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HMG-CoA reductase inhibitor augments the serum total cholesterol-lowering effect of human adipose tissue-derived multilineage progenitor cells in hyperlipidemic homozygous Watanabe rabbits

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ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal codominant disease characterized by high concentrations of proatherogenic lipoproteins secondary to deficiency in low-density lipoprotein (LDL) receptor. We reported recently the use of *in situ* stem cell therapy of human adipose tissue-derived multilineage progenitor cells (hADMPCs) in lowering serum total cholesterol in the homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model of homozygous FH. Here we demonstrate that pravastatin, an HMG-CoA reductase inhibitor, augmented the cholesterol-lowering effect of transplanted hADMPCs and enhanced LDL clearance in homozygous WHHL rabbit. The results suggest the potential beneficial effects of *in situ* stem cell therapy in concert with appropriately selected pharmaceutical agents, in regenerative medicine.

1. Introduction

Familial hypercholesterolemia (FH) is characterized by premature and accelerated development of atherosclerotic lesions caused by elevated levels of cholesterol-rich lipoproteins in plasma. The disease is caused by mutations in the low-density lipoprotein (LDL) receptor gene that result in a significant decrease in receptor-mediated uptake of lipoproteins from the circulation [1–3]. Patients homozygous for defects in LDL receptors have serum cholesterol levels 5–10 times those of normal and suffer as early as the first two decades of life serious complications such as coronary artery disease [4,5]. In homozygous FH patients, conventional drug therapy such as HMG-CoA reductase inhibitors, collectively known as "statins", cannot treat the condition, and therapeutic recourses are limited to chronic plasmapheresis and orthotopic liver transplantation [1]. Although liver transplants lower LDL levels, the procedure is life threatening and, in addition, donor livers are in short supply. A number of gene therapy approaches have shown some promise in animal models and human [6–9]. As an alternative to whole-organ transplantation and/or gene therapy, cellular transplantation has been proposed to provide functional LDL receptors for the treatment of hypercholesterolemia. Transplantation of allogenic and xenogenic hepatocytes is reported to be effective in lowering serum cholesterol in the Watanabe heritable hyperlipidemic (WHHL) rabbit [10–13], which is an animal model of homozygous FH. In this context, we have reported the ability of human adipose tissue-derived multilineage progenitor cells (hADMPCs) to differentiate into hepatocytes both *in vitro* and *in vivo* and to rectify critical liver functions [14,15] similar to reports from other laboratories [16,17]. Various groups have demonstrated the *in vitro* differentiation of hADMPCs into various cell types and confirmed that hADMPCs can be easily and safely obtained in large quantities without serious ethical issues [14,15,18,19]. In homozygous FH patients, HMG-CoA reductase inhibitors have no effect on the condition as mentioned [20]. We hypothesized that HMG-CoA reductase inhibitor can act in concert with *in situ* differentiated hepatocyte-like cells originating from transplanted hADMPCs to lower serum cholesterol

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levels in hyperlipidemia. To test our hypothesis, we tested the effects of treatment with HMG-CoA reductase inhibitor in hADMPC-transplanted homozygous WHHL rabbits.

2. Materials and methods

2.1. Adipose tissue samples

Subcutaneous adipose tissue samples (10–50 g, each) were resected during plastic surgery in 5 females (age, 20–60 years) as excess discards. The study protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine, Kinki University Pharmaceutical Research and Technology Institute and Foundation for Biomedical Research and Innovation. Each subject provided a signed informed consent.

2.2. isolation of hADMPCs

The hADMPCs were prepared as described previously [21] with some modification [14,15,18,19]. Briefly, the resected excess adipose tissue was minced and then digested at 37 °C for 1 h in Hank's balanced salt solution (HBSS, GIBCO Invitrogen, Grand Island, NY) containing 0.075% collagenase type I (Sigma Aldrich, St. Louis, MO). Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 *g* for 10 min. Erythrocytes were excluded using density gradient centrifugation with Lymphoprep ($d = 1.077$; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) with 10% defined fetal bovine serum (FBS, GIBCO Invitrogen) for 24 h at 37 °C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/l ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin (FN)-coated dishes (AGC, Tokyo, Japan) in Stem Cell Medium (Nipro, Osaka, Japan), 1× insulin-transferring selenium (ITS, GIBCO Invitrogen), 1 nM dexamethasone (Sigma–Aldrich), 100 μM ascorbic acid 2-phosphate (Sigma Aldrich), 10 ng/ml epidermal growth factor (EGF, PeproTec, Rocky Hill, NJ), and 5% FBS (GIBCO Invitrogen). After 5–6 passages, the hADMPCs were used for transplantation.

