previous studies have reported that chronic hyperglycemia results in impairments in endothelium-dependent coronary artery vasodilation [15,16], which is consistent with results from the present study. Further, other studies have demonstrated that acute hyperglycemia results in impaired coronary flow via attenuation of mitochondrial ATP—sensitive potassium channel (K_{ATP} channel) activation and via promotion of platelet-dependent thrombus formation [17,18]. By contrast, other investigators have reported that hyperglycemia-induced vascular damage is mediated by increased production of oxygen free radicals, including superoxide anion, from endothelial cells [19–21]. Increased superoxide anion can inactivate nitric oxide and result in enhanced contractility and proliferation of vascular smooth muscle cells with increased vasomotor tone, platelet hyper-reactivity [22], alteration of the adhesive properties of the endothelium [23], and increased production of cytokines [24].

4.2. Hyperglycemia and coronary vascular resistance

Farouque et al. [25] suggested that K_{ATP} channels contribute to basal CVR by demonstrating that inhibition of these channels resulted in impairments in resting CVR. Further, acute hyperglycemia prevents the positive effect of ischemic preconditioning, probably through the attenuation of mitochondrial K_{ATP} channel activity. Barbagallo et al. [26] reported that glucose increased intracellular calcium content in vascular smooth muscle, while other investigators reported that FPG and HbA1c levels are both closely related to fasting basal cytosolic free calcium levels [27]. These observations are consistent with results from the present study and may provide a mechanistic link between why acute hyperglycemia and impaired coronary flow.

In this study, patients with obvious diabetes were excluded. Kawano et al. [28] demonstrated glucose-induced impairments in endothelium-dependent vasodilation in patients with IGT, even when FRG levels were within a normal limit. Further, this effect persisted at 2 h after glucose loading. Thus, repeated exposure to post-prandial hyperglycemia may play an important role in the vascular dysfunction, even in our patients without obvious diabetes.

4.3. Limitations

This study possesses several limitations. First, the study population included only patients with normal or mildly diseased coronary arteries. Thus, the present findings may not be applicable to patients with advanced coronary artery disease. Second, the present study was based on a retrospective analysis of the patients and was only a descriptive study in which we established an association between elevated blood glucose concentrations and an increase of CVR; a more prospective study is required to determine the effect of glycemic control on CVR.

5. Conclusion

Increases in baseline and minimal CVR in association with acute or chronic hyperglycemia may contribute to unfavorable coronary hemodynamics and mortality in non-diabetic patients.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [29].

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STEM CELLS
TISSUE-SPECIFIC STEM CELLS

Allogeneic Injection of Fetal Membrane-Derived Mesenchymal Stem Cells Induces Therapeutic Angiogenesis in a Rat Model of Hind Limb Ischemia

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Key Words. Fetal • Mesenchymal stem cells • Allogeneic • Transplantation • Rat • Angiogenesis

ABSTRACT

Bone marrow-derived mesenchymal stem cells (BM-MSC) have been demonstrated to be an attractive therapeutic cell source for tissue regeneration and repair. However, it remains unknown whether or not allogeneic transplantation of mesenchymal stem cells (MSC) derived from fetal membranes (FM), which are generally discarded as medical waste after delivery, has therapeutic potential. FM-MSC were obtained from Lewis rats and had surface antigen expression and multipotent potential partly similar to those of BM-MSC. Compared with BM-MSC, FM-MSC secreted a comparable amount of hepatocyte growth factor despite a small amount of vascular endothelial growth factor. FM-MSC and BM-MSC both expressed major histocompatibility complex (MHC) class I but not MHC class II antigens and did not elicit allogeneic lymphocyte proliferation in mixed lymphocyte culture. FM-MSC or BM-MSC obtained from Lewis rats were injected into a MHC-mismatched August-Copenhagen-Irish rat model of hind limb ischemia. Three weeks after injection, blood perfusion and capillary density were significantly higher in the FM-MSC and BM-MSC groups than in the phosphate-buffered saline group, and allogeneic FM-MSC and BM-MSC were still observed. In nonischemic hind limb tissues, allogeneic FM-MSC and BM-MSC injection were associated with a comparatively small amount of T lymphocyte infiltration, compared with the injection of allogeneic splenic lymphocytes. In conclusion, allogeneic FM-MSC injection did not elicit a lymphocyte proliferative response and provided significant improvement in a rat model of hind limb ischemia, comparable to the response to BM-MSC. Thus, allogeneic injection of FM-MSC may be a new therapeutic strategy for the treatment of severe peripheral vascular disease. STEM CELLS *2008;26:2625–2633*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSC) reside within bone marrow (BM), adipose tissue, and many other tissues and can differentiate into various cell types [1–3]. These features make MSC an attractive therapeutic tool for tissue regeneration and repair, including the treatment of peripheral artery disease [4]. Although autologous bone marrow-derived mesenchymal stem cells (BM-MSC) are favored to avoid the immunological barrier, there are several limitations of using an autologous cell source, including invasiveness, inadequate cell numbers, and donor site morbidity [5]. Moreover, it has been reported that the differentiation and proliferation potentials of MSC decrease with age [6, 7]. Thus, an alternative source of autologous MSC that can be obtained noninvasively and in sufficient quantity is required.

