Table 1 Continued

Patient #	Sex	Mutation*	Age at First Symptom, yrs	Proband or Relative	Presenting Symptom	Age at Diagnosis, yrs	Aborted Cardiac Arrest	ICD	Age at Baseline, yrs	Drug Therapy at Baseline, mg (mg/kg body weight)	Indication for Starting Flecainide Treatment	Daily Starting/Stable Flecainide Dose, mg (mg/kg body weight)†	Follow-Up, months	Response to Flecainide Treatment	Side Effects of Flecainide
19	F	R420W	33	Proband	Syncope	33	No	Yes	36	Bisoprolol 5 (0.08)	Bigeminy/frequent VPBs	100 (1.5)	17	Complete	None
20	M	R420W	NA	Relative	None	11	No	No	12	Atenolol 25 (0.7)	Couplets	100 (2.6)	23	Complete	None
21‡	F	G3946S	14	Proband	Syncope	15	No	No	34	Nadolol 160 (2.7)	Couplets	200 (3.3)	18	Complete	None
22	F	R420Q	14	Proband	Syncope	15	No	Yes	20	Bisoprolol 1.25 (0.03)	Couplets	200 (4.0)	17	None	None
23‡	F	R2474G	1	Proband	Convulsion without fever	11	No	Yes	18	Atenolol 100 (2.1), verapamil 120 (2.6)	NSVT	150 (3.2)	20	Complete	None
24	F	R420W	NA	Relative	None	20	Yes	No	24	Metoprolol 25 (0.4)#	Bigeminy/frequent VPBs + side effects	100 (1.8)	17	Complete	None
25	F	E1724K	10	Proband	Syncope	31	No	No	39	Carvedilol 2.5 (0.05)	NSVT	100 (2.2)	14	Partial	None
26‡	F	F2215L	5	Proband	Cardiac arrest	10	Yes	No	24	Propranolol 140 (2.8)	NSVT (on Holter recordings) + syncope + palpitations	100 (2.0)	13	None	None
27	F	R4157H	56	Relative	Palpitations	57	No	Yes	57	Bisoprolol 5 (0.08)**	NSVT	150 (2.3)	31	NA**	None
28	F	M3978I	14	Relative	Syncope	15	No	Yes	25	Nadolol 40 (0.7)	Frequent VPBs + syncope	150 (2.5)	31	Complete	Nausea and dizziness
29	F	M3978I	14	Proband	Syncope	14	No	Yes	26	Bisoprolol 5 (0.06)††	Bigeminy/frequent VPBs	150 (3.1)	32	None	None
30	F	M3978I	13	Relative	Syncope	32	No	No	45	None‡‡	Bigeminy/frequent VPBs	150 (2.3)	NA§§	Partial	Nausea and dizziness
31	F	M3978I	13	Relative	Syncope	38	No	No	50	Bisoprolol 5 (0.09)	VPBs + palpitations	100 (1.8)	NA‖‖	None	Nausea and dizziness
32	M	V4771I	4	Proband	Syncope with seizure	18	No	No	18	Sotalol 240 (3.2)	NSVT	200 (2.7)	29 yrs¶¶	Complete	None
33‡	F	R2401H	9	Proband	Syncope	9	No	Yes	17	Nadolol 160 (2.5)	Syncope with VF and arrhythmic storm (recorded on ICD log)	150 (2.3)	40	Complete	None
Total	F: 24 (73%)	*RyR2*: 32 (97%)	Median: 13 (range 1–56)	Probands: 15 (45%)	Symptoms: 21 (64%)	Median: 18 (range 3–57)	Yes: 4 (12%)	Yes: 12 (36%)	Median: 25 (range 7–68)	β-blocker: 31 (94%); Ca^{2+} channel blocker: 4 (12%)	Severe ventricular arrhythmia: 26 (79%); symptoms: 5 (15%)	Median: 100 (range 50–300)/150 (range 100–300)	Median: 20 (range 12–40)	Complete: 14/31 (45%); partial: 10/31 (32%)	Yes: 6 (18%)

**RYR2* mutations unless otherwise indicated. †Stable dose was identical to starting dose when only 1 dose is displayed. ‡Patients who were treated with a first-line β-blocker at an optimal dose (n = 15). §Verapamil was discontinued when flecainide was started. ‖This patient discontinued β-blocker therapy during 3 consecutive pregnancies, and thereafter agreed with her treating cardiologist to permanently discontinue β-blocker therapy and avoid exercise. ¶Flecainide was discontinued within a few days and before exercise testing on flecainide could be performed. #Metoprolol was discontinued and flecainide was started in this patient because of intolerable side effects. **This patient was not included in the primary analysis because the bisoprolol dose was also increased. ††This patient discontinued β-blocker therapy on her own initiative after flecainide treatment was started and before an exercise test on combined therapy could be performed. The ventricular arrhythmia score on flecainide monotherapy did not change compared with that on the baseline exercise test while taking a β-blocker. ‡‡This patient discontinued β-blocker therapy because of side effects. §§This patient discontinued flecainide and restarted β-blocker therapy on her own initiative. ‖‖This patient discontinued flecainide because of side effects after exercise testing while taking a β-blocker and flecainide was performed. ¶¶This patient was excluded from the follow-up calculation.

ICD = implantable cardioverter defibrillator; NA = not applicable; NSVT = nonsustained ventricular tachycardia; SCD = sudden cardiac death; VF = ventricular fibrillation; VPB = ventricular premature beat.

JACC Vol. 57, No. 22, 2011
May 31, 2011:2244–54

ratio of VPBs to sinus beats during the 10-s period with the maximum number of VPBs.

Reaching a ventricular arrhythmia score of 1 was considered complete suppression of ventricular arrhythmias. Other ventricular arrhythmia score improvements were considered partial suppression.

Statistical analysis. Continuous data are presented as mean ± SD or median (range), and categorical variables as number (percentage). Related samples were compared using the paired Wilcoxon signed-rank test for continuous and ordinal variables and the McNemar test for dichotomous variables. Independent continuous variables were compared by means of the Mann-Whitney *U* test. A 2-tailed p value <0.05 was considered statistically significant. Statistical analysis was performed with SPSS software package, version 15.0 (SPSS, Inc., Chicago, Illinois).

Results

Patient characteristics. A total of 33 genotype-positive CPVT patients from 21 families were started on flecainide at 8 tertiary care centers (Table 1). All patients had persistent physical or emotional stress-induced ventricular arrhythmias documented by exercise testing, Holter recordings, or ICD interrogation and/or persistent symptoms of palpitations, syncope, aborted cardiac arrest, or appropriate ICD shocks, while taking β-blockers with or without Ca^{2+}-channel blockers. Twenty-four of the patients (73%) were female. The median age at the start of flecainide therapy was 25 years (range 7 to 68 years). Thirty-one patients (94%) were treated with β-blockers, and 4 (12%) of them also received Ca^{2+}-channel blockers (Table 1).

In 1 patient (Patient #13), flecainide was stopped because of side effects before exercise testing could be repeated; in another patient (Patient #27) the β-blocker dose was increased during flecainide treatment; and 2 patients (Patients #7 and #30) did not receive β-blocker therapy when flecainide was started (Table 1). In the remaining 29 patients, exercise tests on combination therapy of flecainide with conventional drugs at unchanged or lower doses were available for analysis. In 17 patients (59%), baseline exercise testing was performed <48 h before flecainide initiation.

Flecainide therapy reduces exercise-induced ventricular arrhythmias. Flecainide treatment improved the ventricular arrhythmia score in 22 patients (76%) ($p < 0.001$) (Fig. 1A). Fourteen patients (48%) had complete suppression of ventricular arrhythmias (including 7 patients without any VPBs), and 8 (28%) had partial suppression. None of the patients experienced significant (i.e., couplet or VT) worsening of the exercise-induced ventricular arrhythmia score.

Flecainide treatment also significantly improved all other predefined parameters of exercise-induced ventricular arrhythmia (Table 2). For example, patients receiving flecainide therapy achieved significantly higher heart rates before ventricular arrhythmias occurred. Independently, flecainide caused a significant reduction in maximum sinus rate during exercise, even though a higher mean workload was achieved. As expected (28), flecainide prolonged the PR interval (149 ± 21 ms vs. 160 ± 24 ms; $p = 0.003$), and the QRS duration (83 ± 9 ms vs. 89 ± 11 ms; $p = 0.005$), but did not change the QTc interval (399 ± 26 ms vs. 405 ± 19 ms; $p = 0.171$) at rest. These parameters remained within the normal range at rest and during peak exercise in all patients, except for a slightly prolonged resting PR interval (220 ms) in 1 patient (Patient #20).

We next assessed the reproducibility of exercise testing as a measure of the ventricular arrhythmia burden in CPVT. Although not available for all patients, a subset of patients underwent repeated exercise testing either at the same dose of conventional therapy (n = 14) or at the same flecainide dose (n = 16). In both cases, the ventricular arrhythmia score of the second exercise test was not statistically different from that on the first exercise test (Fig. 2). Similarly, all other predefined parameters of exercise-induced ventricular arrhythmia also did not change significantly (e.g., the maximum number of VPBs during a 10-s period was 5 ± 5 on the first exercise test at the stable flecainide dose and 6 ± 6 on the second exercise test at the same flecainide dose [$p = 0.556$]), suggesting that ventricular arrhythmia scores obtained from exercise testing are reproducible measures of drug efficacy in CPVT and that tachyphylaxis was not present.

We found that 14 of the 29 patients included in the primary analysis received drug therapy that could be considered suboptimal (i.e., an unusual β-blocker for CPVT [bisoprolol, carvedilol, or sotalol]) or a relatively low β-blocker dose (atenolol, metoprolol, or nadolol <1 mg/kg body weight daily) (2). These patients had either side effects on other β-blockers and/or a higher β-blocker dose, or nadolol was not available in their country. To assess whether flecainide was also effective in CPVT patients on optimal conventional therapy, we next analyzed the 15 patients who were treated with a first-line β-blocker at an optimal dose (Table 1). Flecainide significantly improved the ventricular arrhythmia score ($p = 0.003$) (Fig. 1B), and all other pre-defined arrhythmia parameters in this subgroup to a similar extent as in the primary analysis.

The ventricular arrhythmia score in the 2 patients (Patients #7 and #30) who did not receive β-blocker therapy when flecainide was started improved from NSVT to couplet and from NSVT to bigeminal VPBs and frequent VPBs, respectively.