2.3. hADMPCs transplantation and immunosuppression/statin treatment regimen

The transplantation procedure was performed as reported previously [15]. Briefly, 8-week-old homozygous WHHL rabbits (Kitayamalabes, Inc., Japan) ($n = 7$) were anesthetized with pentobarbital (50 mg/kg) and an incision distal and parallel to the lower end of the ribcage was made. The peritoneum was incised and hADMPCs (3×10^7 cells) suspended in 3 mL of HBSS (20 °C) with heparin were infused within 5 min into the portal vein via a 18-gauge Angiocath™ (BD, UT) (Fig. 1A). The immunosuppression regimen (Fig. 1B) consisted of the following: (i) intramuscular injection of cyclosporin A (6 mg/kg/day) daily from the day before surgery to sacrifice; (ii) intramuscular injection of rapamycin (0.05 mg/kg/day) daily from the day before surgery to sacrifice; (iii) methylprednisolone at 3 mg/kg/day (day −1 to 7), followed by tapering to 2 mg/kg/day (day 8–14), 1 mg/kg/day (day 15–21) and 0.5 mg/kg/day (day 22 to sacrifice); (iv) intravenous injection of cyclophosphamide (20 mg/kg/day) at day 0, 2, 5 and 7; (v) intramuscular injection of ganciclovir (2.5 mg/kg/day) was also administrated to avoid viral infection in the immunocompromised host. Twelve weeks after hADMPCs transplantation, the rabbits were divided into two groups; the first was treated with low dose pravastatin (0.75 mg/kg/day i.m., $n = 4$), an HMG-CoA reductase inhibitor (treatment group), while the second served as the control and injected intramuscularly with the vehicle ($n = 3$).

2.4. Assay for lipid profiling

Serum samples were obtained from nonfasting rabbits before and after pravastatin treatment (at 12 and 16 weeks). Serum total cholesterol and HDL-cholesterol fraction were measured using assay kits from Wako Pure Chemical Industries (Osaka, Japan)

