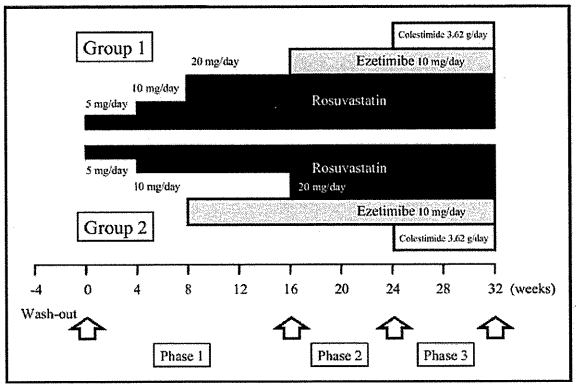

Figure 1. The present study was conducted as a prospective open randomized study to investigate the efficacy and safety of coadministration of rosuvastatin 20 mg/day, ezetimibe 10 mg/day, and granulated colestimide 3.62 g/day. Any lipid-lowering agents had been washed out ≥4 weeks before entry into the study. Study subjects were divided into 2 groups by an envelope method to elucidate the secondary end point of the present study: rosuvastatin 20 mg/day (group 1) versus rosuvastatin 10 mg/day coadministered with ezetimibe 10 mg/day (group 2).

restriction <200 mg/day, and saturated fatty acid restriction <30% of total fatty acid.

Study subjects were divided into 2 groups by an envelope method to elucidate the secondary end point of the present study: rosuvastatin 20 mg/day (group 1) versus rosuvastatin 10 mg/day coadministered with ezetimibe 10 mg/day (group 2) (Figure 1). All participants were started on a 4-week treatment with rosuvastatin 5 mg/day followed by another 4-week treatment of rosuvastatin 10 mg/day. The dose of rosuvastatin in group 1 was increased to 20 mg with an 8-week follow-up, whereas group 2 received ezetimibe 10 mg/day with an 8-week follow-up (phase 1). After phase 1, group 1 received ezetimibe 10 mg/day added to rosuvastatin for 8 weeks, whereas in group 2 the doses of rosuvastatin were increased to 20 mg with an 8-week follow-up (phase 2). In phase 3, groups 1 and 2 were given granulated colestimide 3.62 g (2 times/day before meals, 1 time in the morning and 1 time in the evening) added to the phase 2 treatment regimen. Because all but 1 participant were exposed to the same lipid-lowering drugs for >16 weeks during phases 2 and 3 and the end points were objective laboratory data, the influence of different treatments during phase 1 on the primary end points between groups 1 and 2 were likely to be minimal. Thus, all serum lipid, apolipoprotein, hepatic, muscular, CoQ10, and PCSK9 parameters from the 2 groups at baseline and phase 3 were combined.

Blood samples were obtained after an overnight fast after the wash-out period and phases 1, 2, and 3. Serum cholesterol and triglycerides were measured by an enzymatic method and high-density lipoprotein (HDL) cholesterol levels were measured by a polyamine–polymer/detergent method (Daiichi, Tokyo, Japan). Serum LDL cholesterol levels were calculated using the Friedewald formula.[7] Apolipoproteins A-I, A-II, B, C-II, C-III, and E were determined as described previously.[8] Plasma levels of CoQ10 were determined by a high-performance liquid chromatographic method as described previously.[8] Plasma PCSK9 concentrations were determined using an enzyme-linked immunosorbent assay kit targeting human PCSK9 (Circulex, Nagano, Japan).[9]

The primary end points of the present study were changes in lipid parameters including LDL cholesterol after a combination therapy of rosuvastatin (20 mg/day), ezetimibe (10 mg/day), and granulated colestimide (3.62 g/day) and safety of the therapy. The secondary end points were (1) rate to achieve LDL cholesterol levels below 100 and 120 mg/dl as target LDL cholesterol levels for secondary prevention and primary prevention of high-risk patients of cardiovascular disease as determined by the Japan Atherosclerosis Society[10]; (2) changes in other lipid parameters including serum levels of triglyceride, HDL cholesterol, and apolipoproteins A-I, A-II, B, C-II, C-III, and E; (3) a comparison of lipid parameters and safety between rosuvastatin monotherapy (20 mg/day) and combination therapy using rosuvastatin (10 mg/day) and ezetimibe (10 mg/day).

Values are expressed as mean ± SD unless otherwise stated. Effects of drug therapy on each variable were compared by paired *t* test except for triglycerides and lipoprotein(a). Effects of drug therapy and percent changes of serum levels of triglyceride and lipoprotein(a) were compared by Wilcoxon matched-pair test. All statistical analyses were performed with Prism 4.0a (GraphPad Software, Inc., San Diego, California). A p value <0.05 was considered statistically significant.