Flecainide dose in CPVT. To estimate the optimal dosing of flecainide in CPVT, we analyzed the relationship between starting dose and VT suppression during the first exercise test on flecainide. Patients without suppression of exercise-induced ventricular arrhythmias on the starting flecainide dose received a significantly lower dose (113 ± 39 mg, n = 13; $p = 0.038$) compared with patients with either partial (142 ± 38 mg, n = 6) or complete ventricular arrhythmia suppression (150 ± 60 mg, n = 12). Eight

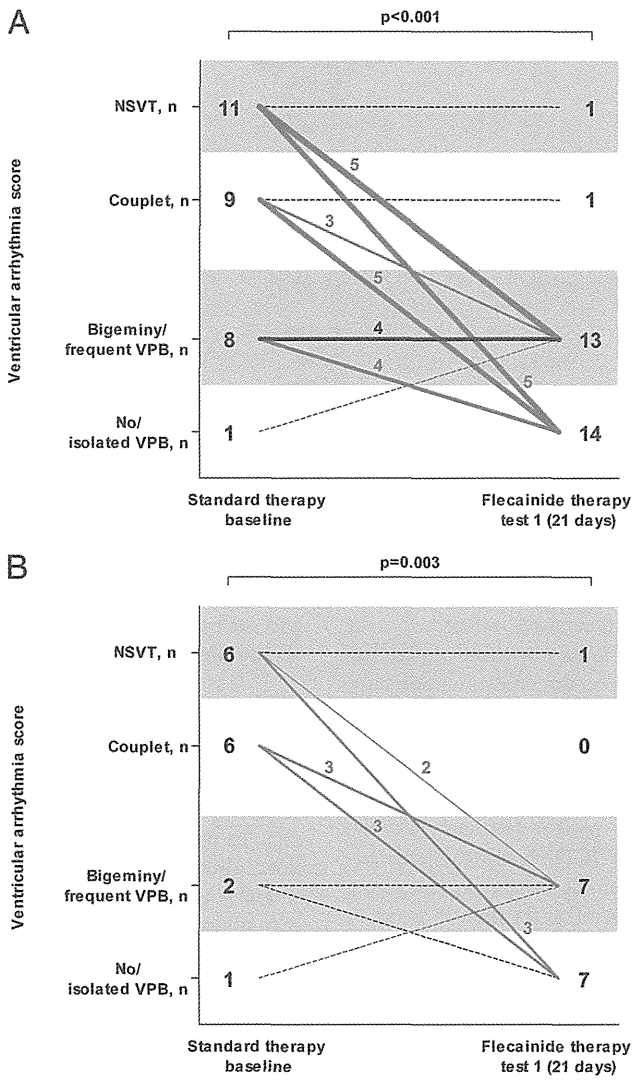

Figure 1 Ventricular Arrhythmia Score on Standard Therapy and on Flecainide

Ventricular arrhythmia score per patient on the baseline exercise test on standard therapy and on the first exercise test on the final (stable) flecainide dose in the entire cohort (n = 29) **(A)** and in the patients who were treated with a first-line β-blocker at an optimal dose (n = 15) **(B)**. The number of patients in each ventricular arrhythmia category and change in ventricular arrhythmia category are shown. The **line thickness** indicates the number of patients, and a **dotted line** represents 1 patient. The median time interval between the 2 tests is shown. All exercise tests were performed on patients receiving an unchanged β-blocker dose. NSVT = nonsustained ventricular tachycardia; VPB = ventricular premature beats.

patients (24%) received an increased flecainide dose after the initial exercise test (Table 1). The dose increased from an average daily dose of 96 ± 28 mg to 178 ± 78 mg (range 100 to 300 mg), which resulted in a significant improvement in the ventricular arrhythmia score (Fig. 3).

Clinical follow-up. Three patients (Patients #13, #30, and #31) discontinued flecainide with <6 months of follow-up due to side effects. One patient (Patient #6) required a pacemaker because flecainide exacerbated pre-existing sinus node dysfunction. Flecainide was resumed after pacemaker implantation, and this patient was included in the study. In 2 patients (Patients #7 and #28), the stable flecainide dose was decreased because of dizziness. All other patients tolerated flecainide well without severe side effects. The β-blocker dose was decreased in 5 patients (Patients #4, #5, #6, #9, and #12) who had a partial suppression of ventricular arrhythmias on flecainide and experienced side effects of β-blocker therapy (in particular, fatigue) before flecainide

JACC Vol. 57, No. 22, 2011
May 31, 2011:2244–54

Table 2 Exercise Test Results of the Baseline Exercise Test on Standard Therapy and on the First Exercise Test on the Final (Stable) Flecainide Dose

	Standard Therapy Baseline (n = 29)	First Exercise Test on Stable Flecainide Dose (n = 29)	p Value
Time after start flecainide, days	—	21 (5–363)	—
Sinus rate at baseline, beats/min	57 ± 10	59 ± 9	0.061
Sinus rate at maximal exercise, beats/min	145 ± 23	133 ± 18	0.002
Maximum workload attained, METs	11 ± 3	12 ± 4	0.042
Sinus rate at onset of ventricular arrhythmias, beats/min	113 ± 19	118 ± 19	0.046*
Maximum no. of VPBs during a 10-s period†	12 ± 5	5 ± 5	<0.001
Ratio of VPBs to sinus beats during the 10-s period with the maximum no. of VPBs†	1.2 ± 0.8	0.4 ± 0.4	<0.001
Isolated VPB	29 (100)	22 (76)	0.016
Bigeminal VPBs	28 (97)	13 (45)	<0.001
Frequent VPBs (>10/min)	27 (93)	14 (48)	0.001
Couplet	20 (69)	2 (7)	<0.001
Nonsustained ventricular tachycardia	11 (38)	1 (3)	0.002
Longest ventricular salvo, VPBs†	5 (3–9)	4	—
Bidirectional NSVT	4 (36)	—	—

Data are mean ± SD, median (range), or n (%). *Only the 22 patients who still had ventricular arrhythmias on the first exercise test at the stable flecainide dose were included in this analysis. †Data were available for 28 patients (not available for Patient #32).
MET = metabolic equivalent; NSVT = nonsustained ventricular tachycardia; VPB = ventricular premature beat.

was started. One patient (Patient #29) refused to take β-blockers during follow-up, with no worsening of exercise-induced ventricular arrhythmias on flecainide monotherapy.

Thus, 30 of 33 patients (91%) continued to receive flecainide and were included in the further analysis of the incidence of arrhythmic events. During a median follow-up of 20 months (range 12 to 40 months, excluding Patient #32), VT recurred in only 1 patient (Patient #1) who experienced several appropriate ICD shocks for polymorphic VT after 8 months of flecainide treatment. Her serum flecainide level was low (0.34 μg/ml) at the time of the event compared with levels obtained previously (0.75 to 0.82 μg/ml), suggesting noncompliance. She was hospitalized for 48 h, nadolol and flecainide were resumed at their previous doses, and no further ventricular arrhythmias occurred during a further follow-up of 17 months. The other 29 patients remained free of arrhythmic events during follow-up. The longest follow-up of 29 years was achieved in Patient #32, who presented with exercise-induced VT in 1981. After unsuccessful trials of multiple antiarrhythmic drugs (including mexilitine, amiodarone, propranolol, sotalol, and Ca^{2+}-channel blockers), flecainide (200 mg/day) was added to sotalol (160 mg/day), which resulted in complete suppression of ventricular arrhythmia during exercise testing. In 2008, an exercise test 48 h after stopping flecainide and sotalol showed NSVT. After restarting the combined therapy, a subsequent exercise test only showed isolated VPBs, but no VT. Subsequent genotyping revealed a mutation in the gene encoding RyR2. In Patient #33, flecainide 150 mg/day was started in 2007 because of 2 episodes of syncope with ventricular fibrillation on the ICD interrogation despite nadolol 240 mg/day. Exercise testing showed complete suppression of ventricular arrhythmias, and she has been free of arrhythmic events on flecainide for 40 months.

Discussion

Main findings. Our study demonstrates that flecainide reduces or prevents exercise-induced ventricular arrhythmias in the majority of CPVT patients receiving conventional drug therapy. These findings are important because several studies have demonstrated a significant failure rate of current drug therapy (1,3,11–16), including potentially fatal arrhythmic events in 11% of CPVT patients over an 8-year period (2). Based on our clinical experience reported here, flecainide in addition to β-blocker therapy should be considered for CPVT patients who otherwise have few alternative therapeutic options. The optimal dose appears to be between 150 and 200 mg/day (range 100 to 300 mg/day). Daily doses <100 mg were associated with a lack of therapeutic response.

Rationale for use of flecainide. CPVT is caused by mutations in the genes encoding RyR2 and cardiac calsequestrin (4,5), 2 proteins that control Ca^{2+} release from the sarcoplasmic reticulum. As a result of the mutations, Ca^{2+} is released prematurely and excessively into the cytosol under conditions of catecholaminergic stimulation, generating repetitive spontaneous Ca^{2+} waves (9,29). The increase in intracellular Ca^{2+} in turn activates the electrogenic Na^{+}/Ca^{2+} exchanger, which produces a transient inward current (I_{Ti}). I_{Ti} generates delayed afterdepolarizations, which can lead to triggered activity, and the initiation of ventricular arrhythmias (30). Flecainide directly targets the molecular defect in CPVT by inhibiting RyR2 channels and preventing arrhythmogenic Ca^{2+} waves (23,24). Flecain-

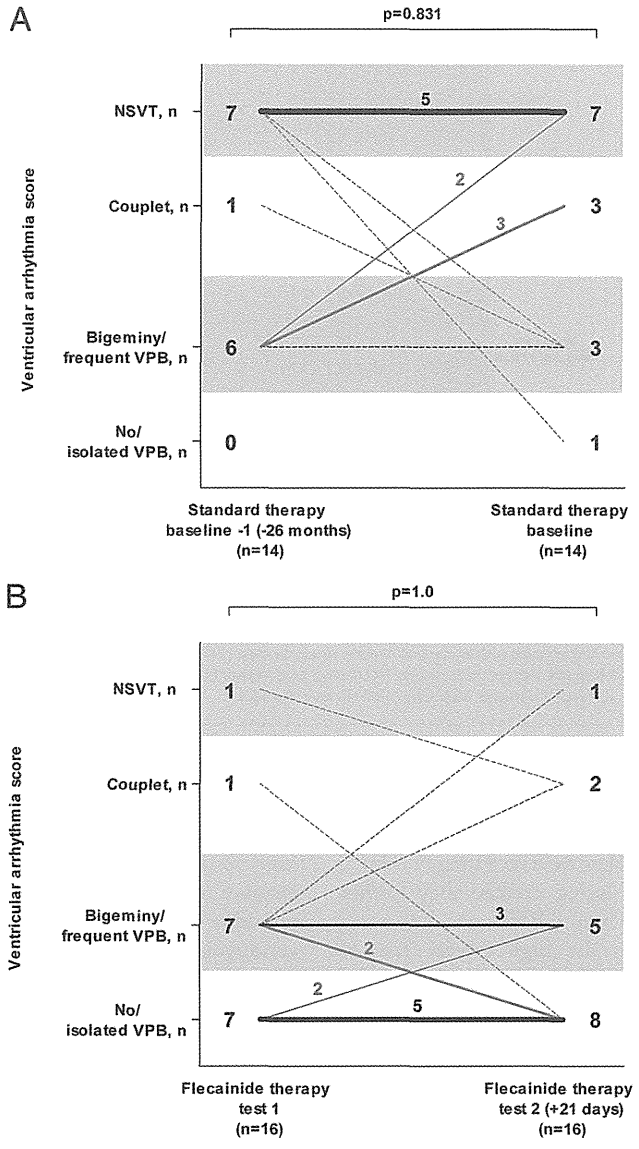

Figure 2 **Reproducibility of Ventricular Arrhythmia Score on Exercise Testing**

Ventricular arrhythmia score per patient on the baseline exercise test and on the previous exercise test at the same standard therapy dose **(A)** and on the first and second exercise tests at the final (stable) flecainide dose **(B)**. The number of patients in each ventricular arrhythmia category and change of ventricular arrhythmia category are shown. The **line thickness** indicates the number of patients, and a **dotted line** represents 1 patient. The median time interval between the 2 tests is shown. The standard therapy exercise tests were performed on patients receiving the same β-blocker dose with or without Ca^{2+}-channel blocker. All exercise tests on patients receiving flecainide were at the same stable flecainide dose in combination with an unchanged or lower β-blocker dose. The sinus rates at maximal exercise on the first and second exercise tests on flecainide were not significantly different (140 ± 19 vs. 144 ± 20; p = 0.245). However, the 2 patients with a ventricular arrhythmia score of 4 and 3 on the second exercise test did reach a significantly higher maximum sinus rate compared with the first exercise test (increase of 32 and 19 beats/min, respectively). Abbreviations as in Figure 1.

ide's Na^+-channel blockade further reduces the rate of triggered beats (23,24). This dual action could explain why flecainide is so effective in severe CPVT and provides a rationale for combination therapy with β-blockers.