Recently, human fetal tissues have been found to be rich sources of MSC [8, 9]. Several studies have shown that MSC can be isolated from human fetal membranes (FM) [10, 11]. FM are generally discarded as medical waste after delivery, and therefore there are no ethical concerns associated with their use. FM-MSC could be used for allogeneic cell transplantation, but it remains possible that they would be rejected by the host immune system. However, FM are known to play a role in preventing rejection of the fetus and are thought to have low immunogenicity [12–14]. Moreover, it has been reported that MSC from various origins fail to trigger allogeneic T-cell pro-

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liferation and induce immune tolerance [15, 16]. Several preclinical and clinical studies have demonstrated that allogeneic BM-MSC prevent graft-versus-host disease in allogeneic BM transplantation [17–19]. These results suggest that allogeneic FM-MSC are a potential alternative to autologous and allogeneic BM-MSC in cell therapy. However, there is little available information on the ability of FM-MSC to evade the allogeneic immune response after transplantation. Furthermore, the therapeutic efficacy of FM-MSC, compared with BM-MSC, in ischemic diseases remains unclear. Thus, the purposes of this study were (a) to isolate and characterize FM-MSC from rats, (b) to investigate whether or not FM-MSC evade T lymphocyte alloreactivity, and (c) to investigate whether allogeneic injection of FM-MSC has a therapeutic effect in a rat model of hind limb ischemia.

MATERIALS AND METHODS

Animals

Different strains of rats were used according to their major histocompatibility complex (MHC) antigen disparity: Lewis rats (MHC haplotype: RT-1^l) (Japan SLC, Hamamatsu, Japan, http://www.jslc.co.jp), ACI rats (MHC haplotype: RT-1^a) (Japan SLC), and green fluorescent protein (GFP)-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Japan). Adult rats, ages 8–12 weeks, were used in all experiments and were maintained in our animal facilities. The experimental protocols were approved by the Animal Care Committee of the National Cardiovascular Center Research Institute.

Isolation and Expansion of FM- and BM-MSC

Isolation and expansion of BM-MSC were performed as described previously [20]. In brief, male Lewis rats were sacrificed, and BM was harvested by flushing the cavity of the femurs and tibias with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, http://www.invitrogen.com). To obtain FM, pregnant Lewis rats (15 days postconception) were sacrificed, and uteri were harvested and placed in PBS. After separation from the placenta, FM were minced with scissors and digested with type II collagenase solution (300 U/ml; Worthington Biochemical, Lakewood, NJ, http://www.worthington-biochem.com) for 1 hour at 37°C in a water shaker. Enzyme activity was neutralized with α-minimal essential medium (α-MEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen). After filtration through a mesh filter (100 μm; BD Biosciences, Bedford, MA, http://www.bdbiosciences.com) and centrifugation at 300*g* for 5 minutes, BM and dissociated FM cells were suspended in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen); plated onto 100-mm noncoated culture dishes; and incubated at 37°C in 5% CO_2. A small number of cells developed into visible symmetric colonies by days 5–7. Nonadherent hematopoietic cells were removed, and the medium was replaced. The adherent, spindle-shaped MSC populations were expanded to more than 50 million cells after approximately 4–5 passages. Isolation of FM- and BM-MSC was repeated three times to evaluate reproducibility. In all experiments, FM- and BM-MSC were used at passages 3–6.

Differentiation of FM- and BM-MSC into Adipocytes, Osteocytes, and Chondrocytes

FM- and BM-MSC were seeded onto six-well plates, and differentiation into adipocytes and osteocytes was induced at 40%–50% confluence. To induce differentiation into adipocytes, MSC were cultured with the following adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Ltd., Osaka, Japan, http://www.wako-chem.co.jp), 1 μM dexamethasone (Wako Pure Chemical Industries, Ltd.), 50 μM indomethacin (Wako Pure Chemical Industries, Ltd.), and 10 μg/ml insulin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) in α-MEM. After 2 weeks of differentiation, adipocytes were identified by the existence of lipid vesicles stained with oil red O (Sigma-Aldrich).

To induce differentiation into osteocytes, MSC were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan, http://www.ds-pharma.co.jp), according to the manufacturer's instructions. After 2 weeks of differentiation, osteocytes were identified by the existence of mineral nodule deposition stained with alizarin red S (Sigma-Aldrich).