Results

Seventeen Japanese subjects with heterozygous FH were enrolled in the present study. Baseline characteristics and concomitant drug therapies are listed in Table 1. Five of 6 diabetic patients (28%) were under hypoglycemic medical therapy and glycohemoglobin concentrations were <7.0%, which varied by only ±0.5% during the study period. No patients were treated with insulin injection therapy. Dosages of coadministered medications were kept constant during the entire study period. A 72-year-old man (patient number 15 in Table 1) in group 2 dropped out during phase 2 because of myalgia without an increase of serum creatinine phosphokinase, which disappeared soon after discontinuing rosuvastatin and ezetimibe.

Serum levels of LDL cholesterol decreased significantly from 296.6 ± 36.8 to 100.1 ± 20.5 mg/dl (−66.4%, p <0.001) with the permitted maximum doses of rosuvastatin, ezetimibe, and granulated colestimide (Figure 2). As a result, serum LDL cholesterol levels of 8 patients (44%) and 16 patients (88%) reached 100 and 120 mg/dl, respectively, which are the target LDL cholesterol levels for the secondary and primary prevention of cardiovascular disease in high-risk patients as determined by the Japan Atherosclerosis Society.[10] No laboratory abnormalities, including from the patient with myalgia, were found such as increased hepatic enzymes (asparate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, and alkaline phosphatase) or creatinine phosphokinase >3 times the normal upper limit.

Serum levels of total cholesterol and triglyceride decreased significantly from 379.6 ± 42.1 to 174.9 ± 27.4 mg/dl (−53.9%, p <0.001) and from 114.1 ± 37.1 to

Table 1
Baseline characteristics of study subjects

Patient Number	Group	Background		Risk Factors			Vascular Complications			Hypolipidemic Medication at Baseline				Concomitant Drugs			
		Age/Sex	BMI	HT	DM	Smoking	CAD	OMI	PAD	Atorvastatin (mg)	Rosuvastatin (mg)	Ezetimibe	Colestimide	Aspirin	CCB	ARB	Diuretics
1	1	47/F	20.1	−	−	−	−	−	−	40	−	+	−	−	−	−	−
2	1	49/M	23.7	+	−	−	+	−	−	−	20	+	+	+	+	+	−
3	1	52/M	24.1	−	−	−	−	−	−	−	20	+	+	−	+	−	−
4	1	58/M	24.2	−	−	−	+	+	−	−	20	+	+	+	−	+	−
5	1	60/F	25.7	+	+	−	−	−	−	−	20	+	+	+	−	+	−
6	1	63/M	25.0	−	+	−	+	+	−	−	20	+	+	+	+	−	−
7	1	63/F	20.6	−	−	−	−	−	−	−	15	−	+	−	−	−	−
8	1	66/M	22.9	+	+	−	+	+	−	−	20	+	+	+	+	+	+
9	1	82/F	22.0	−	−	−	−	−	−	−	20	+	+	+	−	−	−
10	2	60/M	23.9	+	+	−	+	+	+	40	−	+	+	+	+	−	−
11	2	61/M	24.6	−	−	−	+	−	−	−	10	+	+	+	−	+	−
12	2	63/M	25.6	+	−	+	−	−	+	40	−	+	+	−	−	−	−
13	2	65/M	22.1	−	−	−	+	−	−	−	10	+	+	−	−	+	−
14	2	73/M	22.8	+	−	−	+	−	−	−	10	+	+	+	+	+	−
15	2	73/M	23.6	−	−	−	+	−	+	20	−	+	+	+	+	−	−
16	2	75/M	27.8	+	+	−	+	+	−	−	20	+	+	+	+	+	+
17	2	76/F	24.7	+	+	−	−	−	−	−	20	+	+	−	+	+	+
Mean or n		63.9	23.7	8	6	1	10	5	3	4	13	16	16	11	9	9	3
SD or %		7.4	1.4	47%	35%	6%	59%	29%	18%	24%	76%	94%	94%	65%	53%	53%	18%

ARB = angiotensin receptor blocker; BMI = body mass index; CAD = coronary artery disease; CCB = calcium channel blocker; DM = diabetes mellitus; HT = hypertension; OMI = old myocardial infarction; PAD = peripheral artery disease.