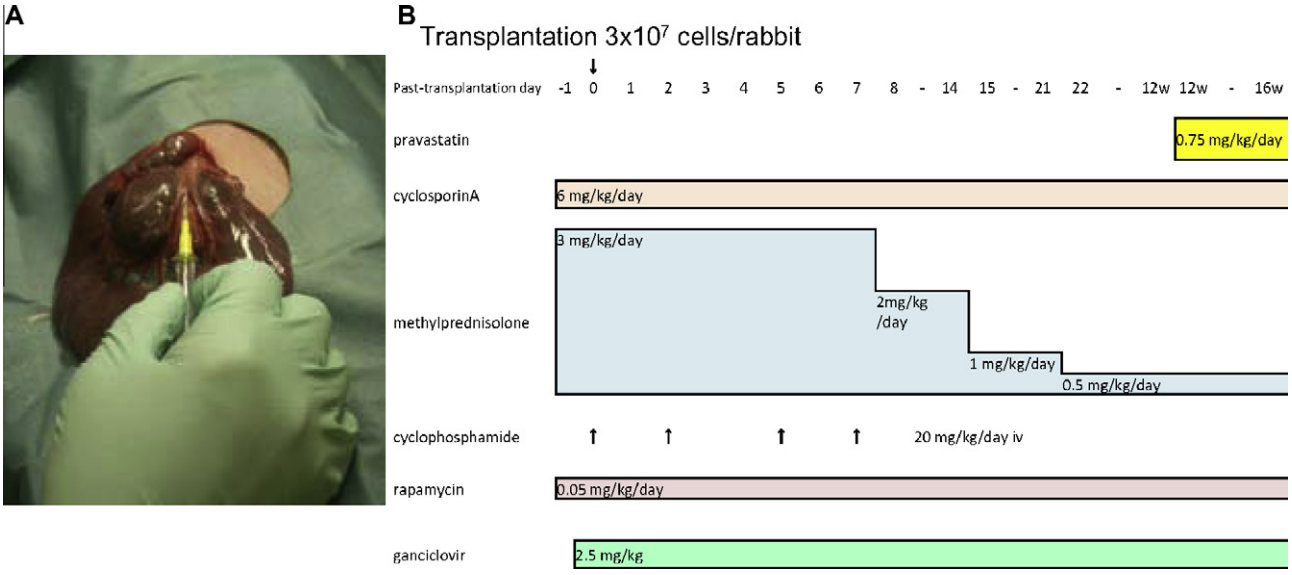

Fig. 1. (A) Surgical procedure. Watanabe heritable hyperlipidemic (WHHL) rabbits were anesthetized with pentobarbital. An incision was made distal and parallel to the lower end of the ribcage. The peritoneum was incised and hADMPCs (3×10^7 cells/rabbit) were infused into the portal vein using an 18-gauge Angiocath. (B) Immunosuppression regimen. Cyclosporin A (6 mg/kg/day) and rapamycin (0.05 mg/kg/day) were administered intramuscularly daily from the day before surgery to sacrifice. Methylprednisolone was administered at 3 mg/kg/day (days 1–7), 2 mg/kg/day (days 8–14), 1 mg/kg/day (days 15–21), and 0.5 mg/kg/day (day 22 to sacrifice). Cyclophosphamide (20 mg/kg/day) was injected intravenously at days 0, 2, 5, and 7. Ganciclovir (2.5 mg/kg/day) was also injected intramuscularly to avoid viral infection in the immunocompromised host. Twelve weeks after hADMPCs transplantation, hADMPC-transplanted WHHL rabbit were divided into two groups; the pravastatin-treated group ($n = 4$) and the control vehicle group ($n = 3$).

and the before and after treatment with/without pravastatin were compared in the two groups.

2.5. Clearance of ^{125}I-LDL from rabbit serum

LDL turnover study was performed as reported previously to examine the clearance of ^{125}I-LDL from rabbit serum [15]. Briefly, at the end of the study (Fig. 1B), the animals were examined for the LDL turnover assay. ^{125}I-human LDL (BT-913R, Biomedical Technologies Inc., Stoughton, MA) was delivered via the marginal ear vein of the WHHL rabbits and normal control rabbits in physiological saline containing 2 mg/mL bovine serum albumin. Blood was collected from the opposite ear after injection at 5 min, 1, 2, 3, 4, 6, 24 and 28 h. ^{125}I-labeled apolipoprotein B-containing LDL was precipitated with 20% of trichloroacetic acid (Wako Pure Chemical Industries) (serum; 320 μL, 100% w/v TCA 80 μL), and then the precipitants were applied for counting.

2.6. Statistical analysis

Values were expressed as mean ± SEM. Differences between mean values before and after treatment in the treated and untreated groups were evaluated using the paired *t*-test or Student's *t*-test. A *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS Statistics 17.0 package (SPSS Inc., Chicago, IL).

3. Results

Pravastatin treatment significantly reduced serum total cholesterol level in hADMPC-transplanted homozygous WHHL rabbits ($n = 4$, before: 410 ± 35, after: 291 ± 46 mg/dL, $p = 0.0382$, Fig. 2), whereas control hADMPC-transplanted WHHL rabbits showed no such fall ($n = 3$, before: 409 ± 63, after: 375 ± 53 mg/dL, Fig. 2). On the other hand, the fall in HDL-cholesterol was not significant in both the pravastatin and control vehicle rabbits (pravastatin group: before 24.3 ± 0.5, after 23.3 ± 0.3 mg/dL, control vehicle group: before 22.8 ± 2.2, after 20.8 ± 2.2 mg/dL, Fig. 2).

Next, we measured human LDL clearance in order to confirm that the fall in serum total cholesterol induced by pravastatin in the hADMPCs-transplanted rabbits was mediated through human LDL receptors on hADMPC-derived hepatocytes (Fig. 3). Pravastatin shifted the LDL-turnover curve to the left ($n = 4$) (Fig. 3A). Furthermore, pravastatin significantly increased the 24-h LDL-clearance rate in the hADMPC-transplanted WHHL rabbits ($n = 4$, 95.0 ± 0.6%) compared to the control ($n = 3$, 90.7 ± 0.2%, $p = 0.0429$, Fig. 3).