To induce differentiation into chondrocytes in three-dimensional culture, the pellet culture method was used. MSC were centrifuged at 150*g* for 5 minutes and resuspended at a density of 1×10^6 cells per milliliter in the hMSC Differentiation BulletKit, Chondrogenic (Cambrex, Walkersville, MD, http://www.cambrex.com), supplemented with transforming growth factor-β3 (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) according to the manufacturer's instructions. Briefly, 5×10^5 cells were placed in a 15-ml polypropylene tube and centrifuged at 150*g* for 5 minutes. Fresh medium was added every 3rd day. After 3 weeks of differentiation, cell pellets were fixed with 4% paraformaldehyde and embedded in paraffin. Differentiation into chondrocytes was identified by the existence of proteoglycan deposition stained with safranin O (Sigma-Aldrich).

Flow Cytometry

Cultured FM- and BM-MSC were analyzed by flow cytometry (FACSCalibur; BD Biosciences). Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), CD45 (clone OX-1; BD Biosciences), CD73 (clone 5F/B9; BD Biosciences), CD90 (clone OX-7; BD Biosciences), $RT1A^{a,b,l}$ (clone B5; BD Biosciences), and RT1B (clone OX-6; BD Biosciences); phycoerythrin-conjugated mouse monoclonal antibodies against rat CD11b/c (clone OX-42; BD Biosciences) and CD31 (clone TLD-3A12; BD Biosciences); and FITC-conjugated hamster anti-rat CD29 monoclonal antibody (clone Ha2/5; BD Biosciences) were used. Isotype-identical antibodies served as controls.

Enzyme-Linked Immunosorbent Assay

The medium from the MSC cultures was collected as described previously [21]. Secreted vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) levels in the medium of the third passage of MSC culture (1×10^6 cells in a 100-mm dish cultured for 48 hours) were measured using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers' protocols (VEGF, R&D Systems; HGF, Institute of Immunology, Tokyo). Absorbance was measured with a microplate reader (Bio-Rad, Hercules, CA, http://www.bio-rad.com) at 450 or 490 nm.

Mixed Lymphocyte Culture

One-way mixed lymphocyte culture (MLC) was used to assess T lymphocyte reactivity against allogeneic cell populations. Spleens obtained from Lewis rats (MHC haplotype: RT-1^l) and August-Copenhagen-Irish (ACI) rats (MHC haplotype: RT-1^a) were minced with scissors in RPMI 1640 medium (Invitrogen) and filtered through a 100-μm mesh filter. Erythrocytes were lysed with 0.83% ammonium chloride for 1 minute, and cells were subsequently washed twice in RPMI 1640. Cell count and viability were assessed by trypan blue dye exclusion. Stimulating Lewis splenic lymphocytes and MSC (1×10^6 cells per milliliter) were treated with 50 μg/ml mitomycin C (Sigma-Aldrich) in the dark at 37°C for 30 minutes and washed three times in 20% FBS-containing RPMI 1640.

A total of 2×10^5 Lewis or MHC-mismatched ACI responding splenic lymphocytes and an equal number of stimulating Lewis splenic lymphocytes or MSC were cocultured in 0.2 ml of tissue culture medium RPMI 1640 supplemented with 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well flat-bottomed plates for 5 days. Proliferation of responding cells was assessed by the Cell Proliferation Biotrak ELISA System (GE Healthcare, Piscataway, NJ, http://www.gehealthcare.com) according to the manufacturer's instructions. Absorbance (Abs) was mea-

sured with a microplate reader (Bio-Rad) at 450 nm. Alloreactive lymphocyte proliferation was presented as the percentage of relative proliferative response, as follows: relative proliferative response (%) = $Absorbance_{Alloproliferation}/Absorbance_{Autoproliferation} \times 100$.

Interleukin-2 (IL-2) is known to play a role in the activation and proliferation of T cells [26]. Secretion of IL-2 was measured in the medium collected from the wells of MLC plates on day 5 post-coculture, using a Quantikine IL-2 Immunoassay Kit (R&D Systems). Absorbance was measured with a microplate reader (Bio-Rad) at 450 nm.

Allogeneic FM- and BM-MSC Injection in Rats with Hind Limb Ischemia

To create a hind limb ischemia model, the left common iliac artery in male ACI rats (220–250 g) was resected under anesthesia by intraperitoneal injection of 50 mg/kg pentobarbital sodium. The distal portion of the saphenous artery and all side branches, as well as the veins, were dissected free and excised. The right hind limb was kept intact and used as a nonischemic limb.