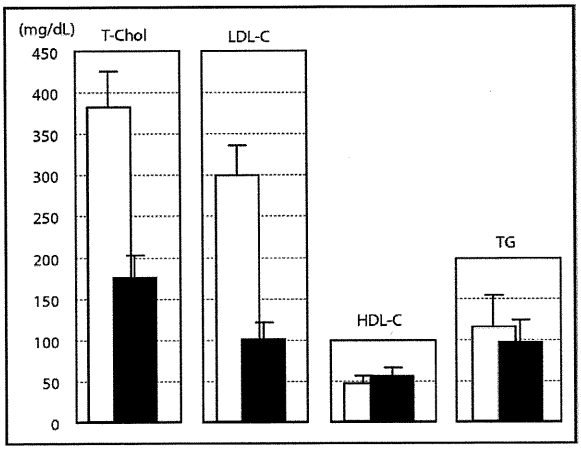

Figure 2. Changes of serum levels of total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride by a combination of permitted maximum doses of rosuvastatin, ezetimibe, and granulated colestimide. The combination of rosuvastatin (20 mg), ezetimibe (10 mg), and granulated coletimide (3.62 g) decreased total cholesterol, low-density lipoprotein cholesterol, and triglyceride concentrations from 379.6 ± 42.1 to 174.9 ± 27.4 mg/dl (−53.9%, p <0.001), from 296.6 ± 36.8 to 100.1 ± 20.5 mg/dl (−66.2%, p <0.001), and from 114.1 ± 37.1 to 96.0 ± 27.1 mg/dl (−15.9%, p = 0.08), respectively, and significantly increased high-density lipoprotein cholesterol concentrations from 46.4 ± 9.0 to 54.8 ± 11.0 mg/dl (18.1%, p <0.001). Pretreatment levels *(white bars)* and levels after combined therapy with the 3 different hypolipidemic drugs *(black bars)* are depicted. HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; T-chol = total cholesterol; TG = triglyceride.

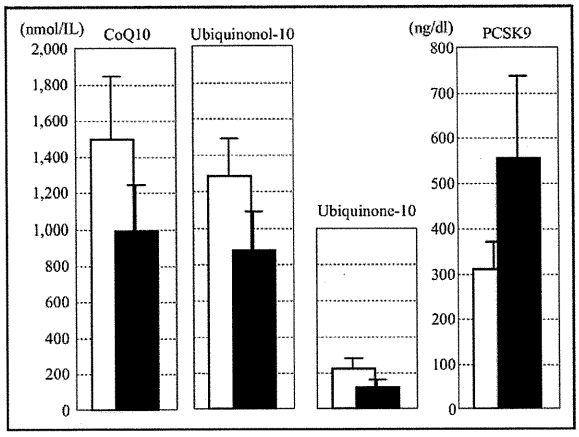

Figure 3. Changes in plasma levels of coenzyme Q10 and proprotein convertase subtilisin/kexin type 9 by a combination of permitted maximum doses of rosuvastatin, ezetimibe, and granulated colestimide. The combination of rosuvastatin (20 mg), ezetimibe (10 mg), and granulated coletimide (3.62 g) significantly decreased plasma levels of coenzyme Q10, ubiquinol-10, and ubiquinone-10 from 1,494 ± 363 to 989 ± 247 nmol/L (−33.8%, p <0.0001), from 1,280 ± 311 to 873 ± 242 nmol/L (−31.8%, p <0.0001), and from 219 ± 70 to 117 ± 33 nmol/L (−46.6%, p <0.0001), respectively. Similarly, plasma levels of proprotein convertase subtilisin/kexin type 9 increased significantly from 309.4 ± 70.6 to 552.6 ± 187.1 ng/dl (+78.6%, p <0.0001) after a combination of the 3 different hypolipidemic drugs.

96.0 ± 27.1 mg/dl (−15.9%, p = 0.027), respectively, and serum levels of HDL cholesterol increased significantly from 46.4 ± 9.0 to 54.8 ± 11.0 mg/dl (18.1%, p <0.001) after treatment with the 3 drugs (Figure 2). Similarly, serum levels of apolipoprotein A-I increased significantly and those of apolipoprotein B, C-II, C-III, and E decreased significantly (data not shown).

Serum levels of LDL cholesterol decreased significantly (−53.8%, p <0.001) after administration of rosuvastatin 20 mg/day, whereas combination therapy using rosuvastatin 10 mg/day and ezetimibe 10 mg/day significantly decreased serum LDL cholesterol levels (−61.1%, p <0.001; comparison between groups, p = 0.046) (Supplementary Table 1). Similarly, monotherapy using rosuvastatin 20 mg/day increased serum HDL cholesterol (+8.2%, p = 0.56) when rosuvastatin 20 mg/day was administered, whereas the combination of rosuvastatin 10 mg/day and ezetimibe 10 mg/day significantly increased serum HDL cholesterol (+17.9%, p = 0.01; comparison between groups, p = 0.036) (Supplementary Table 1). However, changes in apolipoproteins were not statistically different between these 2 regimens (data not shown).

