Methods and Materials

Materials
Probucol was purchased from WAKO Pure Chemical (Osaka, Japan). SQ and DQ were synthesized as previously described. Human apoA-I was purchased from Sigma (St. Louis, MO, USA). HDL was isolated by sequential ultracentrifugation from human plasma. Acetylated human low-density lipoprotein (AcLDL) was prepared according to the methods previously described.

Cell Culture
RAW264.7 cells (Riken Cell Bank, Tsukuba, Japan), a murine macrophage cell line, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were stimulated in 0.3 mmol/L 8-bromoadenocine 3’,5’-cyclic monophosphate (8-Br-cAMP) to induce expression of ABCA1 and ABCG1. All cultures were in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Cell Cholesterol Release
Release of cell cholesterol was determined as previously described. RAW264.7 cells were labeled with 3H-cholesterol (1.0 µCi/mL) in DMEM containing 0.2 % bovine serum albumin (BSA) for 24 hr. The cells were washed with phosphate buffered saline (PBS) and incubated in the same medium in the presence or absence of apoA-I (10 µg/mL) or HDL (50 µg/mL as protein) for 4 hr and 24 hr. Probucol, SQ or DQ was added to the incubation mixture with apoA-I or HDL. After the incubation, radioactivity in the medium and cells was counted in a liquid scintillation counter (LSC). Cholesterol release was calculated as percentage of the initial cell cholesterol count, by dividing the media radioactivity by the sum of that in the media and the cells.

Western Blot Analyses
Cells were harvested and protein extracts prepared as previously described for analysis by Western blotting (10% SDS-PAGE; 15 µg protein per lane), using anti-human ABCA1 rat monoclonal antibodies and rabbit antibodies against ABCG1 (Novus Biologicals), SR-BI (Novus Biologicals) and β-actin (Santa Cruz). The proteins were visualized and quantified using a chemiluminescence method (ECL Plus Western Blotting Detection System; GE Healthcare UK Ltd) and the NIH image analysis software program.

Animals
C57BL/6J and apolipoprotein E (apoE) null mice were obtained from Clea Japan.
(Tokyo, Japan) and Jackson Laboratory (West Grove, PA), and fed a standard chow and high cholesterol (1/25%) diet, respectively. The experiments were performed using mice aged 6-8 weeks. The animal experiment protocol was approved by the National Defense Medical College Institutional Animal Care and Use Committee, and the mice were treated according to its guidelines.

Effects of SQ and DQ on ABC Proteins, Plasma Lipids and Other Biochemical Parameters in Mice

SQ, DQ, 500 mg/kg, or the vehicle was orally administered to six-week-old male C57BL/6 mice. Immediately before and 7 days after the administration, blood samples were taken from the tail vein. Total cholesterol, HDL-C, free cholesterol (FC), triglycerides (TG), phospholipids (PL), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine phosphokinase (CPK) in plasma were determined by enzymatic methods (WAKO, Osaka, Japan). For lipoprotein analysis, 400 µL of plasma from each mouse were pooled for each treatment group, and each pooled plasma was fractionated using a Superose 6 10/300 GL fast protein liquid chromatography (FPLC) column (Amersham Biosciences, Piscataway, NJ). Fractions of 500 µL were collected for lipid measurement. The mice were sacrificed after seven days of the treatment. Peritoneal macrophages were collected by peritoneal lavage with ice-cold PBS and centrifugation at 3000 rpm. Cells obtained as a pellet were re-suspended in DMEM and plated onto 6-well cell culture plates for 1 hr (mouse peritoneal macrophages; MPM). Livers were recovered and perfused with PBS before the analyses. Protein extracts from the liver and MPM were prepared with T-PER (Pierce Chemical Co., Rockford, IL) in the presence of protease inhibitors (Roche Applied Science, Barcelona, Spain), and analyzed by Western blotting as described above.

Real-time Quantitative RT-PCR

Total RNA was extracted from the livers, and first-strand cDNA was synthesized from the total RNA (250 ng) by placing in a Reverse Transcription Reagent (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with a Perkin–Elmer 7900 PCR machine, TaqMan PCR master mix and FAM-labeled TaqMan probes (Assays-on-Demand, Applied Biosystems) for mouse ABCG5, ABCG8, Niemann-Pick C1-Like 1 (NPC1L1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression data were normalized for GAPDH levels.

In Vivo Macrophage RCT Studies
RAW264.7 cells were cultured under RPMI 1640 supplemented with 10% FBS, and then radiolabeled with 5 µCi/mL ³H-cholesterol and enriched with cholesterol by incubating with 100 µg/mL of AcLDL for 48 hr. The cells were washed, equilibrated, detached with cell scrapers, resuspended in RPMI 1640, and pooled. Seven-week-old male C57BL/6 mice (Clea Japan, Tokyo, Japan) were divided into 3 groups (6 mice per group) and caged individually with unlimited access to food and water. The mice were preconditioned by daily administration of 500 mg/kg of SQ, DQ or the vehicle by oral gavage for 7 days. The AcLDL-loaded and ³H-cholesterol-labeled RAW264.7 cells (typically 5.0×10⁶ cells containing 7.5×10⁶ counts per minute (cpm) in 0.5 mL RPMI 1640) were intraperitoneally injected into the mice as described previously⁹. Blood was taken at 24 and 48 hr after the injection, and radioactivity in plasma was counted in an LSC. Feces were collected continuously from 0 to 48 hr and stored at 4°C until counting for radioactivity. At 48 hr after the injection, mice were exsanguinated and livers and bile were collected.