We previously reported that BM-MSC injection caused significantly greater improvement in hind limb ischemia than bone marrow-derived mononuclear cell (MNC) injection [4]. Therefore, BM-MSC derived from same Lewis rats were used as a control in this study. One day after resection of the left common iliac artery, rats were randomized to the following three groups: (a) BM-MSC injection (BM-MSC group; $n = 10$), (b) FM-MSC injection (FM-MSC group; $n = 10$), and (c) PBS injection (PBS group; $n = 10$). In each group, a total of 5×10^6 BM-MSC or FM-MSC obtained from Lewis rats or PBS was injected into the ischemic thigh muscle of MHC mismatched ACI rats with a 26-gauge needle at five different points.

Assessment of Blood Perfusion

A laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, U.K., http://www.moor.co.uk) was used to measure serial blood flow over a period of 3 weeks. Low or no blood perfusion was displayed as dark blue, whereas the highest perfusion was displayed as red. After blood flow had been scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification using stored images. The LDPI index was determined as the ratio of ischemic to nonischemic hind limb blood perfusion [22].

Capillary Density

Rats were sacrificed 3 weeks after injection. Four pieces of ischemic tissue from the adductor and semimembranous muscles were obtained and snap-frozen in liquid nitrogen. Frozen tissue sections were stained with alkaline phosphatase using an indoxyltetrazolium method to detect capillary endothelial cells [23]. Five fields from four tissue sections were randomly selected, and the number of capillaries was counted in each field. To avoid overestimation or underestimation of capillary density as a result of myocyte atrophy or interstitial edema, the capillary number was adjusted for the number of muscle fibers [23].

Assessment of Cell Survival and T Lymphocyte Alloreactivity After Allogeneic FM- and BM-MSC Injection

Allogeneic FM-MSC (5×10^6 cells per animal) derived from GFP-transgenic Lewis rats were injected into the normal or ischemic hind limb muscles of ACI rats. Allogeneic BM-MSC and splenic lymphocytes were used as controls. One or 3 weeks after cell injection, rats were sacrificed, and paraffin-embedded sections were obtained from the hind limb muscles and stained with hematoxylin and eosin (H&E).

To assess cell survival and T lymphocyte infiltration, tissue sections were incubated with rabbit anti-GFP antibody (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) or mouse anti-rat CD3 monoclonal antibody (BD Biosciences) for 40 minutes, followed by incubation with DakoCytomation Envision+ System-horseradish peroxidase (HRP) Labeled Polymer (DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com) or DakoCytomation LSAB2 System-HRP for use on Rat Specimens (DakoCytomation) for 30 minutes. Sections were visualized using 0.5% diaminobenzidine and 0.03% hydrogen peroxide and counterstained with hematoxylin. Five random fields on eight different sections from each rat were photographed (BIOREVO BZ-9000; Keyence, Osaka, Japan, http://www.keyence.com), and the mean numbers of GFP- or CD3-positive cells were counted to assess cell survival and T lymphocyte alloreactivity

Statistical Analysis

Data were expressed as mean ± SEM. Comparisons of two parameters were analyzed by using the unpaired Student's t test. Comparisons of parameters among groups were made by one-way analysis of variance, followed by the Tukey test. Differences were considered significant at $p < .05$.

RESULTS

Characterization of FM- and BM-MSC

In the early passages, FM-derived cells appeared microscopically heterogeneous (Fig. 1A), as did BM-derived cells. However, after several passages, FM-derived cells appeared to form a morphologically homogeneous population of fibroblast-like cells, which was similar to BM-MSC (Fig. 1A).

To evaluate the multipotency of FM-MSC and BM-MSC, we induced differentiation of both types of MSC into adipocytes, osteocytes, and chondrocytes. FM-MSC, as well as BM-MSC, differentiated into adipocytes, osteocytes, and chondrocytes, as demonstrated by oil red O, alizarin red S, and safranin O staining, respectively ($n = 3$) (Fig. 1B).

Flow cytometric analysis of cultured FM-MSC at passage 3 ($n = 3$) demonstrated that FM-MSC were positive for CD29, CD73, and MHC class I molecules (i.e., $RT1A^{a,b,l}$), but negative for CD11b/c, CD31, CD34, CD45, and MHC class II molecules (i.e., RT1B), which satisfied the criteria for identifying MSC [24, 25] (Fig. 1C). However, two distinct subpopulations of FM-MSC were evident in terms of CD90 expression, which was not the case in BM-MSC (66.6% ± 5.4% vs. 98.7% ± 0.4%; $p < .05$). The expression of antigens, except CD90, was equivalent in FM- and BM-MSC.