Plasma levels of CoQ10 decreased significantly from 1,494 ± 363 to 989 ± 247 nmol/L (−33.8%, p <0.001) by maximum doses of rosuvastatin, ezetimibe, and granulated colestimide (Figure 3). Interestingly, the additional use of ezetimibe in group I further decreased LDL cholesterol without significant changes in plasma CoQ10 levels (Supplementary Tables 1 and 2). Moreover, the addition of granulated colestimide resulted in a significant increase in plasma CoQ10 from 841 ± 198 to 989 ± 247 nmol/L (phase 3, +17.7%, p = 0.003), whereas serum levels of LDL cholesterol decreased further from 110.9 ± 16.7 to 100.1 ± 20.5 mg/dl (−9.7%, p = 0.043, data not shown). In contrast, plasma levels of PCSK9 increased significantly from 309.4 ± 70.6 to 552.6 ± 187.1 ng/dl (+78.6%, p <0.0001) with a combination of maximum doses of rosuvastatin, ezetimibe, and granulated colestimide (Figure 3). Interestingly, percent changes of plasma PCSK9 levels in group 1 were significantly greater than those in group 2 during phase 1 (p = 0.03). Moreover, the additional use of granulated colestimide (3.62 g) significantly decreased serum LDL cholesterol as described earlier, whereas plasma levels of PCSK9 did not change significantly (from 562 ± 120 to 575 ± 130 ng/ml).

Discussion

Serum levels of LDL cholesterol were decreased by approximately 1/3 from baseline levels. The target level of serum LDL cholesterol for secondary prevention of coronary artery disease in Japan and the United States is 100 mg/dl, and those for the primary prevention of coronary artery disease in high-risk patients are 120 and 130 mg/dl in Japan and the United States, respectively.[10,11] Thus, we speculated that an adequate introduction of the aggressive medical cholesterol-lowering regimen used in the present study would be sufficient for the primary prevention of coronary artery disease in patients with an extreme hypercholesterolemia such as heterozygous FH. Despite the use of such an aggressive LDL cholesterol-lowering therapy, no

serious side effects were observed except for 1 patient with myalgia without an increase of serum creatinine phosphokinase. Serum levels of total cholesterol of hunter–gatherers and wild primates range from 70 to 140 mg/dl, and corresponding serum LDL cholesterol levels are estimated to be approximately 35 to 70 mg/dl.[12] Moreover, after a single session of LDL apheresis for heterozygous FH, the serum level of LDL cholesterol usually reaches <30 mg/dl without any side effects.[13,14] A recent meta-analysis revealed that any subtype of statin has a neutral effect on any cancer death risk[15]; thus, aggressive LDL cholesterol lowering per se is considered safe therapy.

Statins are the most potent hypolipidemic drugs with compelling evidence for the primary and secondary prevention of cardiovascular disorders and all-cause mortality.[16] In general, a linear dose-dependent LDL cholesterol-lowering effect is not be seen with statin therapy,[17] at least some of which can be explained by a compensatory increase of cholesterol absorption.[18] Tremblay et al[18] reported that induction of atorvastatin increased intestinal mRNA levels of Niemann-Pick C1-like 1 in humans. As previously reported, combination therapy using statin and ezetimibe produces a further reasonable decrease of LDL cholesterol over statin alone.[3] In the present study, combination therapy using rosuvastatin (10 mg/day) and ezetimibe (10 mg/day) was significantly superior to monotherapy with rosuvastatin (20 mg/day) for lipid levels (decrease of LDL cholesterol and increase of HDL cholesterol). However, there was no significant difference in serum apolipoprotein levels after a combination of rosuvastatin (10 mg/day) and ezetimibe (10 mg/day) versus rosuvastatin (20 mg/day) alone. Because each LDL, intermediate-density lipoprotein, and very LDL particle carries a single molecule of apolipoprotein B-100, the total apolipoprotein B value represents the total amount of potentially atherogenic lipoproteins. Recent studies have shown the predictive superiority of apolipoprotein B or A-I over LDL or HDL cholesterol, respectively, for future coronary events.[19] Thus, it is still controversial which regimen is superior to decrease future cardiovascular events. Bile acid sequestering resin also effectively decreases cardiovascular events,[20] and this study is the first to demonstrate the efficacy of a combination of a high-dose strong statin and ezetimibe.

FH strongly indicates a high-risk cardiovascular event; a lifelong aggressive LDL cholesterol-lowering therapy with sufficient safety is needed. Although most participants in the present study had been treated with and could tolerate a combination of a strong statin, ezetimibe, and granulated colestimide, the permitted maximum doses of all 3 drugs proved safe enough with respect to hepatic and muscle toxicity. Inhibition of 3-hydroxyl-3-methylglutaryl coenzyme A reductase results in a decreased synthesis of downstream products other than cholesterol such as CoQ10, which is an essential cofactor in the mitochondrial electron transport chain. In the present study, additional granulated colestimide further decreased serum LDL cholesterol levels without changing plasma CoQ10 levels. Moreover, ezetimibe 10 mg/day in addition to rosuvastatin 20 mg/day in group 1 produced a significant additional 17.9% decrease in serum LDL cholesterol, whereas plasma levels of CoQ10 did not change. These findings may suggest the potential decrease of side effects using hypolipidemic drugs with different mechanisms.