**Liver, Bile and Fecal Analyses**

Liver lipid was extracted as described previously⁹. Briefly, a 50-mg piece of tissue was homogenized in water, and lipid was extracted with a 2:1 (vol/vol) mixture of chloroform/methanol. The organic layer was collected, evaporated, resuspended in a 3:2 (vol/vol) mixture of hexane/isopropanol, and counted for radioactivity. Radioactivity in bile was directly counted in an LSC. The total feces collected from 0 to 48 hr were weighed and soaked in distilled water (1 mL water per 100 mg feces) overnight at 4°C. An equal volume of ethanol was added the next day, and the samples were homogenized. In a 200 µL aliquot of each homogenized sample was counted for radioactivity. Results were expressed as percentage of the cpm injected.

**HDL Metabolic Studies**

Human HDL was isolated from pooled human plasma by sequential ultracentrifugation (density 1.063 < density 1.21 g/ml). Dialyzed human HDL was labeled with ³H-cholesteryl oleate (CEs). Fifty µCi of cholesteryl ether or oleate in toluene were dried down under nitrogen. Ethanol was then added, and the solution taken up by a pipette. This was added to the HDL₃ solution (1.07 ml of dialyzed HDL containing 10 mg of protein) over a period of 5 min while gently shaking with short interruptions for a brief vortex. The HDL solution was incubated for 24 hr at 37°C. The HDL₃ from the solution was re-isolated by ultracentrifugation (40,000 rpm, 48 hr) at the original
density and the $^3$H-HDL was dialyzed overnight against PBS containing 0.01% EDTA. Finally, the $^3$H-HDL was filter-sterilized and stored at 4°C until injection.

For the metabolic study, 1 million dpm of HDL labeled with $^3$H-CEs were injected intravenously via tail veins into the mice at day 7 after administration of SQ/DQ or the vehicle. Feces were collected continuously up to 48 hr and then analyzed for tracer radioactivity. Results were expressed as a percentage of the injected dose.

**HDL Cholesterol Efflux Capacity Assay**

J774 macrophages were purchased from RIKEN (Tsukuba, Japan) and cultured in RPMI1640 medium containing 10% FBS and kept under constant conditions of 5% CO$_2$ and 37°C. J774 cells were plated in 24-well plates and grown until 80% confluency, and radiolabeled with 2 µCi/mL of $^3$H-cholesterol. Apolipoprotein B–depleted sera were prepared by incubating with 13% polyethylene glycol 6000 solution (Wako Pure Chemicals). We confirmed depletion of apolipoprotein B by fast protein liquid chromatography and western blot analysis. Subsequently, efflux mediums containing 2.8% apolipoprotein B (apoB)–depleted sera obtained from the mice treated with SQ/DQ or the vehicle were added and incubation conducted for 4 hr. All procedures were performed in the presence of the acyl–coenzyme A: cholesterol acyltransferase inhibitor; Sandoz 58-035 (2 µg/mL) (Sigma) and 8-bromoadenocine 3’,5’-cyclic monophosphate (0.3 mmol/L) (Sigma). An LSC was used to quantify the efflux of radioactive cholesterol from the cells. The quantity of radioactive cholesterol incorporated into cellular lipids was calculated by means of hexane: isopropanol (v:v/1:1) extraction of control wells not exposed to the serum (blank). For each 24-wells in the culture plate, 3 were used for the blank. Percent efflux was calculated by the following formula \([(\text{cpm of } ^3\text{H-cholesterol in media containing } 2.8\% \text{ apoB-depleted plasma – cpm of } ^3\text{H-cholesterol in plasma-free media)/cpm of } ^3\text{H-cholesterol in cells extracted before the efflux step} ] \times 100.

**Atherosclerotic Lesion Analysis**

Forty apoE mice were fed with a high cholesterol diet (containing 1.25% cholesterol) with or without SQ (0.1%) for 12 weeks. Two mice in the control group died due to unknown reasons. The entire aorta was dissected, opened longitudinally, and stained with Sudan IV. Total areas of the aorta and intimal atherosclerotic plaques were measured from NIH images. The areas of atherosclerotic lesions as percentages of the aortic wall were calculated by dividing atherosclerotic area by total area of the aorta.
**Statistical Analysis**

The Student’s t-test was performed as appropriate. A p value of less than 0.05 was considered to be statistically significant. Values are expressed as mean ± SD.

**References**

density lipoprotein with cellular lipid in an abca1- or abca7-dependent manner. *J Lipid Res.* 2006;47:1542-1550