Secretion of Angiogenic Factors from Cultured FM- and BM-MSC

To investigate the secretion of major angiogenic factors from cultured FM-MSC, we performed ELISAs for VEGF and HGF. As shown, FM-MSC secreted a significant amount of growth factors, including VEGF and HGF (Fig. 2). Compared with BM-MSC, FM-MSC secreted less VEGF (82.5 ± 3.8 and 881.5 ± 58.7 pg/ml, respectively; $p < .05$ vs. BM-MSC; $n = 8$), but they secreted a comparable amount of HGF (351.0 ± 123.1 pg/ml) compared with BM-MSC (337.0 ± 120.0 pg/ml; $n = 8$).

T Lymphocyte Proliferative Response to Allogeneic FM- and BM-MSC in MLC

We performed MLC assays to investigate whether FM-MSC are able to evade alloreactive lymphocyte proliferation in vitro. Compared with autologous splenic lymphocytes, the lymphocyte proliferative response to allogeneic splenic lymphocytes (230.7% ± 9.4% increase in proliferation) was significantly higher, whereas negligible responses were observed to FM-MSC and BM-MSC (115.1% ± 4.5% and 94.7% ± 4.2%,

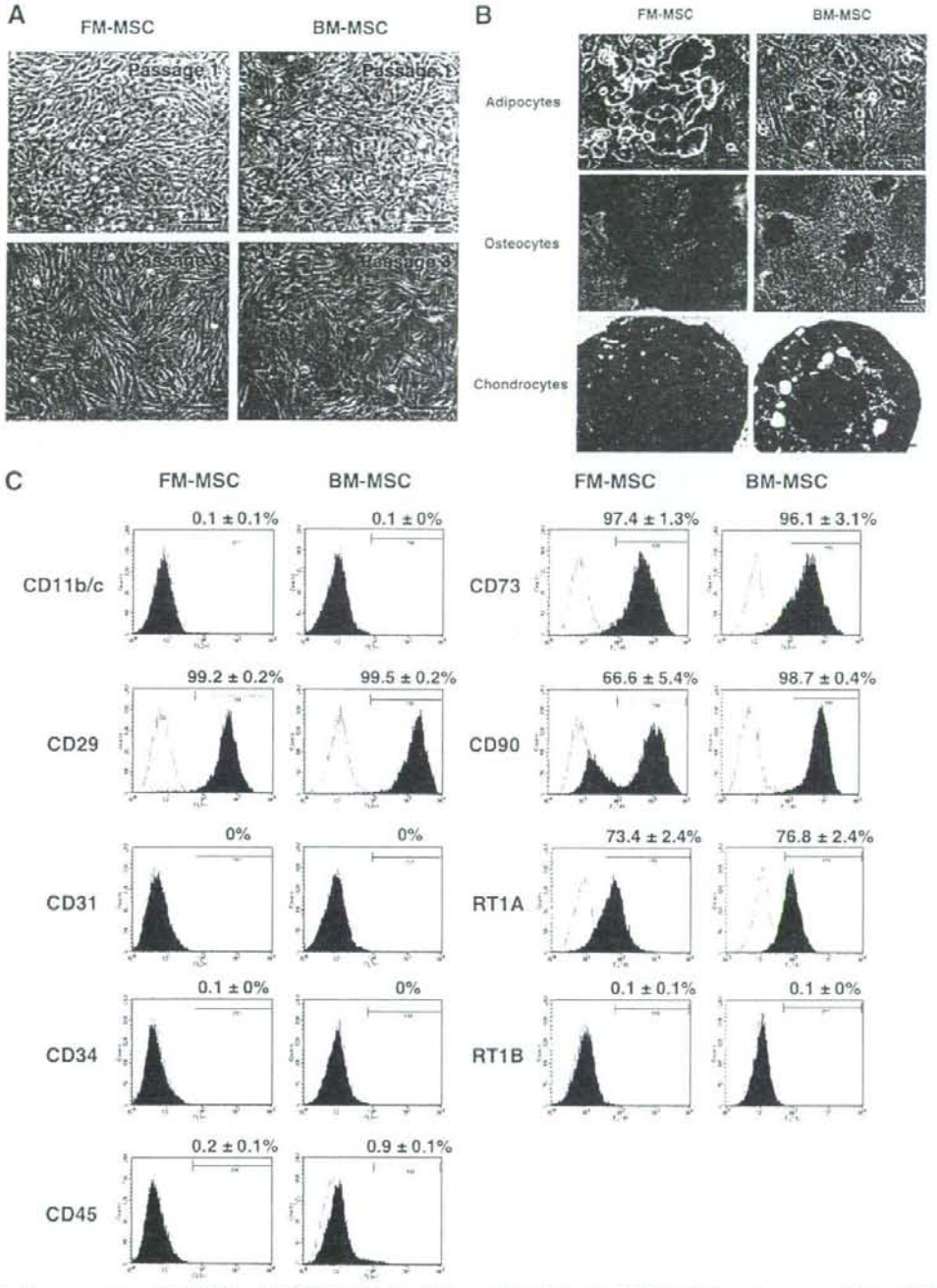

Figure 1. Characterization of FM-MSC and BM-MSC. **(A):** Morphology of FM-MSC and BM-MSC. In the early passages, FM- and BM-MSC appeared microscopically heterogeneous. After several passages, these cells formed a morphologically homogeneous population of fibroblast-like cells, which was similar to BM-MSC. Scale bars = 100 μm. **(B):** Multipotency of FM-MSC and BM-MSC. Differentiation into adipocytes was observed by oil red O staining. Differentiation into osteocytes was observed by alizarin red S staining. Differentiation into chondrocytes was observed by safranin O staining (n = 3 each). Scale bars = 50 μm. **(C):** Flow cytometric analysis of FM-MSC and BM-MSC at passage 3 (n = 3 each). Filled areas indicate staining with a specific antibody, whereas open areas represent staining with isotype control antibodies. Bar represent percentages of positive cells. Abbreviations: BM-MSC, bone marrow-derived mesenchymal stem cells; FM-MSC, fetal membrane mesenchymal stem cells.