Another potential mechanism to decrease the dose-dependent LDL cholesterol-lowering effect of statins is an increase in PCSK9 because of sterol regulatory element binding protein 2 activation, resulting in degradation of the LDL receptor.[5] In the present study, plasma PCSK9 levels increased by combination hypolipidemic drug therapy (Figure 3). Interestingly, an additional dose of rosuvastatin 10 mg/day supplementing the combined use of rosuvastatin 10 mg/day and ezetimibe 10 mg/day (phase 2, group 2) produced a significant 16.9% increase of serum PCSK9 level without changing serum LDL cholesterol levels. In contrast, granulated colestimide produced an additional significant 15.6% decrease of serum LDL cholesterol without changing plasma PCSK9 levels. This may also explain the advantage of combination hypolipidemic drug therapy. Inhibition of PCSK9 may be an alternative target to further decrease LDL cholesterol when combined with statins.[21] All findings considered, a combination of these 3 different hypolipidemic drug therapies is considered effective with sufficient safety.

This study has some limitations: (1) a small number of participants because of the rarity of the disease (diagnosed genetic heterozygous FH) and (2) apparent differences of mean age, gender distribution, and baseline lipid levels between groups by chance.

Acknowledgment: We are indebted to Kazuko Honda and Sachio Yamamoto for their outstanding technical assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.amjcard.2011.09.019.

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Atherosclerosis 219 (2011) 663–666

Contents lists available at ScienceDirect

Atherosclerosis

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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).

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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.

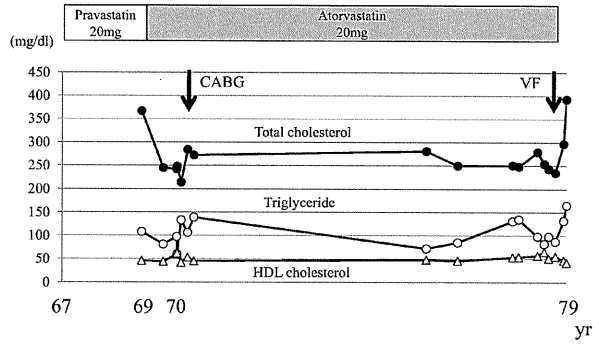

Fig. 1. Clinical course of the proband. Plasma concentration of the total cholesterol (solid circle), triglyceride (open circle), and HDL-C (open triangle) in the proband, and the major clinical events were illustrated. CABG; coronary artery bypass grafting, VF; ventricular fibrillation.

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.

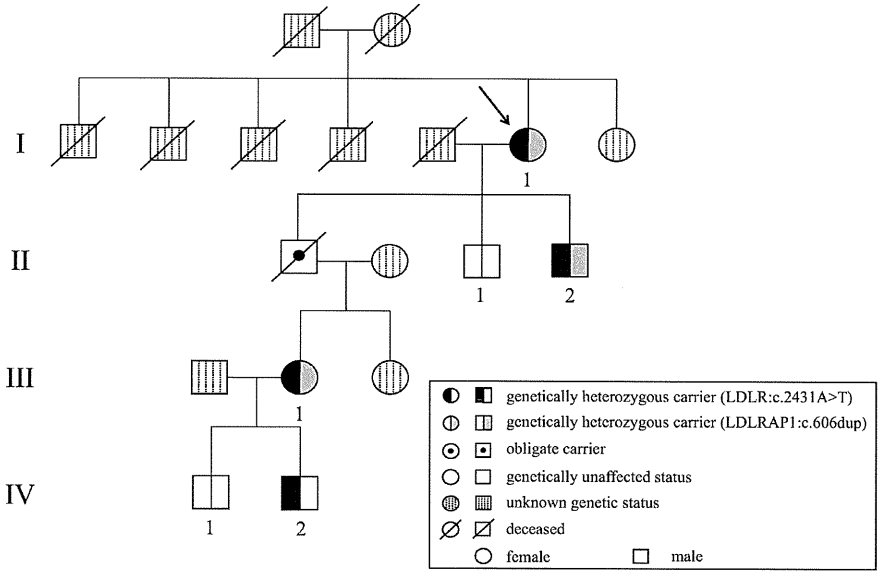

Fig. 2. Pedigree of the proband. Half-filled by black squares or circles indicate the heterozygous mutation carrier in LDLR (c.2431A > T). Half-filled by brown squares or circles indicate the heterozygous mutation carrier in LDLRAP1 (c.606dup). Square with a dot indicates the obligate carrier. Open squares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