4. Discussion

The main finding of this study was that pravastatin enhanced the lipid-lowering effects and the LDL-clearance rate of transplanted hADMPCs in spontaneously hyperlipidemic homozygous WHHL rabbits.

An important issue in cellular therapy is the cell source selected for clinical application. The major advantages of hADMPCs are their availability through simple harvesting surgical procedure and lack of ethical obstacles. In fact, a simple liposuction surgery yields massive amount of lipoaspirate adipose tissue, ranging from 100 ml to >3 L [22]. Our previous study in homozygous WHHL rabbits showed that human LDL binds to the receptors on hADMPC-derived hepatocytes and such human LDL receptors compensate the non-functional mutant LDL receptors in the WHHL rabbit [15]. Moreover, hepatocytes derived from hADMPCs have the advantage of expressing LDL receptor from an endogenous gene with intact regulatory sequences. These findings prompted us to test the effect of HMG-CoA reductase inhibitor on serum total cholesterol in hyperlipidemic rabbits transplanted with hADMPCs.

Among the numerous enzymes involved in the cholesterol biosynthesis pathway, HMG-CoA reductase plays an essential in cholesterol synthesis. Inhibition of the HMG-CoA enzyme by pravastatin decreases LDL-cholesterol by the following mechanisms: *de novo* decrease in cholesterol synthesis, simultaneous increase in

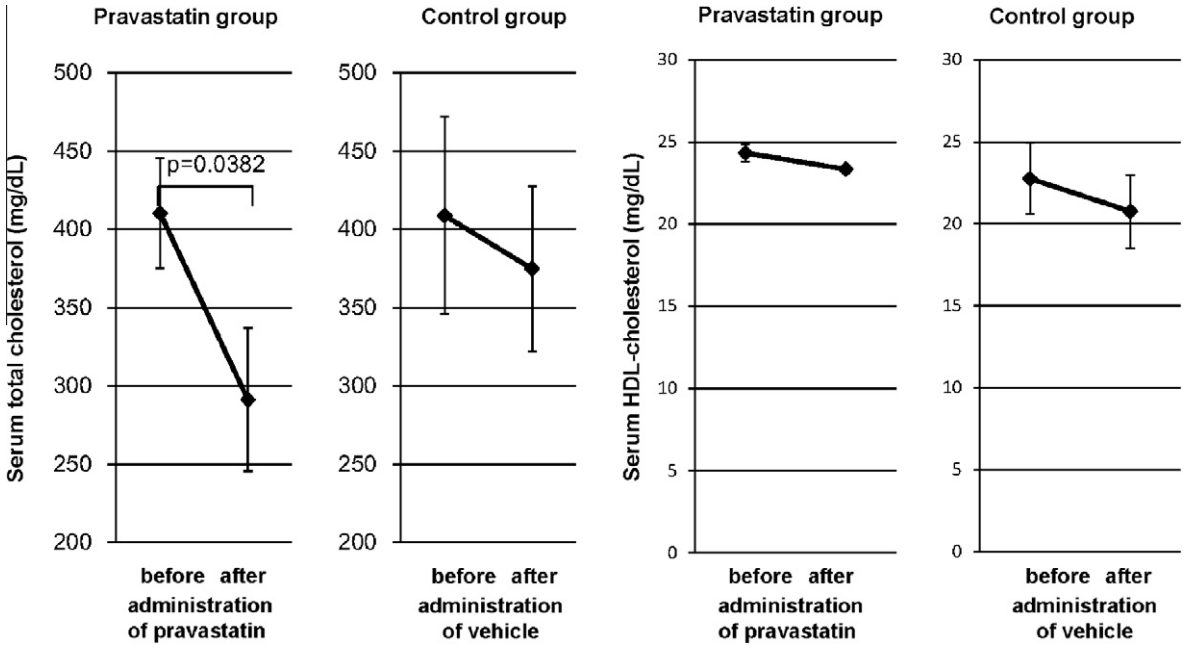

Fig. 2. Serum total cholesterol (*left*) and serum HDL-cholesterol (*right*) in hADMPC-transplanted homozygous WHHL rabbits before and after administration of pravastatin ($n = 4$) or the vehicle ($n = 3$). Data are mean ± SEM. Differences between mean values before and after administration of in the pravastatin or the vehicle were evaluated using the paired *t*-test. A *P* value less than 0.05 was considered statistically significant.