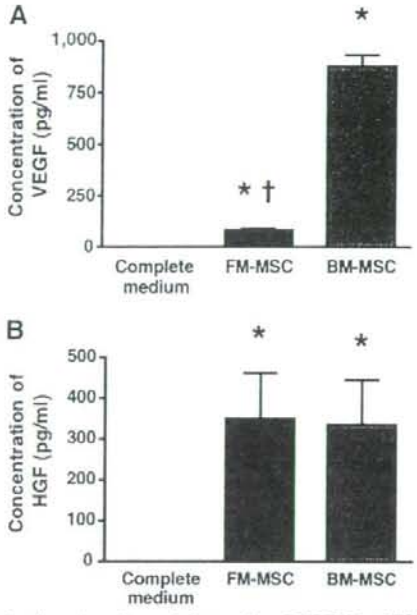

Figure 2. Secretion of growth factors from FM-MSC and BM-MSC. VEGF (**A**) and HGF (**B**) in conditioned medium obtained from FM-MSC and BM-MSC were measured by enzyme-linked immunosorbent assay ($n = 8$ each). Data are mean ± SEM. *, $p < .05$ versus complete medium; †, $p < .05$ versus BM-MSC. Abbreviations: BM-MSC, bone marrow-derived mesenchymal stem cells; FM-MSC, fetal membrane mesenchymal stem cells; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor.

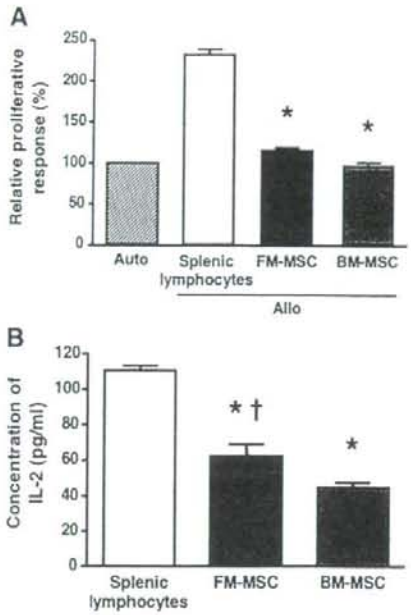

Figure 3. T lymphocyte proliferative responses to Allo FM-MSC and BM-MSC in mixed lymphocyte culture (MLC). (**A**): The Allo lymphocyte proliferative response to FM-MSC was significantly lower than that to splenic lymphocytes and equivalent to BM-MSC ($n = 8$ each). T lymphocyte proliferation induced by Auto cells was assigned a value of 100%. (**B**): Supernatants from MLC were examined for secretion of IL-2 by enzyme-linked immunosorbent assay. Secretion of IL-2 from MLC of Allo FM-MSC and BM-MSC was significantly lower than that of Allo splenic lymphocytes ($n = 8$ each). Data are mean ± SEM. *, $p < .05$ versus splenic lymphocytes; †, $p < .05$ versus BM-MSC. Abbreviations: Allo, allogeneic; Auto, autologous; BM-MSC, bone marrow-derived mesenchymal stem cells; FM-MSC, fetal membrane mesenchymal stem cells; IL, interleukin.

respectively; $p < .05$ vs. allogeneic splenic lymphocytes; $n = 8$) (Fig. 3A).

IL-2 is known to play a role in the activation and proliferation of T cells [26]. Supernatants of MLC were therefore examined for IL-2 secretion using ELISA. Secretion of IL-2 by FM-MSC (62.4 ± 7.3 pg/ml) and BM-MSC (44.5 ± 3.6 pg/ml) was significantly lower than that by splenic lymphocytes (110.5 ± 3.3 pg/ml; $p < .05$ vs. splenic lymphocytes; $n = 8$) (Fig. 3B).