RyR2-mediated sarcoplasmic reticulum Ca^{2+} release importantly regulates the beating rate of sinoatrial nodal cells (31), especially in response to catecholamines (32), and flecainide reduces the rate of spontaneous sarcoplasmic

JACC Vol. 57, No. 22, 2011
May 31, 2011:2244–54

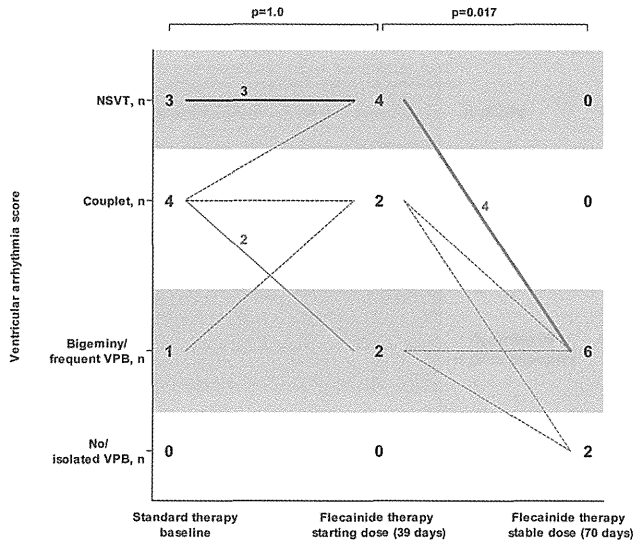

Figure 3 Dose Dependence of Flecainide in 8 CPVT Patients Who Had an Increase in Flecainide Dose

The number of patients in each ventricular arrhythmia category and change in ventricular arrhythmia category on the last exercise test at the flecainide starting dose (96 ± 28 mg; range 50 to 150 mg) and on the first exercise test at the final (stable) flecainide dose (178 ± 78 mg; range 100 to 300 mg) is shown. The **line thickness** indicates the number of patients, and a **dotted line** represents 1 patient. The median time interval from the start of flecainide therapy is shown. All exercise tests were performed with the patients receiving an unchanged β-blocker dose. Abbreviations as in Figure 1.

reticulum Ca^{2+} release in myocytes (24). This mechanism may explain why maximum hearts rates were significantly lower in flecainide-treated patients even though workloads were higher compared with baseline exercise testing (Table 2). The reduction in sinus rate during exercise may further contribute to flecainide's efficacy in CPVT.

Clinical implications. Given the high fatality rate of untreated CPVT patients (1,2), adequate treatment is mandatory and potentially life-saving. β-blockers are considered first-line therapy. In the largest published series of patients with CPVT, the risk of cardiac arrest (defined as aborted cardiac arrest, appropriate ICD shocks, and sudden cardiac death), despite β-blocker therapy during a mean follow-up period of 8 years, was 11% (2). Others have reported very diverse fatal or near-fatal event rates despite β-blocker therapy (1,3,11–16), although the highest event rates may be explained by the predominance of (symptomatic) probands and underdosing of β-blockers. An ICD was recommended for CPVT patients who were survivors of cardiac arrest, or when syncope or sustained VT persisted despite maximum tolerable β-blockade (33). Yet, ICDs have a potentially harmful effect in CPVT patients (17,18). Moreover, many CPVT patients are children, in whom ICD implantation can lead to significant complications (34). Thus, to avoid ICD implantation and prevent ICD shocks in patients with ICDs, controlling ventricular arrhythmias is of great clinical importance. Alternative therapies are needed for CPVT patients.

Left cardiac sympathetic denervation is an effective alternative when symptoms persist despite β-blockade, but requires surgery, is not universally available, and has only been tested in small cohorts (19–22). The use of Ca^{2+}-channel blockers in addition to β-blockade has been reported to decrease ventricular ectopy in CPVT patients with continuous symptoms and/or exercise-induced ventricular arrhythmias (12,27,35), but is not effective in all patients (27,35,36). From the original 6 patients treated with verapamil and β-blockers after failure of β-blockers alone, reported by Rosso et al. (27) in 2007, 3 had clinically significant ventricular arrhythmias during 37 ± 6 months of follow-up (36). Other pharmacological agents, including Na^{+}-channel blockers, amiodarone, and magnesium, lack of efficacy in CPVT patients (1,12).

In this analysis of all consecutive patients started on flecainide at 8 international centers, adding flecainide to standard therapy was effective in further reducing exercise-induced VT and preventing arrhythmic events CPVT patients. To suppress CPVT, adequate dosing of flecainide seems critical. An increased dose may be effective when the initial dose of flecainide fails to suppress VT. Based on these results, flecainide could be added to β-blocker therapy when symptoms or either spontaneous or exercise-induced ventricular arrhythmias persist despite β-blocker.

In our young patient population with no structural heart disease, the proarrhythmic effect of flecainide as documented in patients with ischemia and impaired left ventric-

ular function (37) may not be applicable. Consistent with this hypothesis, flecainide did not cause arrhythmic events during a median follow-up of 20 months, which is longer than the mean follow-up of 10 months in the CAST (Cardiac Arrhythmia Suppression Trial). The only arrhythmic event was associated with low flecainide serum levels, suggesting that the event was due to the underdosing and not toxicity.

Study limitations. This study reports on our experience of using flecainide in a clinical setting. The number of patients is relatively small because CPVT is a rare condition and only patients without other treatment alternatives were started on flecainide. However, it is the largest evaluation of a new therapeutic strategy in CPVT patients refractory to current drug therapy, with a median of 20 months follow-up. One patient has received flecainide for 29 years with continuous VT suppression on unchanged doses, and another severely symptomatic patient has been free of arrhythmic events on flecainide for 40 months. Nevertheless, long-term follow-up in more patients would further support the clinical utility of flecainide in CPVT.

Another potential limitation is that we only quantified the effect of flecainide on exercise-induced ventricular arrhythmias, which may not accurately predict fatal arrhythmic events. However, exercise testing is clinically used to guide therapy in CPVT. In a previous study including 70 CPVT patients, exercise-induced couplets or more successive VPBs were significantly associated with future arrhythmic events (sensitivity, 0.62; specificity, 0.67) (2).

Furthermore, we cannot exclude potential bias introduced by the variability of exercise test results on unchanged treatment, as illustrated in Figure 2. Finally, in 14 patients, conventional therapy may be considered suboptimal because they received an unusual β-blocker for CPVT or a low β-blocker dose for reasons previously outlined. However, flecainide was equally effective in the subgroup of CPVT patients who were treated with a first-choice β-blocker at an adequate dose (Fig. 1B).

Conclusions

Our results suggest that flecainide is a safe and effective therapy to reduce ventricular arrhythmias in the majority of CPVT patients who have exercise-induced ventricular arrhythmias despite conventional therapy.

Reprint requests and correspondence: Dr. Arthur A. M. Wilde, Academic Medical Center, University of Amsterdam, Department of Cardiology, Heart Failure Research Center, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands. E-mail: a.a.wilde@amc.uva.nl. OR Dr. Björn C. Knollmann, Oates Institute for Experimental Therapeutics, Vanderbilt University School of Medicine, Division of Clinical Pharmacology, Medical Research Building IV, Room 1265, 2215B Garland Avenue, Nashville, Tennessee 37232-0575. E-mail: bjorn.knollmann@vanderbilt.edu.

REFERENCES

1. Leenhardt A, Lucet V, Denjoy I, Grau F, Ngoc DD, Coumel P. Catecholaminergic polymorphic ventricular tachycardia in children. A 7-year follow-up of 21 patients. Circulation 1995;91:1512–9.
2. Hayashi M, Denjoy I, Extramiana F, et al. Incidence and risk factors of arrhythmic events in catecholaminergic polymorphic ventricular tachycardia. Circulation 2009;119:2426–34.
3. Priori SG, Napolitano C, Memmi M, et al. Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. Circulation 2002;106:69–74.
4. Priori SG, Napolitano C, Tiso N, et al. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. Circulation 2001;103:196–200.
5. Lahat H, Pras E, Olender T, et al. A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in Bedouin families from Israel. Am J Hum Genet 2001;69:1378–84.
6. Medeiros-Domingo A, Bhuiyan ZA, Tester DJ, et al. The RYR2-encoded ryanodine receptor/calcium release channel in patients diagnosed previously with either catecholaminergic polymorphic ventricular tachycardia or genotype negative, exercise-induced long QT syndrome: a comprehensive open reading frame mutational analysis. J Am Coll Cardiol 2009;54:2065–74.
7. Jiang D, Xiao B, Yang D, et al. RyR2 mutations linked to ventricular tachycardia and sudden death reduce the threshold for store-overload-induced Ca2+ release (SOICR). Proc Natl Acad Sci U S A 2004; 101:13062–7.
8. di Barletta MR, Viatchenko-Karpinski S, Nori A, et al. Clinical phenotype and functional characterization of CASQ2 mutations associated with catecholaminergic polymorphic ventricular tachycardia. Circulation 2006;114:1012–9.
9. Knollmann BC, Chopra N, Hlaing T, et al. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. J Clin Invest 2006;116:2510–20.
10. Cerrone M, Noujaim SF, Tolkacheva EG, et al. Arrhythmogenic mechanisms in a mouse model of catecholaminergic polymorphic ventricular tachycardia. Circ Res 2007;101:1039–48.
11. Bauce B, Rampazzo A, Basso C, et al. Screening for ryanodine receptor type 2 mutations in families with effort-induced polymorphic ventricular arrhythmias and sudden death: early diagnosis of asymptomatic carriers. J Am Coll Cardiol 2002;40:341–9.
12. Sumitomo N, Harada K, Nagashima M, et al. Catecholaminergic polymorphic ventricular tachycardia: electrocardiographic characteristics and optimal therapeutic strategies to prevent sudden death. Heart 2003;89:66–70.
13. Postma AV, Denjoy I, Kamblock J, et al. Catecholaminergic polymorphic ventricular tachycardia: RYR2 mutations, bradycardia, and follow up of the patients. J Med Genet 2005;42:863–70.
14. Lahat H, Eldar M, Levy-Nissenbaum E, et al. Autosomal recessive catecholamine- or exercise-induced polymorphic ventricular tachycardia: clinical features and assignment of the disease gene to chromosome 1p13-21. Circulation 2001;103:2822–7.
15. Swan H, Piippo K, Viitasalo M, et al. Arrhythmic disorder mapped to chromosome 1q42-q43 causes malignant polymorphic ventricular tachycardia in structurally normal hearts. J Am Coll Cardiol 1999;34: 2035–42.
16. Haugaa KH, Leren IS, Berge KE, et al. High prevalence of exercise-induced arrhythmias in catecholaminergic polymorphic ventricular tachycardia mutation-positive family members diagnosed by cascade genetic screening. Europace 2010;12:417–23.
17. Mohamed U, Gollob MH, Gow RM, Krahn AD. Sudden cardiac death despite an implantable cardioverter-defibrillator in a young female with catecholaminergic ventricular tachycardia. Heart Rhythm 2006;3:1486–9.
18. Pizzale S, Gollob MH, Gow R, Birnie DH. Sudden death in a young man with catecholaminergic polymorphic ventricular tachycardia and paroxysmal atrial fibrillation. J Cardiovasc Electrophysiol 2008;19: 1319–21.
19. Wilde AA, Bhuiyan ZA, Crotti L, et al. Left cardiac sympathetic denervation for catecholaminergic polymorphic ventricular tachycardia. N Engl J Med 2008;358:2024–9.