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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]

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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

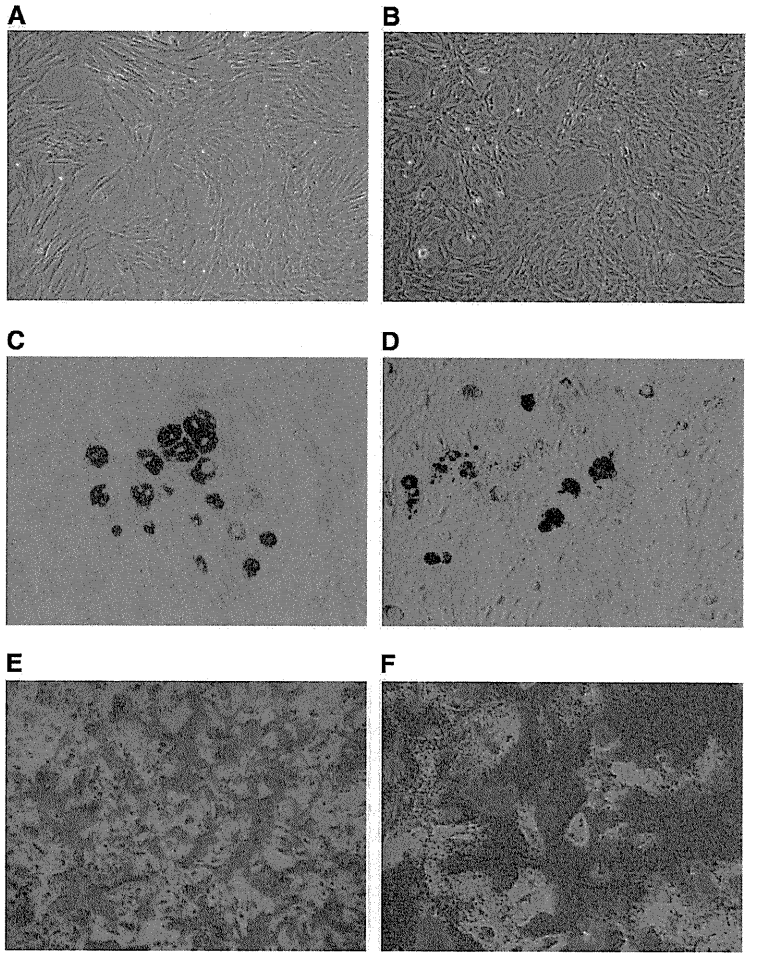

Figure 1. Morphology and differentiation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC). Cultured ASC (**A**) and BM-MSC (**B**) at passage 2 (×100). Their typical morphology is a similar spindle shape, but ASC are slightly longer than BM-MSC. 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).

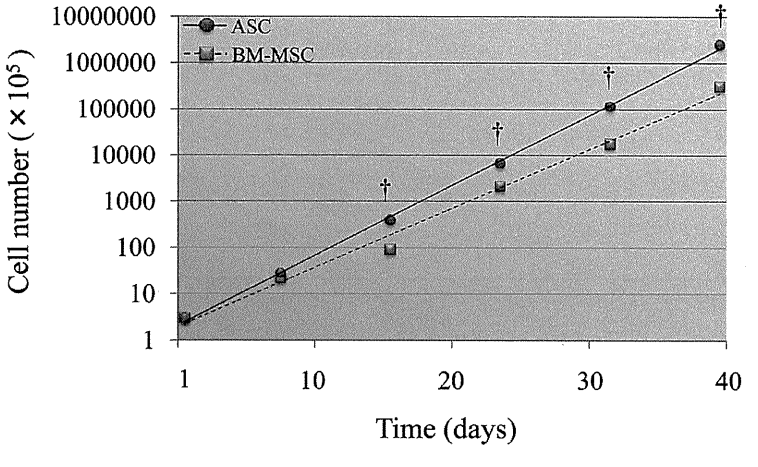

Figure 2. Proliferation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC) shown as a growth chart. Vertical axis, cell number; horizontal axis, day after first passage. Day 1 is the first day at passage 1. Values are mean±SEM. *P<0.05, †P<0.01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