Angiogenesis After Allogeneic FM- and BM-MSC Injection into Ischemic Hind Limbs

We intramuscularly injected FM-MSC obtained from Lewis rats into MHC-mismatched ACI rats with hind limb ischemia. We previously reported that BM-MSC injection caused significantly greater improvement in hind limb ischemia than bone marrow-derived MNC injection [4]. Therefore, BM-MSC derived from the same Lewis rats were used as a control in this study. Blood perfusion of the ischemic hind limb was considerably impaired 3 weeks after surgery (PBS group; Fig. 4A). An improvement in blood perfusion was observed in the FM-MSC and BM-MSC groups compared with the PBS group (Fig. 4A). The LDPI index in the FM-MSC group (53.7 ± 4.4 on day 14, 58.6 ± 2.6 on day 21; both $p < .05$ vs. PBS group) was comparable to that in the BM-MSC group (50.9 ± 2.6, 62.7 ± 6.4 on day 21; $p < .05$ vs. PBS group) and was significantly higher than that in the PBS group (39.9 ± 3.4, 42.6 ± 4.2) on days 14 and 21 ($n = 10$ in each group) (Fig. 4B). Histological analysis on day 21 demonstrated a large number of capillaries in the ischemic muscles of the FM-MSC and BM-MSC groups compared with the PBS group (Fig. 4C), and quantitative analysis showed that the capillary/muscle fiber ratio of ischemic muscle was significantly higher in the FM-MSC (1.8 ± 0.1; $p < .05$ vs. PBS) and BM-MSC (1.5 ± 0.1; $p < .05$ vs. PBS) groups, compared with the PBS group (1.0 ± 0.1; $n = 10$) (Fig. 4D).

Engraftment of Injected Allogeneic FM- and BM-MSC in Ischemic Hind Limb Tissues

To investigate the survival of allogeneic FM-MSC after injection, we injected FM-MSC or BM-MSC obtained from GFP-transgenic Lewis rats into the ischemic hind limb muscle of MHC-mismatched ACI rats. One week after cell injection, GFP-positive allogeneic FM-MSC and BM-MSC were obviously present in the hind limb tissues (Fig. 5A). Quantitative analysis demonstrated that comparable numbers of GFP-positive allogeneic FM-MSC or BM-MSC were observed (FM-MSC, 195.0 ± 27.6 cells per mm^2; BM-MSC, 147.5 ± 32.9 cells per mm^2; $n = 8$ each) (Fig. 5B). A few GFP-positive allogeneic FM-MSC or BM-MSC were still observed at the injection site 3 weeks after cell injection (FM-MSC, 5.8 ± 3.5 cells per mm^2; BM-MSC, 3.3 ± 2.3 cells per mm^2; $n = 8$ each) (Fig. 5A, 5B).

T Lymphocyte Alloreactivity After Allogeneic FM- and BM-MSC Injection into Hind Limb Muscle

To investigate the degree of T lymphocyte alloreactivity after FM-MSC injection, we injected FM-MSC, BM-MSC, or