20. Odero A, Bozzani A, De Ferrari GM, Schwartz PJ. Left cardiac sympathetic denervation for the prevention of life-threatening arrhythmias: the surgical supraclavicular approach to cervicothoracic sympathectomy. Heart Rhythm 2010;7:1161–5.
21. Makanjee B, Gollob MH, Klein GJ, Krahn AD. Ten-year follow-up of cardiac sympathectomy in a young woman with catecholaminergic polymorphic ventricular tachycardia and an implantable cardioverter defibrillator. J Cardiovasc Electrophysiol 2009;20:1167–9.
22. Collura CA, Johnson JN, Moir C, Ackerman MJ. Left cardiac sympathetic denervation for the treatment of long QT syndrome and catecholaminergic polymorphic ventricular tachycardia using video-assisted thoracic surgery. Heart Rhythm 2009;6:752–9.
23. Watanabe H, Chopra N, Laver D, et al. Flecainide prevents catecholaminergic polymorphic ventricular tachycardia in mice and humans. Nat Med 2009;15:380–3.
24. Hilliard FA, Steele DS, Laver D, et al. Flecainide inhibits arrhythmogenic Ca2+ waves by open state block of ryanodine receptor Ca2+ release channels and reduction of Ca2+ spark mass. J Mol Cell Cardiol 2010;48:293–301.
25. Anastasiou-Nana MI, Anderson JL, Stewart JR, et al. Occurrence of exercise-induced and spontaneous wide complex tachycardia during therapy with flecainide for complex ventricular arrhythmias: a probable proarrhythmic effect. Am Heart J 1987;113:1071–7.
26. Katritsis D, Rowland E, O'Nunain S, Shakespeare CF, Poloniecki J, Camm AJ. Effect of flecainide on atrial and ventricular refractoriness and conduction in patients with normal left ventricle. Implications for possible antiarrhythmic and proarrhythmic mechanisms. Eur Heart J 1995;16:1930–5.
27. Rosso R, Kalman JM, Rogowski O, et al. Calcium channel blockers and beta-blockers versus beta-blockers alone for preventing exercise-induced arrhythmias in catecholaminergic polymorphic ventricular tachycardia. Heart Rhythm 2007;4:1149–54.
28. Roden DM, Woosley RL. Drug therapy. Flecainide. N Engl J Med 1986;315:36–41.
29. George CH, Higgs GV, Lai FA. Ryanodine receptor mutations associated with stress-induced ventricular tachycardia mediate increased calcium release in stimulated cardiomyocytes. Circ Res 2003;93:531–40.
30. Schlotthauer K, Bers DM. Sarcoplasmic reticulum Ca(2+) release causes myocyte depolarization. Underlying mechanism and threshold for triggered action potentials. Circ Res 2000;87:774–80.
31. Maltsev VA, Lakatta EG. Dynamic interactions of an intracellular Ca2+ clock and membrane ion channel clock underlie robust initiation and regulation of cardiac pacemaker function. Cardiovasc Res 2008;77:274–84.
32. Vinogradova TM, Bogdanov KY, Lakatta EG. beta-Adrenergic stimulation modulates ryanodine receptor Ca(2+) release during diastolic depolarization to accelerate pacemaker activity in rabbit sinoatrial nodal cells. Circ Res 2002;90:73–9.
33. Zipes DP, Camm AJ, Borggrefe M, et al. ACC/AHA/ESC 2006 guidelines for management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: a report of the American College of Cardiology/American Heart Association Task Force and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Develop Guidelines for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death). J Am Coll Cardiol 2006;48:e247–346.
34. Sherrid MV, Daubert JP. Risks and challenges of implantable cardioverter-defibrillators in young adults. Prog Cardiovasc Dis 2008;51:237–63.
35. Swan H, Laitinen P, Kontula K, Toivonen L. Calcium channel antagonism reduces exercise-induced ventricular arrhythmias in catecholaminergic polymorphic ventricular tachycardia patients with RyR2 mutations. J Cardiovasc Electrophysiol 2005;16:162–6.
36. Rosso R, Kalman J, Rogowsky O, et al. Long-term effectiveness of beta blocker and calcium blocker combination therapy in patients with CPVT. Heart Rhythm 2010;7:S423.
37. Echt DS, Liebson PR, Mitchell LB, et al. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The Cardiac Arrhythmia Suppression Trial. N Engl J Med 1991;324:781–8.

Key Words: antiarrhythmia agents ▪ catecholaminergic polymorphic ventricular tachycardia ▪ ventricular arrhythmia.

Atherosclerosis 219 (2011) 663–666

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Short communication

A novel type of familial hypercholesterolemia: Double heterozygous mutations in LDL receptor and LDL receptor adaptor protein 1 gene

Hayato Tada[a,*], Masa-aki Kawashiri[a], Rumiko Ohtani[b], Tohru Noguchi[c], Chiaki Nakanishi[a], Tetsuo Konno[a], Kenshi Hayashi[a], Atsushi Nohara[c], Akihiro Inazu[b], Junji Kobayashi[c], Hiroshi Mabuchi[c], Masakazu Yamagishi[a]

[a] Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, Kanazawa, Japan
[b] Department of Laboratory Science, Molecular Biochemistry and Molecular Biology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan
[c] Department of Lipidology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form 1 August 2011
Accepted 1 August 2011
Available online 10 August 2011

Keywords:
Autosomal recessive hypercholesterolemia
Low density lipoprotein receptor adopter protein 1
Familial hypercholesterolemia
PCSK9

ABSTRACT

Background: Autosomal recessive hypercholesterolemia (ARH) is an extremely rare inherited hypercholesterolemia, the cause of which is mutations in low-density lipoprotein (LDL) receptor adaptor protein 1 (LDLRAP1) gene.
Methods: A total of 146 heterozygous familial hypercholesterolemic (FH) patients with a mutation in LDLR gene were screened for genes encoding proprotein convertase subtilisin/kexin type 9 (PCSK9) and LDLRAP1.
Results: Among the 146 subjects, we identified a 79-year-old Japanese female with double mutations in LDLR gene (c.2431A>T) and LDLRAP1 gene (c.606dup). Two other relatives with double mutations in those genes in her family were also identified. Although the proband exhibited massive Achilles tendon xanthoma and coronary and aortic valvular disease, serum LDL-C level of subjects with double mutations was similar with that of subjects with single LDLR mutation (284.0 ± 43.5 versus 265.1 ± 57.4 mg/dl).
Conclusion: Additional mutation in LDLRAP1 may account for severer phenotype in terms of xanthoma and atherosclerotic cardiovascular disease in FH patients.

1. Introduction

Familial hypercholesterolemia (FH) is an inherited disease characterized by the triad of (1) hypercholesterolemia due to a high level of plasma LDL, (2) tendon xanthomas and (3) premature coronary artery disease [1]. Patients with homozygous FH have been defined as who have two mutant alleles of either of three following FH-associated genes: LDLR, apolipoprotein B (ApoB) gene and proprotein convertase subtilisin/kexin type 9 (PCSK9) [2]. Previously, we identified several homozygous FH patients who possessed double heterozygous mutations in LDLR gene and PCSK9 gene in relatively mild phenotypic patients compared with those with double mutations in LDLR gene [3]. In addition to autosomal dominant types of FH, recessive form of FH-associated gene was identified in 1992 [4]. The null mutations in the LDL receptor adaptor protein 1 (LDLRAP1) gene, which serves as an adaptor for LDLR endocytosis in the liver, causes autosomal recessive hypercholesterolemia (ARH) [5]. It is described that several heterozygous LDLRAP1 mutation carrier showed elevated LDL-C levels [6,7]. However, there is no data on clinical significance of adding a mutation in LDLRAP1 gene onto single LDLR gene mutation.

2. Methods

2.1. Study subjects

This study was approved by the Ethics Committee of Graduate School of Medical Science, Kanazawa University, and all study subjects gave their written informed consent to participate. We examined consecutive unrelated 146 subjects with a single mutation in the LDLR gene (male = 96, mean age = 56.5 ± 16.0, mean LDL-C = 265.6 ± 57.7 mg/dl) since 2003 to 2008. All the participants were free from unstable or acute cardiovascular diseases. All the lipid-lowering therapy had been transiently suspended for one to three months to diagnose lipid disorders correctly. Although it has been described the existence of the rebound effect after transient suspension of statin therapy [8], it is also reported that short-term suspension of statins is safe for at least patients with stable

* Corresponding author at: Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8641, Japan. Tel.: +81 76 265 2000x2251; fax: +81 76 234 4251.
E-mail addresses: ht240z@med.kanazawa-u.ac.jp, ht240z@sa3.so-net.ne.jp (H. Tada).

0021-9150/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2011.08.004

Table 1
Characteristics of the screened FH subjects.

Age (year)	56.5 ± 16.0
Sex (male/female)	96/50
BMI (kg/m^2)	23.2 ± 3.8
ATT (mm)	12.5 ± 3.5
TC (mg/dl)	330.1 ± 43.1
TG (mg/dl)	114.6 ± 35.1
HDL-C (mg/dl)	42.3 ± 8.7
LDL-C (mg/dl)	265.6 ± 57.7
ApoA-I (mg/dl)	121.8 ± 29.4
ApoB (mg/dl)	189.6 ± 25.8

Values are mean ± SD.

cardiovascular disease [9]. Complications related to this short-term suspension of lipid-lowering therapy have not been observed so far in our institute. The characteristics of the study subjects were listed in Table 1 and Supplementary Table S1.

2.2. Biochemical analysis

Serum concentrations of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically. LDL-C concentrations were derived using the Friedewald formula. Apolipoprotein E (ApoE) phenotype was separated by isoelectric focusing and detected by Western blot with apoE polyclonal antibody (phenotyping apoE IEF system, JOKOH, Tokyo, Japan). Plasma cholesteryl ester transfer protein (CETP) levels were determined by a specific ELISA [10].

2.3. Genetic analysis

Genomic DNA was isolated from peripheral blood white blood cells according to standard procedures and was used for PCR. Primers for the study were as used previously [3,11]; PCR products were purified by Microcon (Millipore Corp., Bedford, MA) and used as templates for direct sequencing. DNA sequencing was carried out according to the manufacturer's instructions using a dye terminator method (ABI PRISM™ 310 Genetic Analyzer (PerkinElmer Biosystems, Waltham, MA). We screened the study subjects for all coding region of PCSK9 and LDLRAP1 genes as candidate genes that could affect their lipid profile and clinical phenotype. In addition, we analyzed the two common mutations of the CETP gene (c.1321 + 1G > A, previously described as Int14A and c.1376A > G, previously described as D442G) among Japanese population as previously described [12].

3. Results

3.1. Biochemical analysis

Serum lipids and apolipoproteins in the proband and her pedigree are presented in Table 2.

3.2. Sequence of LDLR gene

Mutation in LDLR gene of the proband (c.2431A > T) was one of the most common mutations in Japan [13] (Supplementary Fig. S1A).

3.3. Sequence analysis of candidate genes for inherited hypercholesterolemia

Although there was no genetic abnormality in her PCSK9 gene, we identified another heterozygous mutation in her LDLRAP1 gene (c.606dup, Supplementary Fig. S1B).

3.4. Clinical course of the proband

At the age of 67, she was diagnosed as FH due to severe hypercholesterolemia with Achilles' tendon thickness (Supplementary Fig. S2). Initial levels of TC, TG, and HDL-C concentrations were 367, 108, and 46 mg/dl, respectively under statin therapy (pravastatin 20 mg daily). She underwent coronary artery bypass graft surgery at the age of 70 due to angina pectoris. The more intensive cholesterol lowering therapy using atorvastatin 20 mg daily was introduced for secondary prevention of cardiovascular disease. She was referred to our hospital for further examination of her hypercholesterolemia and coronary artery disease at the age of 78. Although her coronary atherosclerosis including bypass grafts did not progress substantially during 8 years (Supplementary Fig. S3), severe aortic valve stenosis developed causing her chest pain (Supplementary Fig. S4). Although aortic valve replacement surgery was recommended, she refused due to potential complications derived from extreme high age (Fig. 1).

3.5. Family study

Family study was performed as intensively as possible to find another family member with LDLR or LDLRAP1 mutation. We identified two other relatives with double mutations, and one obligate carrier who died suddenly probably due to cardiac event in his forties (Fig. 2).

Table 2
Clinical data of the pedigree.

Subject (gender)	I-1 (female)	II-1 (male)	II-2 (male)	III-1 (female)	IV-1 (male)	IV-2 (male)
LDLR genotype	W/M1	W/W	W/M1	W/M1	W/W	W/M1
LDLRAP1 genotype	W/M2	W/W	W/M2	W/M2	W/W	W/W
Age (year)	79	51	45	32	3	2
ATT (mm)	24	n.d.	n.d.	13	n.d.	n.d.
TC (mg/dl)	393	224	365	392	166	286
TG (mg/dl)	165	46	63	60	39	92
HDL-C (mg/dl)	42	97	96	61	59	62
LDL-C (mg/dl)	318	118	235	299	99	205
ApoA-I (mg/dl)	114	n.d.	n.d.	136	136	141
ApoB (mg/dl)	232	n.d.	n.d.	174	68	129
ApoE phenotype	3/3	3/3	3/3	3/3	3/3	3/3
CETP (μg/ml)	4.2	2.0	3.2	2.6	n.d.	n.d.