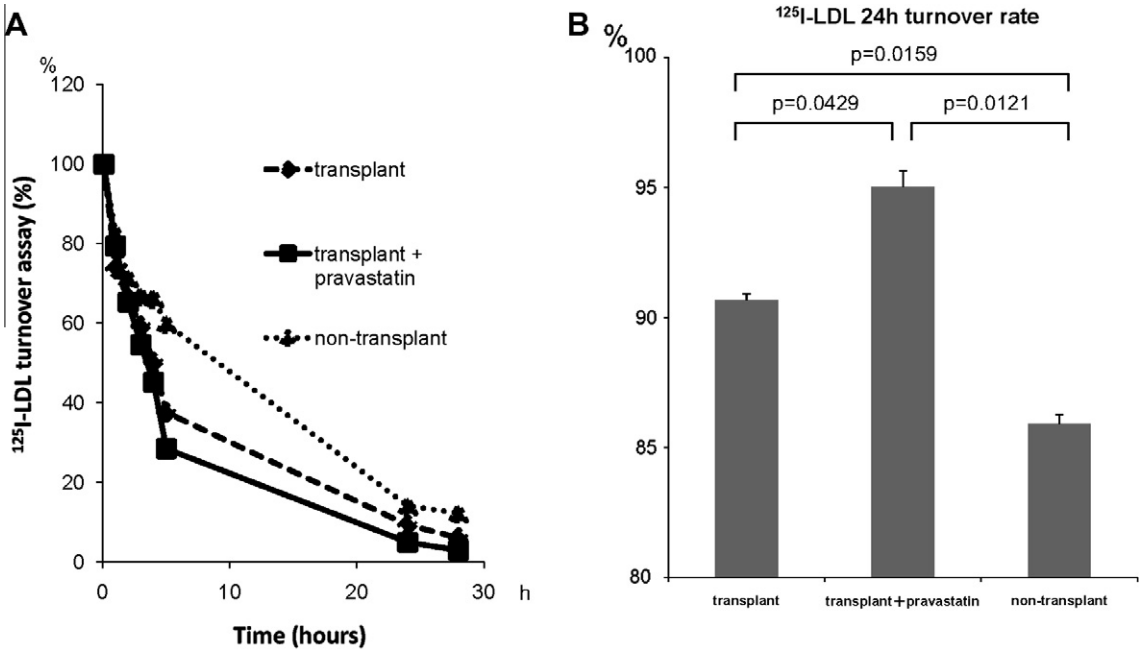

Fig. 3. (A) Rate of clearance of LDL from the serum of WHHL rabbits with and without transplantation of hADMPCs. Animals were injected with ^{125}I-labeled human LDL, and the time course of clearance was monitored following trichloroacetic acid precipitation of serum at time 5 min, 1 h, 2 h, 4 h, 6 h, 24 h and 28 h. Residual ^{125}I-LDL was expressed as percentage of that at 5 min. (B) Differences in the 24-h LDL-clearance rate in pravastatin-treated hADMPC-transplanted group ($n = 4$), hADMPC-transplanted control group ($n = 3$), and non-transplanted control homozygous WHHL group ($n = 3$). Data are mean ± SEM. Differences between mean values before and after treatment in the treated and untreated groups were evaluated using the Student's t-test. A P value less than 0.05 was considered statistically significant.

the LDL receptor synthesis on hepatocytes, thus enhancing the clearance of LDL-cholesterol from the circulation, resulting in lowering serum cholesterol levels [20]. For these reason, pravastatin fail to act in patients with homozygous FH who have no LDL receptor due to the genetic abnormality, and also in the homozygous hyperlipidemic WHHL rabbit, in which pravastatin could show cholesterol-lowering effects in much higher doses of 50 mg/kg/day [23–26]. The substantial fall in serum cholesterol and increased LDL-clearance from the circulation by pravastatin noted in the present study suggest that the hADMPC-derived hepatocyte-like cells both internalize LDL and metabolize cholesterol more efficiently and in concert with pravastatin. The relationship between hypercholesterolemia and coronary heart disease has been well documented, and a reduction in serum total cholesterol of the magnitude demonstrated in the present study is likely to reduce morbidity and mortality rates in patients with homozygous FH [27]. Further studies are needed to tests the potential usefulness of hADMPC-transplantation and simultaneous treatment pravastatin in these patients.

Acknowledgments

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