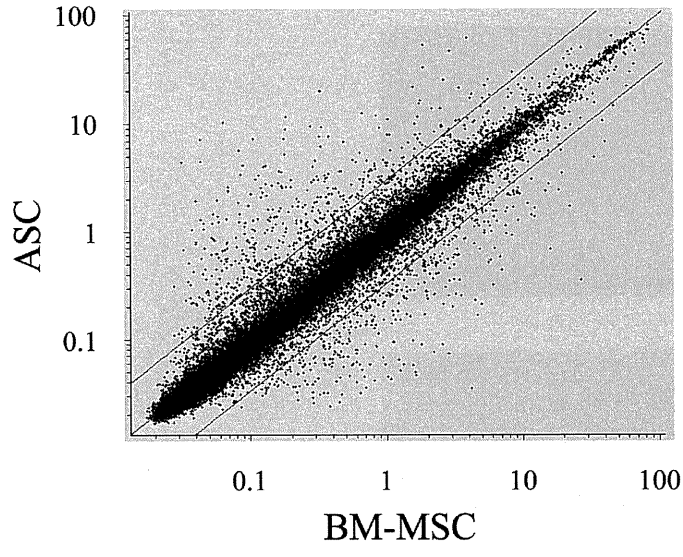

Figure 3. Gene expression profiles of adipose tissue-derived mesenchymal stem cells (ASC, vertical axis) and bone marrow-derived mesenchymal stem cells (BM-MSC, horizontal axis) by microarray. The scatter plot shows normalized microarray datasets of ASC and BM-MSC. All 31,099 gene probes are represented 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

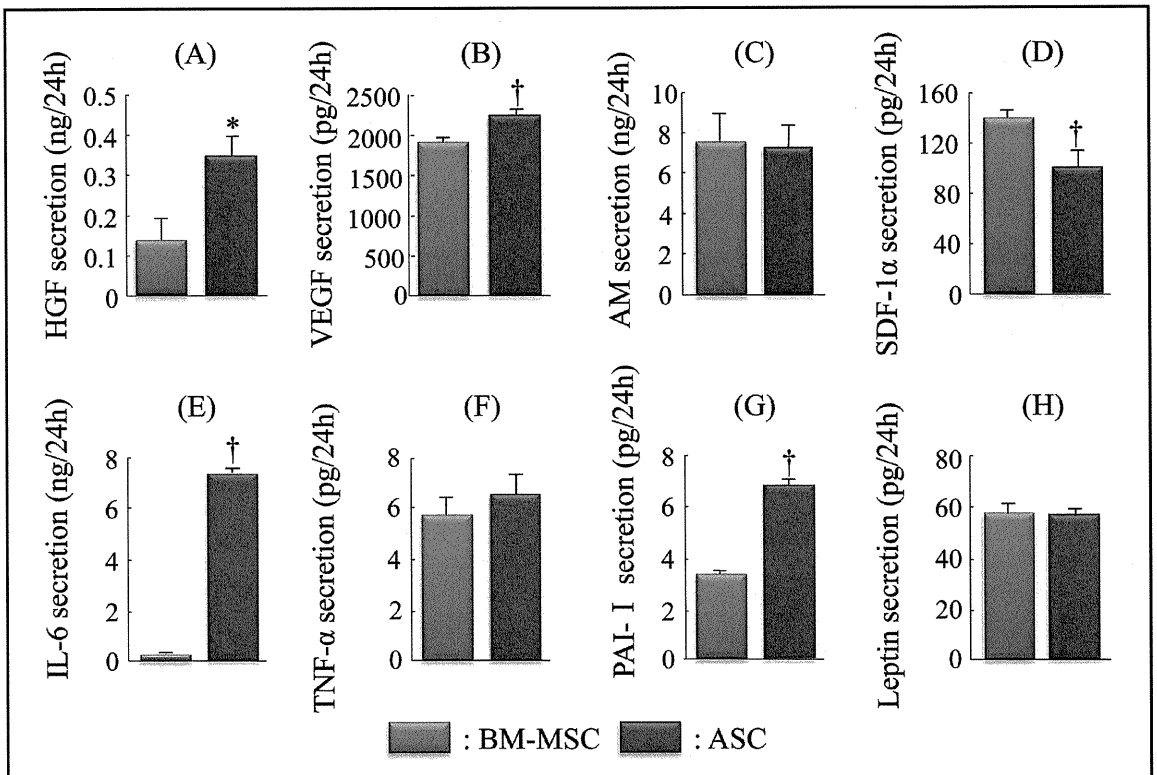

Figure 4. Secretory proteins from adipose tissue-derived mesenchymal stem cells (ASC: red) and bone marrow-derived mesenchymal stem cells (BM-MSC: blue). Conditioned media from ASC and BM-MSC were collected after incubation for 24 h in complete medium. Hepatocyte growth factor (HGF, **A**), vascular 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.