LDLR genotype: W = wild type, M1 = c.2431A > T; LDLRAP1 genotype: W = wild type, M2 = c.606dup.

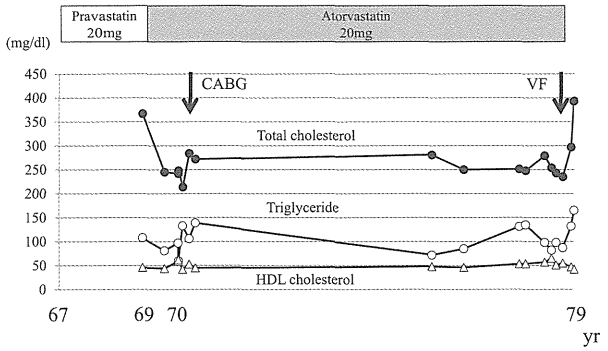

Fig. 1. Clinical course of the proband. Plasma concentration of the total cholesterol (solid circle), triglyceride (open circle), and HDL-C (open triangle) in the proband, and the major clinical events were illustrated. CABG; coronary artery bypass grafting, VF; ventricular fibrillation.

3.6. Genetic analysis for CETP gene

There was no carrier for both of common CETP gene mutation in this family.

4. Discussion

Patients with homozygous FH have two mutant alleles of either of three FH-associated genes (FH genes), namely LDLR, apolipoprotein B-100 and PCSK9 genes. In addition to those dominant form inherited gene mutation recessive form of null mutations in LDLRAP1 gene also causes FH (autosomal recessive hypercholesterolemia:ARH). There are few published data about the clinical characteristics of LDLRAP1 heterozygous mutation carriers because of rarity of this disorder. Previously, we have shown that c.606dup mutation carriers in LDLRAP1 gene had elevated LDL-C concentrations compared with non-carrier family members [14], suggesting that "autosomal recessive hypercholesterolemia" is not necessarily a correct term.

In this paper, we report the first family which exhibit double mutations in LDLR and LDLRAP1 gene with severe xanthomas and coronary artery disease as well as the episode of ventricular fibrillation due to aortic valve stenosis. Besides the proband, we found two other relatives in her family with the same double mutations in LDLR and LDLRAP1 gene.

Some of the pedigrees, including double mutation carriers exhibit relatively high HDL-C level. Previously, we reported that the CETP gene mutations causing higher HDL-C levels are common in Japan [12]. However, there was no carrier of two common CETP gene mutations (c.1321 + 1G > A and c.1376A > G) among this family member. The plasma levels of CETP of this family member were within normal limit, suggesting absence of CETP deficiency. It has been reported that the causes of high HDL-C level were quite heterogeneous [15]. Thus, we cannot exclude the possibility that unknown genetic factors may be involved in their high HDL-C levels. Another possibility of higher HDL-C is their excessive alcohol drinking. The pedigrees whose HDL-C levels were more than 90 mg/dl (II-1 and II-2) were both heavy drinkers (ethanol > 120 g/day).

In conclusion, we report the first family with double mutation in LDLR and LDLRAP1 genes associated with autosomal dominant and recessive form of hypercholesterolemia. Although the proband exhibited massive Achilles tendon xanthoma and severe coronary and aortic valvular disease, serum LDL-C level of subjects with double mutations was similar with that of subjects with single LDLR mutation. We suggest that an additional mutation in LDLRAP1 may account for severer phenotype in terms of xanthoma and atherosclerotic cardiovascular disease in FH patients.

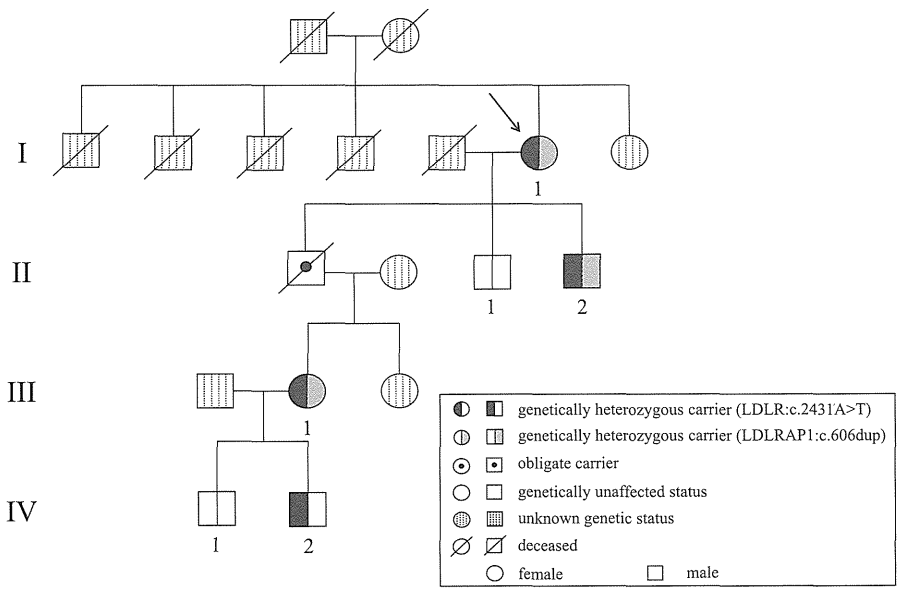

Fig. 2. Pedigree of the proband. Half-filled by black squares or circles indicate the heterozygous mutation carrier in LDLR (c.2431A > T). Half-filled by brown squares or circles indicate the heterozygous mutation carrier in LDLRAP1 (c.606dup). Square with a dot indicates the obligate carrier. Open squares or circles indicate unaffected subjects. Hatched squares or circles indicate the genetically unknown subjects.

Sources of funding

None declared.

Conflict of interest statement

The authors have no conflict of interest.

Acknowledgements

We express our special thanks to Kazuko Honda and Sachio Yamamoto (staff of Kanazawa University) for their outstanding technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.08.004.

References

[1] Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease, 8the ed., vol. 2. New York: McGraw-Hill; 2001. p. 2863–913.
[2] Soutar AK, Naoumova RP. Mechanisms of disease: genetic causes of familial hypercholesterolemia. Nat Clin Pract Cardiovasc Med 2007;4:214–25.
[3] Noguchi T, Katsuda S, Kawashiri MA, et al. The E32K variant of PCSK9 exacerbates the phenotype of familial hypercholesterolemia by increasing PCSK9 function and concentration in the circulation. Atherosclerosis 2010;210:166–72.
[4] Harada-Shiba M, Tajima S, Yokoyama S, et al. Siblings with normal LDL receptor activity and severe hypercholesterolemia. Arterioscler Thromb 1992;12:1071–8.
[5] Garcia CK, Wilund K, Arca M, et al. Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. Science 2001;292:1394–8.
[6] Pisciotta L, Oliva CP, Pes GM, et al. Autosomal recessive hypercholesterolemia (ARH) and homozygous familial hypercholesterolemia (FH): a phenotypic comparison. Atherosclerosis 2006;188:398–405.
[7] Harada K, Miyamoto Y, Morisaki H, et al. A novel Thr56Met mutation of the autosomal recessive hypercholesterolemia gene associated with hypercholesterolemia. J Atheroscler Thromb 2010;17:131–40.
[8] Daskalopoulou SS. When statin therapy stops: implications for the patient. Curr Opin Cardiol 2009;24:454–60.
[9] McGowan MP, Treating to New Target (TNT) Study Group. There is no evidence for an increase in acute coronary syndromes after short-term abrupt discontinuation of statins in stable cardiac patients. Circulation 2004;110:2333–5.
[10] Kiyohara T, Kiriyama R, Zamma S, et al. Enzyme immunoassay for cholesteryl ester transfer protein in human serum. Clin Chim Acta 1998;271:109–18.
[11] Barbagallo CM, Emmanuele G, Cefalù AB, et al. Autosomal recessive hypercholesterolemia in a Sicilian kindred harboring the 432insA mutation of the ARH gene. Atherosclerosis 2003;166:395–400.
[12] Inazu A, Jiang XC, Haraki T, et al. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. J Clin Invest 1994;94:1872–82.
[13] Tada H, Kawashiri MA, Noguchi T, et al. A novel method for determining functional LDL receptor activity in familial hypercholesterolemia: application of the CD3/CD28 assay in lymphocytes. Clin Chim Acta 2009;400:42–7.
[14] Tada H, Kawashiri MA, Noguchi T, et al. Clinical impact of heterozygous carrier of autosomal recessive hypercholesterolemia on asymptomatic hyperlipidemic patients: evidence from familial gene analysis. Circulation 2008;118:S405.
[15] Inazu A, Mabuchi H. High-HDL syndrome. In: Ganten D, Ruckpaul K, editors. Encyclopedic reference of genomic and proteomics in molecular medicine, 1st ed., part 8. Springer; 2006. p. 792–5.

Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Regenerative Medicine

Gene and Protein Expression Analysis of Mesenchymal Stem Cells Derived From Rat Adipose Tissue and Bone Marrow

Chiaki Nakanishi, MD; Noritoshi Nagaya, MD, PhD; Shunsuke Ohnishi, MD, PhD; Kenichi Yamahara, MD, PhD; Shu Takabatake, MD; Tetsuo Konno, MD, PhD; Kenshi Hayashi, MD, PhD; Masa-aki Kawashiri, MD, PhD; Toshinari Tsubokawa, MD, PhD; Masakazu Yamagishi, MD, PhD

Background: Mesenchymal stem cells (MSC) are multipotent and reside in bone marrow (BM), adipose tissue and many other tissues. However, the molecular foundations underlying the differences in proliferation, differentiation potential and paracrine effects between adipose tissue-derived MSC (ASC) and BM-derived MSC (BM-MSC) are not well-known. Therefore, we investigated differences in the gene and secretory protein expressions of the 2 types of MSC.

Methods and Results: ASC and BM-MSC were obtained from subcutaneous adipose tissue and BM of adult Lewis rats. ASC proliferated as rapidly as BM-MSC, and had expanded 200-fold in approximately 2 weeks. On microarray analysis of 31,099 genes, 571 (1.8%) were more highly (>3-fold) expressed in ASC, and a number of these genes were associated with mitosis and immune response. On the other hand, 571 genes (1.8%) were more highly expressed in BM-MSC, and some of these genes were associated with organ development and morphogenesis. In secretory protein analysis, ASC secreted significantly larger amounts of growth factor and inflammatory cytokines, such as vascular endothelial growth factor, hepatocyte growth factor and interleukin 6, whereas BM-MSC secreted significantly larger amounts of stromal-derived factor-1α.