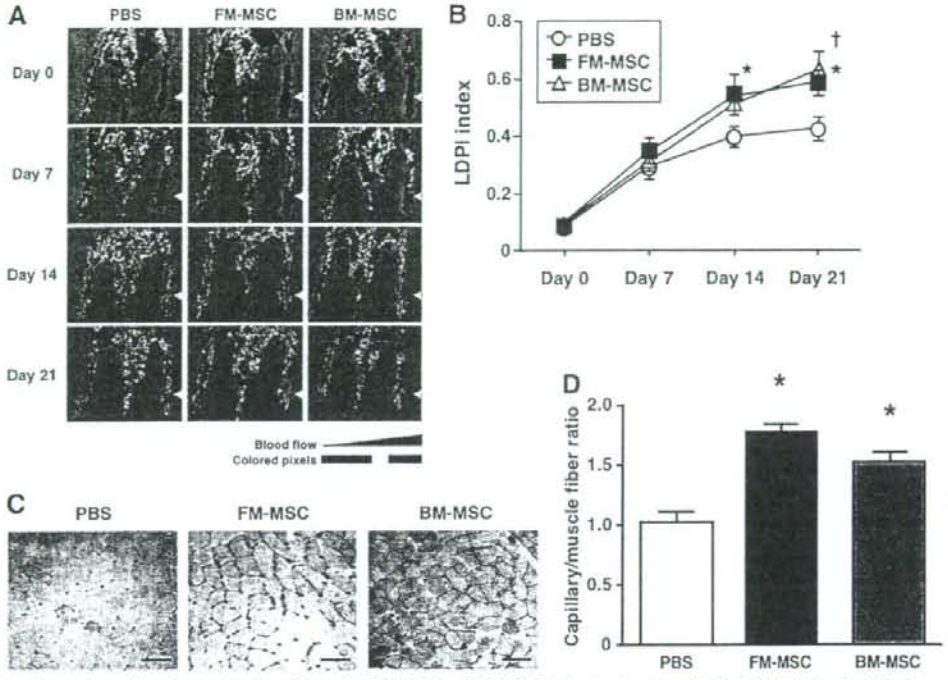

Figure 4. Comparison of angiogenesis after allogeneic FM-MSC and BM-MSC injection in rats with hind limb ischemia. **(A):** Representative examples of serial LDPI. Blood perfusion of the ischemic hind limb was markedly increased in the FM-MSC and BM-MSC group 3 weeks after cell injection (red to orange) (n = 10 each group). **(B):** Quantitative analysis of hind limb blood perfusion. LDPI index was significantly higher in the FM-MSC and BM-MSC groups than in the PBS group 3 weeks after cell injection. Data are mean ± SEM. *, $p < .05$ FM-MSC versus PBS; †, $p < .05$ BM-MSC versus PBS. **(C):** Representative examples of alkaline phosphatase staining of ischemic hind limb muscles. In the FM-MSC and BM-MSC groups, the number of capillaries was increased compared with that in the PBS group. Scale bars = 50 μm. **(D):** Quantitative analysis of capillary density in ischemic hind limb muscles. Capillary density is shown as capillary/muscle fiber ratio. The capillary/muscle fiber ratio of ischemic hind limb muscle was significantly higher in the FM-MSC and BM-MSC groups compared with that in the PBS group. Data are mean ± SEM. *, $p < .05$ versus PBS. Abbreviations: BM-MSC, bone marrow-derived mesenchymal stem cells; FM-MSC, fetal membrane mesenchymal stem cells; LDPI, laser Doppler perfusion image; PBS, phosphate-buffered saline.

splenic lymphocytes obtained from GFP-transgenic Lewis rats into the hind limb tissue of MHC-mismatched ACI rats. Ligation of the femoral artery causes severe inflammation in the ischemic hind limbs. Because of severe T-cell infiltration in the ischemic hind limb tissues, we therefore assessed T lymphocyte alloreactivity using nonischemic hind limb tissues. One week after cell injection, a comparatively small number of T lymphocytes had infiltrated in the GFP-positive allogeneic FM-MSC (963.8 ± 147.8 cells per mm^2; $p < .05$ vs. splenic lymphocytes) and BM-MSC (1,447.5 ± 94.6 cells per mm^2; $p < .05$ vs. splenic lymphocytes) groups compared with the allogeneic splenic lymphocyte (3,222.5 ± 322.7 cells per mm^2) group (Fig. 6A, 6C; n = 8). In addition, a number of GFP-positive transplanted allogeneic FM-MSC and BM-MSC survived, but no injected allogeneic splenic lymphocytes were found in hind limb muscles 1 week after injection (280.0 ± 49.4 and 347.5 ± 92.9 cells per mm^2, respectively; $p < .05$ vs. splenic lymphocytes) (n = 8; Fig. 6B).

Discussion

In this study, we demonstrated that (a) FM-MSC were multipotent and expressed surface antigens almost similar to those of BM-MSC, (b) FM-MSC evaded T lymphocyte alloreactivity both in vitro and in vivo, and (c) allogeneic injection of FM-MSC enhanced therapeutic angiogenesis in a rat model of hind limb ischemia in a manner equivalent to BM-MSC. MSC are multipotent cells and have been isolated from various tissues, including BM, adipose tissue, and fetal tissues [1–3]. Some groups have recently reported that MSC could be isolated from human FM [10, 11]. However, there are no reports regarding the isolation and characterization of MSC obtained from rat FM and application to disease model. We demonstrated that isolated rat FM-MSC were multipotent and expressed surface antigens similar to those of rat BM-MSC. We compared the results of FM-MSC with those of BM-MSC, because the effects of BM-MSC transplantation have been well established by previous investigations, including those in our laboratory [4, 21, 27].

The immunophenotype of MSC is positive for MHC I and negative for MHC II and for costimulatory factors, such as CD40, CD80, and CD86, and MSC are thus considered to be nonimmunogenic [1]. MHC I may activate T cells, but in the absence of costimulatory factors, a secondary signal would not engage, leaving T cells anergic [1, 28]. In addition, MSC have been reported to have immunosuppressive properties, such as modulation of T-cell function [1, 29, 30]. Furthermore, a recent study demonstrated that human MSC modulate the function of dendritic cells and natural killer cells, suggesting that MSC could be a therapeutic tool for reduction of graft-versus-host disease and modulation of inflammation [31]. In several pre-