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The Journal of International Medical Research
2011; 39: 549 – 557

Impact of Severe Coronary Disease Associated or Not Associated with Diabetes Mellitus on Outcome of Interventional Treatment Using Stents: Results from HERZ (Heart Research Group of Kanazawa) Analyses

K UCHIYAMA, H INO, K HAYASHI, K FUJIOKA, S TAKABATAKE, J YOKAWA, M NAMURA, S MIZUNO, R TATAMI, H KANAYA, Y NITTA, I MICHISHITA, H HIRASE, K UEDA, T AOYAMA, K OKEIE, T HARAKI, K MORI, T ARAKI, M MINAMOTO, H OIWAKE, T KONNO, K SAKATA, M KAWASHIRI AND M YAMAGISHI; ON BEHALF OF THE HEART RESEARCH GROUP OF KANAZAWA (HERZ)

Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

Percutaneous coronary intervention (PCI) using a drug-eluting stent (DES) leads to less re-stenosis than PCI using a bare metal stent (BMS), however there is still controversy whether use of a DES for severe coronary disease leads to an acceptable outcome in patients with diabetes mellitus (DM). In this study 8159 lesions were treated in 6739 patients (mean age 68.9 years) with coronary artery disease. Use of a DES significantly decreased the re-stenosis rate compared with BMS in both DM (9.6% versus 21.3%) and non-DM (9.5% versus 17.1%) patients. The re-stenosis rate was significantly higher in DM than in non-DM patients in the BMS group but not in the DES group. There was no statistically significant difference in event-free survival after stenting of patients with left main coronary artery (LMCA) disease between the BMS and DES groups. It was concluded that, compared with BMS, DES reduced re-stenosis in patients with DM, however, we advise careful treatment after using DES for severe coronary disease, including LMCA lesions, in patients with DM.

KEY WORDS: PERCUTANEOUS CORONARY INTERVENTION; DRUG-ELUTING STENT; BARE METAL STENT; CORONARY ARTERY DISEASE; LEFT MAIN CORONARY ARTERY DISEASE; DIABETES MELLITUS; MAJOR ADVERSE CARDIOVASCULAR EVENTS; CLINICAL OUTCOME

Part of this work was presented at the 74th Annual Scientific Meeting of the Japanese Circulation Society, 5 – 7 March 2010, Kyoto, Japan.

Introduction

Introduced about a decade after coronary artery bypass grafting, percutaneous coronary intervention (PCI) has come to be preferred, because it is less invasive, and it is now widely used to treat coronary artery disease.[1,2] Technological improvements, especially the development of coronary stents, have made it possible to treat complex lesions.[3 - 5] However, in previous randomized trials coronary artery bypass grafting was superior to PCI for patients with diabetes or multivessel coronary artery disease when using plain balloon angioplasty or a bare metal stent (BMS).[6,7] The differences in outcome between coronary artery bypass grafting and PCI were mainly associated with re-stenosis of the treated lesions and target lesion revascularization (TLR).

The recent advent of drug-eluting stents (DES) has dramatically reduced the rate of re-stenosis after PCI, and DES are particularly superior to BMS in PCI for small vessels, whether or not the coronary disease is associated with diabetes mellitus (DM). Several trials, however, have demonstrated that DES did not reduce subsequent rates of major adverse clinical events or mortality, although they did reduce the rate of target lesion revascularization compared with the use of a BMS.[8,9] The objective of the present study was to evaluate the clinical outcomes of PCI with a stent in the presence and absence of DM in Japanese patients with coronary artery disease.

Patients and methods

PATIENTS, SUCCESS CRITERIA AND TREATMENTS

Consecutive patients who underwent PCI for coronary artery disease at Kanazawa University Hospital and affiliate hospitals (see Appendix for a list of affiliate hospitals) between January 2006 and December 2008 were eligible for enrolment into the study. The indications for PCI included stable angina pectoris, unstable angina and acute myocardial infarction. Patients who underwent PCI but were treated without stenting, using only balloon dilatation, thrombectomy or directional coronary atherectomy, were excluded.

All procedural decisions, including device selection and adjunctive pharmacotherapy, were made at the discretion of the individual PCI operator. Intravascular ultrasonography was used at the operator's discretion. Procedural (angiographic) success was defined as residual stenosis of < 25%. The definition of clinical success included angiographic success and the in-hospital absence of acute myocardial infarction, congestive heart failure and cardiac death. Procedural and clinical success were evaluated at the time the patient was discharged.

All patients who underwent PCI with a stent received dual antiplatelet therapy comprising aspirin (100 mg/day) and clopidogrel (75 mg/day) or ticlopidine (200 mg/day), and continued this therapy for an appropriate period.

This study was certified by the Ethics Committee of Kanazawa University.

CLINICAL FOLLOW-UP

All patients were evaluated clinically during a follow-up visit to the outpatient clinic and were recommended to receive follow-up coronary angiography at least 6 months after the PCI procedure. Angiographic results and clinical symptoms were used to evaluate the clinical outcome. Binary re-stenosis was defined as ≥ 50% stenosis at the target lesion.

The occurrence of major adverse cardiac events (MACE), defined as sudden cardiac death, acute coronary syndrome-related