Conclusions: There are significant differences between ASC and BM-MSC in the cytokine secretome, which may provide clues to the molecule mechanisms associated with tissue regeneration and alternative cell sources.
(*Circ J* 2011; **75:** 2260–2268)

Key Words: Cell therapy; Mesenchymal stem cells; Microarray; Secretory protein

Mesenchymal stem cells (MSC) are multipotent cells that reside within various tissues, including bone marrow (BM), adipose tissue and many other tissues,[1,2] and can differentiate into a variety of cell types of mesodermal lineage.[1,3] MSC can be expanded in vitro over the short term, and they are thought to be an attractive tool for cell therapy. It has been demonstrated in animal and human studies of cardiovascular disease that transplanted BM-MSC induce neovascularization and differentiate into functional cells.[4–8] In addition, recent studies suggest that MSC exert tissue regeneration, secreting various kinds of angiogenic and cytoprotective factors.[6,9,10]

Editorial p 2060

Subcutaneous adipose tissue can be harvested more safely and noninvasively than BM, and ASC have emerged as a possible alternative cell source to BM-MSC.[9,11] We and others have demonstrated that ASC transplantation induces neovascularization in animal models of myocardial infarction and hindlimb ischemia.[12,13] ASC are similar to BM-MSC in terms of morphology and surface marker expression.[14] However, few data exist regarding their differences in biological activity, such as proliferative activity, differentiation potential and productive ability. Using microarray and enzyme-linked immunosorbent

Received March 6, 2011; revised manuscript received April 27, 2011; accepted May 10, 2011; released online July 12, 2011 Time for primary review: 17 days
Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, Kanazawa (C.N., S.T., T.K., K.H., M.K., T.T., M.Y.); Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Suita (N.N., K.Y.); and Department of Gastroenterology, Hokkaido University Graduate School of Medicine, Sapporo (S.O.), Japan
Part of this work was presented at the Annual Scientific Session of the American College of Cardiology, Orland, 2009.
Mailing address: Masakazu Yamagishi, MD, PhD, Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8641, Japan. E-mail: myamagi@med.kanazawa-u.ac.jp
ISSN-1346-9843 doi:10.1253/circj.CJ-11-0246

assay (ELISA), we have performed a comprehensive analysis to evaluate both the differences between ASC and BM-MSC, and their usage as an effective transplanted cell source from the point of view of the gene and protein expression profile of the 2 MSC sources.

Methods

Isolation and Culture of ASC and BM-MSC

All protocols were performed in accordance with the guidelines of the Animal Care Committee of the National Cardiovascular Center Research Institute and Kanazawa University. MSC isolation and culture were performed according to previously described methods.[15] In brief, we harvested BM from male Lewis rats (Japan SLC, Hamamatsu, Japan) weighing 200–250 g by flushing their femoral cavities with phosphate-buffered saline. Subcutaneous adipose tissue was harvested from the inguinal region and minced with scissors, then digested with 0.1% type I collagenase (300 U/ml; Worthington Biochemical, Lakewood, NJ, USA) for 1 h at 37°C in a water-bath shaker. After filtration with 100-μm filter mesh (Cell Strainer; Becton Dickinson, Bedford, MA, USA) and centrifugation at 1,240 g for 5 min, MSC were cultured in complete culture medium: α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). A small number of cells developed visible symmetric colonies by days 5–7. Nonadherent hematopoietic cells were removed, and the medium was replaced. The adherent, spindle-shaped MSC population expanded to >5×10^7 cells within 3–5 passages after the cells were first plated.

Cell Proliferation

We compared the proliferative activity of ASC and BM-MSC in cell culture, as reported previously.[16] In brief, cells (3×10^5 cells/dish) at passage 1 were cultured in a 10-cm dish with complete culture medium, and harvested at 70–90% confluency at each passage. Cell number was counted with a hemocytometer (n=5).

Differentiation of ASC and BM-MSC Into Adipocytes and Osteoblasts

MSC (1×10^5 cells/well) were seeded onto 12-well plates, and differentiation into adipocytes and osteocytes was induced when MSC were 70–80% confluent. MSC were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan) according to the manufacturer's instructions. After 14–17 days of differentiation, cells were fixed and stained with Alizarin Red S (Sigma-Aldrich, St Louis, MO, USA). To induce differentiation into adipocytes, MSC were cultured with adipocyte differentiation medium: 0.5 mmol/L 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μmol/L dexamethasone (Wako Pure Chemical Industries), 50 μmol/L indomethacin (Wako Pure Chemical Industries), and 10 μg/ml insulin (Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% FBS. After 21 days of differentiation, adipocytes were stained with Oil Red O (Sigma-Aldrich). In order to measure lipid accumulation, isopropyl alcohol was added to the stained culture plate, the extracted dye was immediately collected, and the absorbance was measured spectrophotometrically at 490 nm (Bio-Rad, Hercules, CA, USA).

Microarray Analysis of ASC and BM-MSC

To compare the gene expression of ASC and BM-MSC, microarray analysis was performed according to previously reported methods.[17] Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by spectrometry, and its quality was confirmed by gel electrophoresis. Double-stranded cDNA was synthesized from 10 μg of total RNA, and in-vitro transcription was performed to produce biotin-labeled cRNA using GeneChip One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. After fragmentation, 10 μg of cRNA was hybridized with a GeneChip Rat Genome 230 2.0 Array (Affymetrix) containing 31,099 genes. The GeneChips were then scanned in a GeneChip Scanner 3000 (Affymetrix). Normalization, filtering and Gene Ontology analysis of the data were performed with GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA, USA). The raw data from each array were normalized as follows: each CEL file was preprocessed with RMA, and each measurement for each gene was divided by the 80th percentile of all measurements. Genes showing at least a 3-fold change were then selected.

Quantitative Real-Time Reverse-Transcription–Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cultured BM-MSC and ASC as described, and 5 μg of total RNA was reverse-transcribed into cDNA using a QuantiTect reverse-transcription kit (Qiagen) according to the manufacturer's instructions. PCR amplification was performed in 50 μl containing 1 μl of cDNA and 25 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, amplified from the same samples, served as an internal control. After an initial denaturation at 95°C for 10 min, a 2-step cycle procedure was used (denaturation at 95°C for 15s, annealing and extension at 60°C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). Gene expression levels were normalized according to that of GAPDH.

ELISA

To investigate differences in protein secretion between ASC and BM-MSC, we measured the levels of various bioactive proteins, including proliferative and anti-apoptotic factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and adrenomedullin (AM); chemokines such as stem cell-derived factor-1α (SDF-1α); inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6); and adipokines such as leptin and plasminogen activator inhibitor-1 (PAI-1). Protein levels were measured in conditioned medium 24 h after medium replacement. MSC (1×10^6 cells/dish) were plated in 10-cm dishes and cultured in complete culture medium. After 24 h, conditioned medium (n=6) was collected and centrifuged at 2,000 g for 10 min, and the supernatant was filtered through a 0.22-μm filtration unit (Millipore, Bedford, MA, USA). Angiogenic and growth factors were measured by ELISA according to each of the manufacturer's instructions (VEGF, TNF-α: R&D Systems, Minneapolis, MN, USA; HGF: Institute of Immunology, Tokyo, Japan; AM: Phoenix Pharmaceuticals, Burlingame, CA, USA; IL-6: Pierce, Rockford, IL, USA; adiponectin: AdipoGen, Seoul, Korea; PAI-1, Oxford Biomedica Reseach, Oxford, CT, USA).

Statistical Analysis

Data are expressed as mean±standard error of the mean. Comparisons of parameters among groups were made by 1-way ANOVA, followed by Newman-Keuls' test. Differences were

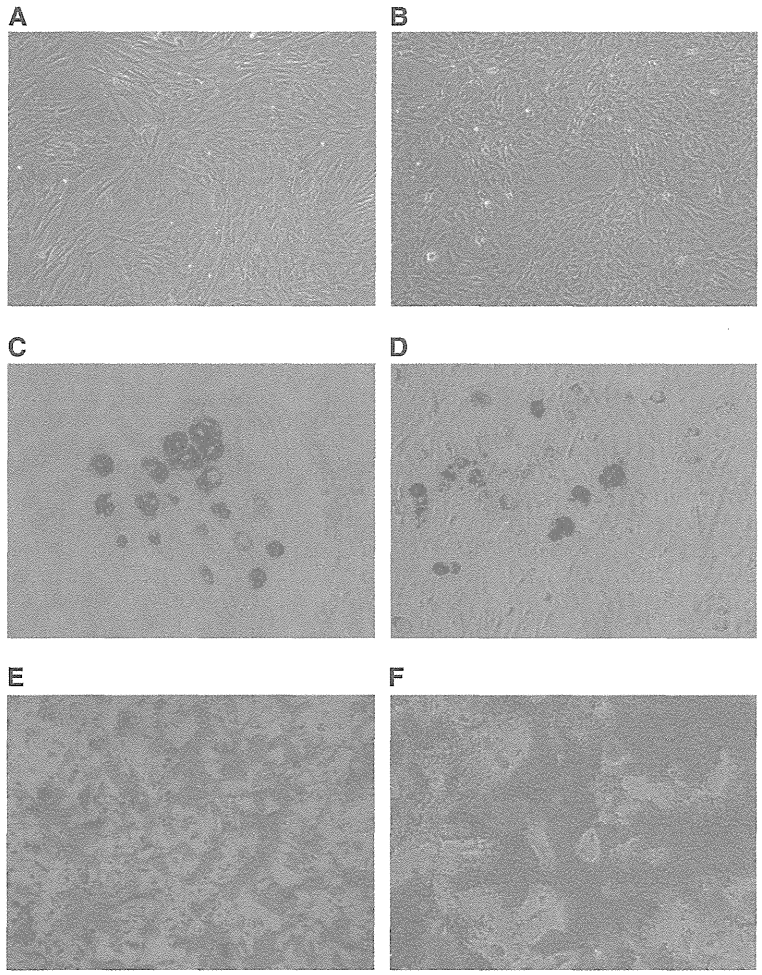

Figure 1. Morphology and differentiation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC). Cultured ASC (**A**) and BM-MSC (**B**) at passage 2 (×100). Their typical morphology is a similar spindle shape, but ASC are slightly longer than BM-MSC. ASC (**C**) and BM-MSC (**D**) differentiated into adipocytes (×100; Oil Red O). ASC (**E**) and BM-MSC (**F**) differentiated into osteoblasts (×100; Alizarin Red S).

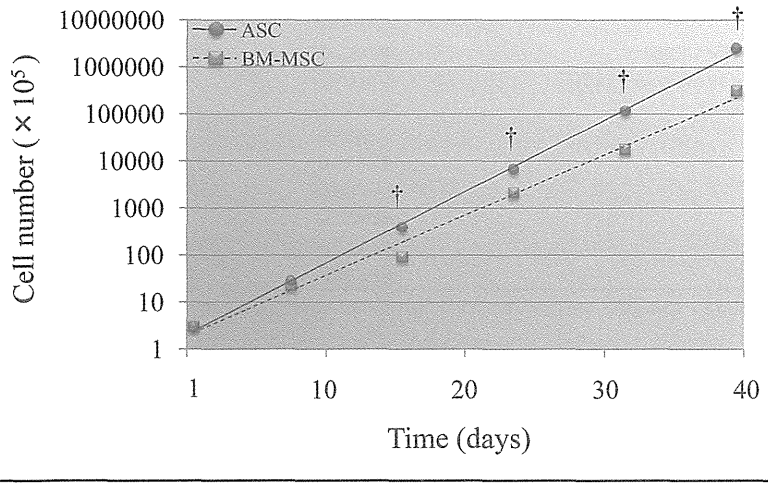

Figure 2. Proliferation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC) shown as a growth chart. Vertical axis, cell number; horizontal axis, day after first passage. Day 1 is the first day at passage 1. Values are mean±SEM. *P<0.05, †P<0.01 vs. BM-MSC.

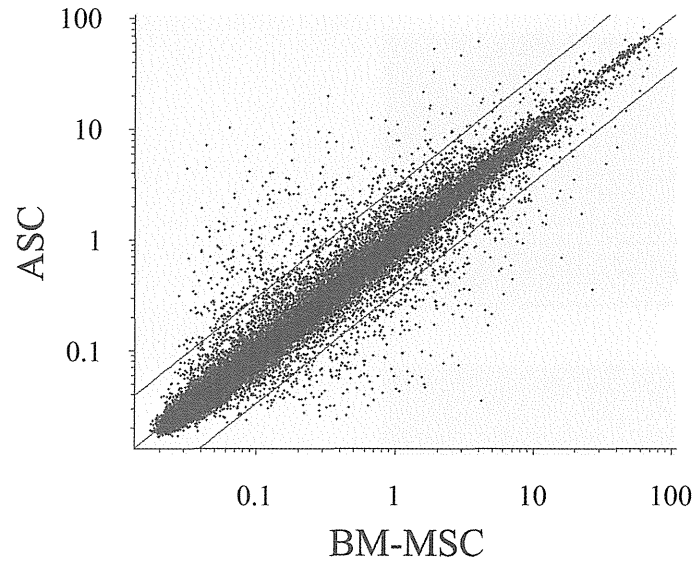

Figure 3. Gene expression profiles of adipose tissue-derived mesenchymal stem cells (ASC, vertical axis) and bone marrow-derived mesenchymal stem cells (BM-MSC, horizontal axis) by microarray. The scatter plot shows normalized microarray datasets of ASC and BM-MSC. All 31,099 gene probes are represented in these plots. The outer lines indicate a 3-fold difference; the central line represents equality.

Table 1. Genes Upregulated in ASC in Comparison With BM-MSC (>10-Fold Upregulation)

Gene name	GenBank Acc. no.	Fold change
Interleukin 1α (Il1a)	NM017019	38.1
Interleukin 1 receptor, type II (Il1r2)	NM053953	21.7
Chemokine (C-X-C motif) ligand 1 (Cxcl1)	NM030845	21.6
Lipocalin 2 (Lcn2)	NM130741	21.5
Fast myosin alkali light chain (Rgd:620885)	NM020104	20.6
Interleukin 6 (Il6)	NM012589	20.5
Chemokine (C-C motif) ligand 20 (Ccl20)	AF053312	17.6
Twist homolog 2 (Twist2)	NM021691	17.5
RAS, dexamethasone-induced 1 (Rasd1)	AF239157	17.1
Complement component 3 (C3)	NM016994	16.9
NADPH oxidase 1 (Nox1)	NM053683	16.3
Matrix metallopeptidase 9 (Mmp9)	NM031055	15.2
Colony-stimulating factor 3 (Csf3)	NM017104	14.5
Prostaglandin E synthase (Ptges)	AB048730	12.8
Adenosine A2B receptor (Adora2b)	NM017161	12.5
Oxidized low-density lipoprotein receptor 1 (Oldlr1)	NM133306	12.4
Uterine sensitization-associated gene 1 protein (Sostdc1)	AA892798	12.1
Chemokine (C-X-C motif) ligand 5 (Cxcl5)	NM022214	11.9
Neuregulin 1 (Nrg1)	U02315	11.8
CD24 antigen (Cd24)	BI285141	11.6
Cathepsin c (Ctsc)	AA858815	11.2
Lymphocyte antigen 68 (C1qr1)	BI282932	11.2
Interleukin 1 receptor antagonist (Il1rn)	NM022194	11.1
Chemokine (C-C motif) ligand 2 (Ccl2)	NM031530	10.8

ASC, adipose tissue-derived mesenchymal stem cells; BM-MSC, bone marrow-derived mesenchymal stem cells.

considered significant at $P<0.05$.

Results

Proliferation and Differentiation of ASC and BM-MSC

Both ASC and BM-MSC could be expanded on a plastic dish, and they exhibited a similar fibroblast-like morphology (**Figures 1A,B**). To examine the potential of ASC and BM-MSC to differentiate into adipocytes, the cells were cultured in adipogenesis medium for 21 days (**Figures 1C,D**). Although lipid droplets were not observed in undifferentiated ASC or BM-MSC, ASC and BM-MSC cultured in adipogenesis

Table 2. Genes Upregulated in BM-MSC in Comparison With ASC (>10-Fold Upregulation)

Gene name	GenBank Acc. no.	Fold change
WNT1 inducible signaling pathway protein 2 (Wisp2)	NM031590	202.5
Complement component factor H (Cfh)	NM130409	81.9
Osteomodulin (Omd)	NM031817	67.4
Solute carrier organic anion transporter family, member 2a1 (Slco2a1)	AI407489	65.8
Dynein, cytoplasmic, intermediate chain 1 (Dncic1)	NM019234	64.8
3-α-hydroxysteroid dehydrogenase (RGD:708361)	BF545626	37.7
Preproenkephalin, related sequence (Penk-rs)	NM017139	29.3
Fc receptor, IgG, low affinity lib (Fcgr2b)	X73371	29.3
Actin, γ 2 (Actg2)	NM012893	25.9
α–2-macroglobulin (A2m)	NM012488	23.2
Lysozyme (Lyz)	L12458	22.2
Jagged 1 (Jag1)	NM019147	19.3
Phospholamban (Pln)	BI290034	17.6
Procollagen, type XI, α 1 (Col11a1)	BM388456	16.2
Gamma sarcoglycan (RGD:1359577)	AA850867	15.3
Pleiomorphic adenoma gene-like 1 (Plagl1)	NM012760	15.0
Matrix metallopeptidase 12 (Mmp12)	NM053963	14.7
Cyclin D2 (Ccnd2)	L09752	14.4
Transforming growth factor, β 2 (Tgfb2)	NM031131	14.3
Solute carrier family 29, member 1 (Slc29a1)	NM031684	14.1
Tissue inhibitor of metalloproteinase 3 (Timp3)	AA893169	13.2
Procollagen, type XI, α 1 (Col11a1)	BM389291	13.1
Down syndrome critical region gene 1-like 1 (Dscr1l1)	AI138048	12.8
Bone morphogenetic protein 4 (Bmp4)	NM012827	12.7
Matrix metallopeptidase 13 (Mmp13)	M60616	11.8
Macrophage galactose N-acetyl-galactosamine specific lectin 1 (Mgl1)	NM022393	11.2
Glycoprotein nmb (Gpnmb)	NM133298	10.7
Aquaporin 1 (Aqp1)	AA891661	10.6
Cadherin 13 (Cdh13)	NM138889	10.5
Selenoprotein P, plasma, 1 (Sepp1)	AA799627	10.5
Secreted frizzled-related protein 4 (Sfrp4)	AF140346	10.4
Cellular retinoic acid binding protein 2 (Crabp2)	U23407	10.2

ASC, adipose tissue-derived mesenchymal stem cells; BM-MSC, bone marrow-derived mesenchymal stem cells.

medium stained positively with Oil Red O in 3 weeks. To quantify lipid accumulation, the absorbance of the extracted cells was measured; however, there was no difference in the absorbance between differentiated ASC and BM-MSC. In addition, both ASC and BM-MSC differentiated identically into osteocytes (**Figures 1E,F**). ASC proliferated more rapidly than BM-MSC; the number of ASC was approximately 10-fold higher than that of BM-MSC at the 40th day (**Figure 2**). In approximately 2 weeks, ASC had expanded almost 200-fold, whereas BM-MSC had expanded nearly 30-fold.

Differences in the Gene Expression of ASC and BM-MSC

Of 31,099 genes analyzed, 571 (1.8%) were more highly (>3-fold) expressed in ASC, whereas 571 genes (1.8%) were more highly (>3-fold) expressed in BM-MSC (**Figure 3**). The genes showing the most enriched expression (>10-fold) in ASC and BM-MSC are listed in **Table 1**. Of note, the genes that were highly expressed in ASC included various types of molecules involved in inflammation, such as IL-1α and IL-6, and chemotaxis, such as chemokine (C-C motif) ligand 20 and chemokine (C-X-C motif) ligand 5 (**Table 1**). The genes that were highly expressed in BM-MSC included differentiation-associated genes, such as WNT1-inducible signaling pathway protein 2 (Wisp2), osteomodulin and jagged1 (**Table 2**). Furthermore, the differential expression patterns of 5 representative genes in ASC and BM-MSC obtained by microarray were confirmed by qRT-PCR, which gave the relative expression of IL-1α as 438.2±560.9 (ratio ASC/BM-MSC, n=5), IL-6 as 54.0±26.6, MMP9 as 3.9±2.2, VEGF 1.8±0.4, and Wisp2 as 7.0±2.2.

To evaluate the genes upregulated in ASC, 571 genes that were more highly expressed in ASC were classified by functional annotation using gene ontology terms (**Table 3**). The 31 terms listed had a P-value <0.00001, and included mitosis (eg, pituitary tumor-transforming 1, cyclin B1, cyclin-dependent kinase 2), immune response (eg, chemokine (C-C motif) ligand 20, cathepsin C and IL-1α) and response to stress (glutathione peroxidase 2, superoxide dismutase 2 and metallothionein). In BM-MSC, 22 terms were listed for the 571 enriched genes, and included regulation of organ development (eg, Wisp2, osteomodulin and bone morphogenetic protein 4), morphogenesis (cadherin 13, elastin and Neuropillin 2) and cell migration (chemokine (C-X3-C motif) ligand 1 and chemokine (C-X-C motif) receptor 4) (**Table 4**).

Differences Between ASC and BM-MSC in Secretory Proteins Determined by ELISA

In previous reports, MSC evoked a cell protective effect and induced angiogenesis via secretion of various cytokines, includ-

Table 3. Classification of Highly (>3-Fold) Expressed Genes in ASC According to Gene Ontology Terms

Category	% of genes in category	% of genes in list in category	P value
0007067: Mitosis	1.3	11.4	4.43×10^{-24}
0000279: M phase	1.8	12.4	6.87×10^{-22}
0000278: Mitotic cell cycle	2.2	12.7	2.53×10^{-19}
0007049: Cell cycle	7.0	21.1	3.23×10^{-16}
0007059: Chromosome segregation	0.31	4.14	6.51×10^{-12}
0006260: DNA replication	1.3	7.32	9.94×10^{-12}
0007088: Regulation of mitosis	0.34	3.82	3.10×10^{-10}
0000070: Mitotic sister chromatid segregation	0.15	2.86	3.11×10^{-10}
0051301: Cell division	0.79	5.41	3.30×10^{-10}
0006955: Immune response	5.7	14.9	1.15×10^{-9}
0007017: Microtubule-based process	1.6	7.32	1.60×10^{-9}
0007093: Mitotic checkpoint	0.13	2.54	2.01×10^{-9}
0000074: Regulation of progression through cell cycle	4.5	12.7	2.77×10^{-9}
0006259: DNA metabolism	4.8	13.1	5.12×10^{-9}
0006952: Defense response	6.2	15.2	7.53×10^{-9}
0009613: Response to pest, pathogen or parasite	3.5	10.8	8.31×10^{-9}
0000075: Cell cycle checkpoint	0.44	3.82	9.71×10^{-9}
0009607: Response to biotic stimulus	6.6	15.6	1.32×10^{-8}
0043207: Response to external biotic stimulus	3.7	10.8	1.73×10^{-8}
0006950: Response to stress	9.2	19.1	3.41×10^{-8}
0031577: Spindle checkpoint	0.084	1.91	7.56×10^{-8}
0007018: Microtubule-based movement	0.87	4.77	8.72×10^{-8}
0006954: Inflammatory response	1.6	6.05	9.52×10^{-7}
0009605: Response to external stimulus	5.9	12.7	4.21×10^{-6}
0050896: Response to stimulus	16	25.8	4.24×10^{-6}
0031649: Heat generation	0.046	1.27	4.68×10^{-6}
0007052: Mitotic spindle organization and biogenesis	0.153	1.91	5.28×10^{-6}
0000226: Microtubule cytoskeleton organization and biogenesis	0.649	3.51	5.55×10^{-6}
0007010: Cytoskeleton organization and biogenesis	4.39	10.1	8.14×10^{-6}
0000067: DNA replication and chromosome cycle	0.0993	1.59	8.43×10^{-6}
0007051: Spindle organization and biogenesis	0.168	1.91	9.76×10^{-6}

ASC, adipose tissue-derived mesenchymal stem cells.

ing VEGF, HGF and SDF-1α.[4,5,10] To compare the proteins secreted by cultured ASC and BM-MSC, we used ELISA to investigate the production of several angiogenic and growth factors from ASC and BM-MSC cultures (**Figure 4**). As compared with BM-MSC, ASC secreted significantly larger amounts of not only HGF and VEGF, which are growth and angiogenic factors, but also PAI-1 and IL-6, which are adipokines. On the other hand, BM-MSC secreted significantly larger amounts of SDF-1α, which is a cell migration-related chemokine, than ASC. There was no significant difference between ASC and BM-MSC for several secreted adipokines, such as adiponectin and TNF-α.

Discussion

In this study, we examined the differences between ASC and BM-MSC in proliferation, differentiation, gene expression and secreted proteins. We showed that (1) ASC are more proliferative than BM-MSC, although there is no difference in differentiation into adipocytes or osteocytes; (2) genes associated with mitosis, inflammation and stress response are highly expressed in ASC; (3) genes associated with regulation of organ development, morphogenesis and cell migration are highly expressed in BM-MSC; and (4) ASC secrete significantly larger amounts of growth factors and inflammatory cytokines than BM-MSC, although BM-MSC secrete significantly larger amounts of chemokine than ASC.

In terms of differentiation, both ASC and BM-MSC differentiated into adipocytes and osteocytes, and there was no difference between them in adipogenesis in our quantitative analysis. A previous report demonstrated that BM-MSC had distinct osteogenic differentiation capability in comparison with ASC,[18] although we did not evaluate difference in osteogenesis between ASC and BM-MSC. Indeed, osteomodulin, which is an osteogenesis-related gene, was upregulated in BM-MSC in comparison with ASC (**Table 2**). Therefore, BM-MSC might have more osteogenic potential than ASC. These findings suggest that ASC and BM-MSC have multilineage potential and an equivalent potential to differentiate into unfavorable cells. Under these conditions, we found that ASC proliferated more rapidly than BM-MSC, and expanded 4-fold as much BM-MSC in approximately 2 weeks. Lee et al compared the proliferation and gene expression profile of human ASC and BM-MSC,[19] and also demonstrated that ASC differ from BM-MSC in terms of proliferation according to culture medium. A large number of MSC are needed for cell transplantation, so rapid proliferation of ASC ex vivo is thought to be a favorable source of transplanted cells in the acute clinical setting, although there remain prob-

Table 4. Classification of Highly (>3-Fold) Expressed Genes in BM-MSC According to Gene Ontology Terms

Category	% of genes in category	% of genes in list in category	P value
0048513: Organ development	8.86	21.9	5.02×10^{-12}
0008283: Cell proliferation	5.07	15.4	1.77×10^{-11}
0040007: Growth	2.18	9.62	4.23×10^{-11}
0009653: Morphogenesis	8.46	20.6	5.90×10^{-11}
0007275: Development	21.1	37.1	1.64×10^{-10}
0016049: Cell growth	1.53	7.56	6.82×10^{-10}
0016477: Cell migration	1.88	8.24	1.29×10^{-9}
0001558: Regulation of cell growth	1.31	6.52	8.83×10^{-9}
0007155: Cell adhesion	5.82	14.7	1.47×10^{-8}
0001501: Skeletal development	1.73	7.21	3.42×10^{-8}
0000902: Cellular morphogenesis	4.19	11.3	1.92×10^{-7}
0040008: Regulation of growth	1.64	6.52	3.21×10^{-7}
0009887: Organ morphogenesis	3.96	10.6	5.31×10^{-7}
0050678: Regulation of epithelial cell proliferation	0.0687	1.71	6.13×10^{-7}
0051674: Localization of cell	2.87	8.59	1.10×10^{-6}
0007626: Locomotory behavior	3.16	8.93	1.92×10^{-6}
0050673: Epithelial cell proliferation	0.084	1.71	2.17×10^{-6}
0006952: Defense response	6.27	13.7	2.26×10^{-6}
0009607: Response to biotic stimulus	6.59	14.1	3.12×10^{-6}
0045785: Positive regulation of cell adhesion	0.045	1.37	3.46×10^{-6}
0042127: Regulation of cell proliferation	3.32	8.93	4.56×10^{-6}
0050874: Organismal physiological process	16.7	27.1	4.75×10^{-6}

BM-MSC, bone marrow-derived mesenchymal stem cells.

lems concerning tumorigenesis and instability.

In this study, we carried out a comprehensive analysis in rat ASC and BM-MSC using microarrays. Interestingly, there was a considerable difference between the gene profile of our data and that of Lee et al,[19] who demonstrated that highly expressed genes in ASC accounted for less than 1% of all genes, and keratin 18, thrombospondin 1 and heat shock protein were included in the list of genes upregulated in ASC as compared with BM-MSC. Their human study was of 16–84-year-old patients undergoing arthroplasty and abdominoplasty, whereas we used 6-week-old rats. It is possible that differences in species and culture conditions, as well as age, contributed to these differences in gene expression.

We demonstrated that many of the genes that were highly expressed in ASC could be classified into categories such as mitosis, cell cycle and inflammatory cytokines, suggesting that ASC are more proliferative than BM-MSC. Thus, ASC transplant may not be superior to BM-MSC in terms of improvement of cardiac function in acute myocardial infarction, although it might be expected that ASC would contribute more to cell proliferation because of their secretion of VEGF and HGF. Also, ASC might initiate a stronger inflammatory response, because of the significantly increased upregulation of genes associated with inflammation as compared with BM-MSC. On the other hand, many of the genes that were highly expressed in BM-MSC were classified into categories such as organ development and morphogenesis. BM-MSC upregulated the expression of genes associated with cardiogenesis and angiogenesis, such as Wisp2, jagged1 and insulin-like growth factor binding protein 4 (IGFBP4). In particular, jagged1 and IGFBP4 have been reported to induce cardiogenesis and angiogenesis, respectively, via activation of notch signals and inhibition of Wnt signals.[20,21] Indeed, a previous report demonstrated that BM-MSC transplantation into the infarcted heart induces cardiogenesis and angiogenesis.[22–24] On the other hand, ASC are also reported to be able to differentiate into cardiomyocytes.[25] Therefore, ASC and BM-MSC both might improve cardiac function by supplementing cardiomyocytes, as well as in a paracrine manner, although we did not investigate differences in differentiation into cardiomyocytes between them.

BM-derived mononuclear cells and MSC have been used for therapeutic angiogenesis in ischemic disease.[26,27] MSC are thought to be more effective than mononuclear cells as a source of transplanted cells because MSC secrete larger amounts of growth factors.[26] Recent studies suggest that MSC exert tissue regeneration not only by differentiation into specific cell types, but also through paracrine actions, secreting various kinds of angiogenic and cytoprotective factors,[5,10] as shown in the present study. A recent report has shown that the combination of VEGF and MSC can enhance angiogenesis after acute myocardial infarction in rats.[28] Additionally, a previous study demonstrated that BM-MSC activate cardiac progenitor cells, which have the ability to differentiate into cardiomyocytes, in a paracrine manner in vitro and in vivo.[29,30] HGF and SDF-1α improve cardiac function via the activation of cardiac progenitor cells.[31] In our study, both ASC and BM-MSC secreted various cytokines and chemokines that are related to angiogenesis and cardiogenesis.

Although ASC are used as an adequate transplanted cell type for the treatment of ischemic limb disease,[32] ASC secrete larger amounts of not only inflammatory cytokines, such as IL-6, but also PAI-1 which promotes coagulation. In our gene analysis, several genes associated with other inflammatory cytokines and chemokines were upregulated in ASC. Not only the gene analysis but also the ELISA results suggested that ASC evoke more inflammation and thrombogenesis than BM-MSC. Therefore, ASC transplantation might be a more useful

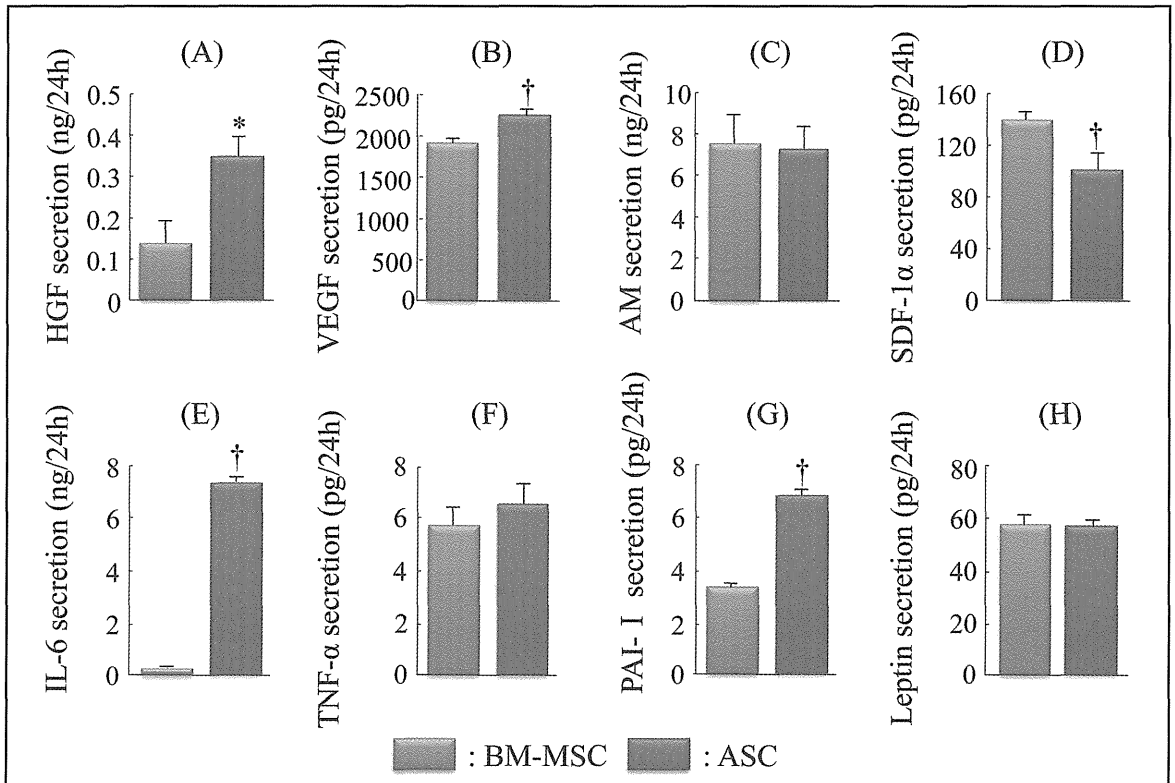

Figure 4. Secretory proteins from adipose tissue-derived mesenchymal stem cells (ASC: red) and bone marrow-derived mesenchymal stem cells (BM-MSC: blue). Conditioned media from ASC and BM-MSC were collected after incubation for 24 h in complete medium. Hepatocyte growth factor (HGF, **A**), vascular endothelial growth factor (VEGF, **B**), adrenomedullin (AM, **C**), stem cell-derived factor-1α (SDF-1α, **D**), interleukin-6 (IL-6, **E**), tumor necrosis factor-α (TNF-α, **F**), plasminogen activator inhibitor-1 (PAI-1, **G**), and leptin (**H**) in conditioned media were measured by enzyme-linked immunosorbent assay. Values are mean±standard error of the mean. *P<0.05, †P<0.01 vs. BM-MSC.

treatment for chronic ischemia without severe inflammation.

In this study, we investigated ASC and BM-MSC obtained from young, 6-week-old rats, and we did not examine differences among various generations of rats. A previous report showed that MSC are subject to molecular genetic changes, such as alterations in p53, HGF and VEGF, during aging.[33] Our results might reflect the character of MSC obtained from young rats, contributing to difference from results in humans.[18] We need to further investigate differences between ASC and BM-MSC not only derived from rats but also derived from humans of various ages.

Conclusion

We have demonstrated difference in proliferation and gene expression between ASC and B-MSC, and accordingly, we suggest the importance of selecting the appropriate cell type for transplantation according to the therapeutic indication.

Acknowledgments

This work was supported by research grants for Human Genome Tissue Engineering 009 from the Ministry of Health, Labor and Welfare, and the Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284:** 143–147.
2. Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. *Exp Biol Med* 2001; **226:** 507–520.
3. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997; **276:** 71–74.
4. Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, et al. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *Am J Physiol* 2004; **287:** 2670–2676.
5. Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. *Circulation* 2005; **112:** 1128–1135.
6. Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, et al. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* 2004; **109:** 1543–1549.
7. Chen S, Liu Z, Tian N, Zhang J, Yei F, Duan B, et al. Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery. *J Invasive Cardiol* 2006; **18:** 552–556.
8. Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* 2004; **94:** 92–95.
9. De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu