Cholesterol transport between red blood cells and lipoproteins contributes to cholesterol metabolism in blood

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Abstract Lipoproteins play a key role in transport of cholesterol to and from tissues. Recent studies have also demonstrated that red blood cells (RBCs), which carry large quantities of free cholesterol in their membrane, play an important role in reverse cholesterol transport. However, the exact role of RBCs in systemic cholesterol metabolism is poorly understood. RBCs were incubated with autologous plasma or isolated lipoproteins resulting in a significant net amount of cholesterol moved from RBCs to LDL, while cholesterol from LDL moved in the opposite direction. Furthermore, the bi-directional cholesterol transport between RBCs and plasma lipoproteins was saturable and temperature-, energy-, and time-dependent, consistent with an active process. We did not find LDLR, ABCG1, or scavenger receptor class B type 1 in RBCs but found a substantial amount of ABCA1 mRNA and protein. However, specific cholesterol efflux from RBCs to isolated apoA-I was negligible, and ABCA1 silencing with siRNA or inhibition with vanadate and Probucol did not inhibit the efflux to apoA-I, HDL, or plasma. Cholesterol efflux from and cholesterol uptake by RBCs from Abca1+/+ and Abca1−/− mice were similar, arguing against the role of ABCA1 in cholesterol flux between RBCs and lipoproteins. Bioinformatics analysis identified ABCA7, ABCG5, lipoprotein lipase, and mitochondrial translocator protein as possible candidates that may mediate the cholesterol flux. Together, these results suggest that RBCs actively participate in cholesterol transport in the blood, but the role of cholesterol transporters in RBCs remains uncertain.

Supplementary key words adenosine 5′-triphosphate binding cassette transporter A1 • apolipoprotein A-I • cholesterol/metabolism • cholesterol flux • erythrocyte • high density lipoprotein • low density lipoprotein • lipidomics

LDL and HDL carry cholesterol between liver and peripheral tissues. Impairment of LDL and/or HDL metabolism causes accumulation of cholesterol in the arterial wall, leading to the development of atherosclerosis. Measurements of plasma levels of LDL-C and HDL-C are commonly used to predict the risk of atherosclerosis. Although very useful and reliable, these traditional biomarkers do not always fully reflect the risk of cardiovascular events. Furthermore, when plasma levels of lipoproteins are targeted by lipid-lowering therapies, they reduce but do not eliminate cardiovascular risk despite normalizing the plasma levels of lipoproteins. Existence of what has been termed a “residual risk” strongly suggests that other players that modulate systemic cholesterol metabolism may also contribute to cardiovascular disease risk (1).

Over decades, systemic lipoprotein metabolism has been thoroughly investigated, with most findings derived from studies using blood plasma, thus ignoring a possible contribution of blood cells. Red blood cells (RBCs) occupy almost half of the total blood volume and carry large quantities of free (unesterified) cholesterol in their plasma membrane. Potentially, this cholesterol can be transferred to or from lipoproteins through nonspecific exchange or by active transport. The total cholesterol (TC) content of RBC membrane was found to be higher in patients with acute coronary syndrome (2) and correlated with severity of coronary artery disease (3, 4). Recent studies demonstrated that trafficking of free cholesterol (FC) (as opposed to esterified cholesterol) may be a major contributor to reverse cholesterol transport (5) and demonstrated cholesterol exchange between RBCs and lipoproteins in healthy volunteers (6). Hung et al. (7) injected cholesterol-labeled foam cells into apoA-I-deficient and anemic mice to demonstrate that, at least in this model, cholesterol can be transported from the peripheral tissue to feces via RBCs. These findings indicate that the pool of cholesterol in RBCs may be an important contributor to systemic cholesterol metabolism.

In this context, it is reasonable to hypothesize that cholesterol can be transferred between RBCs and plasma lipoproteins affecting distribution of cholesterol among lipoproteins and their metabolism. Further understanding of the role of RBCs in systemic cholesterol metabolism may unveil novel pathways of systemic cholesterol trafficking.
that can be potentially targeted for therapeutic purposes. In this study, we investigated the trafficking of cholesterol between RBCs and lipoproteins, the impact of this pathway on lipoprotein metabolism, and the possible involvement of cholesterol transporters in RBCs.

MATERIALS AND METHODS

Ethics statement

Human blood samples were collected with informed written consent of the participants. This study was approved by the ethics committee of the Faculty of Medicine, Tokyo Medical and Dental University (No. M2000-1790), and Baker Heart and Diabetes Institute (No. 573/17). This study has been performed in accordance with the rules of the World Medical Association and the principles of the Declaration of Helsinki.

All animal procedures were approved by the National Institutes of Health Institutional Animal Care and Use Committee (protocol H-0050R).

Animal studies

Creation and characterization of ABCA1-KO mice with C57Bl/6N genetic background has been described previously (8). Normal C57Bl/6N mice were from Taconic. Mice were bled from the retroorbital sinus to small heparin-coated capillary tubes following the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines for survival bleeding of mice. The RBCs were obtained after centrifugation of blood at 1,500 g for 15 min. At the end of the study, the mice were euthanized by exposure to carbon dioxide.

Collection and preparation of blood samples

Blood samples were collected from the antecubital vein of healthy adult volunteers. To prepare packed RBCs and plasma, whole blood (WB) containing 1.8 mg/ml EDTA-2K (unless stated otherwise) was centrifuged at 400 g for 15 min. Plasma and buffy coat were removed, and RBCs were washed three times with saline. Plasma was centrifuged at 2,500 g for 30 min to separate the buffy coat. The packed RBCs were kept in mannitol-adenine-phosphate solution (natrium citricum, 5.10 mmol/l; citric acid, 0.95 mmol/l; NaCl, 85 mmol/l; NaH2PO4, 6.03 mmol/l; adenine, 1.04 mmol/l; mannitol, 80 mmol/l; and glucose, 40 mmol/l) at 4°C until used (9). All packed RBCs were used within 2 weeks after collection. RBC ghosts were prepared as described previously (10) and stored at −80°C.

Purification of lipoproteins and apolipoproteins

VLDL (d < 1.006 g/ml), LDL (d = 1.006–1.030 g/ml), HDL (d = 1.033–1.063 g/ml), and lipoprotein-depleted plasma (LDP) (d > 1.210 g/ml) were isolated by sequential density ultracentrifugation (11). Isolated apoA-I and apoA-II were prepared as described (12).

Measurement of TC and FC

The concentrations of TC, FC, HDL-C, and LDL-C were determined by using commercial enzymatic assay kits:Determinar L TC II, Determinar L FC, MetaboLead HDL-C, and Determinar L LDL-C, respectively (Kyowa Medex, Japan).

Analysis of plasma cholesterol after incubation with RBCs

Packed RBCs and plasma obtained from the same individual were mixed at indicated ratios and incubated at indicated temperatures for 4–72 h. Similarly, the packed RBCs were mixed with lipoprotein fractions (VLDL, LDL, HDL, or LPD at their plasma concentrations), apoA-I (1.2 or 2.4 mg/ml), or apoA-II (0.4 and 0.8 mg/ml) and incubated at 37°C for 4–24 h. After removing RBCs, cholesterol levels in plasma or lipoprotein fractions were determined. When indicated, RBCs and plasma were incubated at 37°C in the presence or absence of 0.5 mM DNPB. To observe morphological change in RBCs after incubation with plasma, a smear was prepared on a glass slide and stained with May-Grünwald-Giemsa by automated slide maker SP-10 (Sysmex).

Lipidomic analysis

Lipidomic analysis was performed as described previously (15). RBC ghosts were isolated, sonicated on ice, and lipids were extracted using a single phase CHCl3:CH3OH method. The analysis was performed by LC-MS/MS analysis using an Agilent 1200 LC system (Agilent Technologies), an API 4000 Q/Trap mass spectrometer, and Analyst 1.5 and MultiQuant data systems (AB SCIEX). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding standard. Lipid concentrations are presented relative to the phosphatidylcholine levels.

Electrophoresis and Western blotting

Membrane proteins in RBCs were analyzed by SDS-PAGE using 18% polyacrylamide gel under nonreducing condition for glycoporphin or 4–12% gradient polyacrylamide gel (Invitrogen) for ABC transporters. Briefly, the proteins separated by electrophoresis were transferred to PVDF membranes (Millipore). After blocking, the membranes were incubated with the following antibodies: polyclonal goat anti-glycophorin A (GAPDH) antibody (Merck, #SAB2500470), monoclonal mouse anti-ABCA1 antibody (Abcam, #ab18180), polyclonal anti-ABCG1 antibody (Novus Biologicals, NB400-132), and monoclonal mouse anti-GAPDH antibody (Calbiochem, #CB1001), as primary antibodies, and peroxidase-conjugated polyclonal rabbit anti-goat antibody (Medical and Biological Laboratories, #IS-546) and peroxidase-conjugated polyclonal goat anti-mouse antibody (Southern Biotech, #1020-05) as secondary antibodies. The change of HDL particle size was evaluated by native gel electrophoresis using 8% polyacrylamide gel followed by staining with Coomassie brilliant blue.

Cholesterol uptake and efflux assays

3H-cholesterol solution (PerkinElmer, 0.5 μCi/ml) was combined with either methyl-β-cyclodextrin (MBCD, Sigma Aldrich)/cholesterol complex [MBCD:cholesterol molar ratio of 8:1 (14)] or indicated concentrations of apoA-I, HDL, or plasma diluted in buffer A (20 mmol/l HEPES, 3.3 mmol/l NaH2PO4, 2.9 mmol/l KCl, 1 mmol/l MgCl2, 138 mmol/l NaCl, and 1 mg/ml glucose (pH 7.4)) as previously described (15).

Packed RBCs (from the same individual as the plasma) were mixed with each uptake solution (final concentrations: 5 x 10^6 cells/ml RBCs and 0.5 μCi/ml 3H-cholesterol with 0.1 mmol/l MBCD, 2.5 μg/ml apoA-I, HDL, or 0.05% plasma) and incubated at 37°C for 0.5–3 h. Supernatant was collected and RBCs were lysed with distilled water. The uptake rate was calculated as follows: uptake rate (%) = [DPM of RBC lysate/(DPM of supernatant + DPM of RBC lysate)] x 100. Cholesterol uptake by murine RBCs was performed using the same methodology.

Cholesterol efflux was done as described previously (16). Briefly, cells were labeled by incubation in serum-containing medium supplemented with [3H]cholesterol (75 kBq/ml; American Radiolabeled Chemicals) for 72 h. Cells were washed and incubated for 6 h in serum-free medium. Acceptors were added in a serum-free medium at indicated concentrations and incubated...
for 2 h, and the radioactivity in the cells and medium was determined. Cholesterol efflux was calculated as a proportion of radioactivity moved from cells to medium; nonspecific efflux (i.e., the efflux to the medium without acceptor) was subtracted.

**ABCA1 silencing**

RBCs were transfected with Silencer Select ABCA1 siRNA (Thermo Fisher Scientific; siRNA ID s848) or 100 nM ALLStars negative control scrambled siRNA (Qiagen) using Viomer Blue transfection reagent (Lipocalyx GmbH, Germany) according to manufacturer’s instructions. Briefly, 5.0 × 10⁵ RBCs were added to RPMI-1640 containing 10% FBS without antibiotics, and incubated with Viromer-siRNA duplex at 37°C for 6 h. The transfection mixture was then replaced with RPMI-1640 containing 10% FBS and RBCs and incubated for 72 h at 37°C.

**Statistical analysis**

All data are shown as mean ± SD unless stated otherwise. Statistical significance of the differences was assessed in SPSS by a general linear model or ANOVA followed by Dunnet’s test and Bonferroni’s post hoc comparisons tests or unpaired Student’s t-test. Experiments were conducted in triplicate or quadruplicate and repeated two to five times.

### RESULTS

**Transfer of cholesterol between RBCs and plasma**

Packed RBCs obtained from WB collected into EDTA-2K or heparin vacutainer tubes were mixed with plasma from the same individual. For experiments with WB, blood was collected into vacutainer tubes with clot activator, and blood cells were not removed. WB or mixtures of RBCs with plasma were incubated at 37°C for 4–24 h, and the cholesterol level in serum or plasma was measured after separating them from cells. Serum and plasma cholesterol levels increased in a time-dependent manner (Fig. 1A). After 24 h incubation of serum or plasma with packed RBCs, the cholesterol level significantly increased by 13.2 ± 8.9 mg/dl (7.2%, P < 0.01 vs. 0 h), 20.8 ± 1.8 mg/dl (12.3%, P < 0.001 vs. 0 h), and 17.1 ± 1.0 mg/dl (9.7%, P < 0.001 vs. 0 h) for serum, EDTA-2K, and heparin plasma, respectively (initial cholesterol concentrations were 183.5 ± 37.9 mg/dl, 169.8 ± 31.5 mg/dl, and 175.6 ± 33.2 mg/dl). There was no statistically significant difference in cholesterol content changes between serum and plasma and between plasmas collected into different anticoagulants at any of the time points.

Next, packed RBCs were mixed with plasma at various ratios (30, 50, and 70% of RBCs by volume) and incubated at the same conditions. After 24 h incubation with 70% and 50% RBCs, plasma cholesterol levels were significantly elevated compared with plasma incubated with 30% RBCs (P < 0.01 and P < 0.05, respectively) (Fig. 1B). To establish the rate-limiting step of the cholesterol transfer, after incubation of the plasma/RBC mixture (50%) at 37°C for 12, 24, or 36 h, 80% of the conditioned plasma was replaced with fresh plasma sample every 12 h, and the mixture was further incubated for a total of 48 h. Figure 1C shows a difference in TC content in plasma between each time point and the previous time point (i.e., how much cholesterol was added to plasma in 12 h). After replacement with fresh plasma at 12 h, cholesterol transfer was significantly increased in the subsequent 12 h (at the 24 h time point, P < 0.05) and 24 h incubations (at the 36 h time point, P < 0.02, Fig. 1C). By the 48 h time point, most of the available cholesterol from RBCs has been removed (Fig. 1C). In a reciprocal experiment, RBCs were replaced with fresh RBCs after 12, 24, 36, 48, and 60 h. TC concentration in the plasma was continuously increasing for at least 72 h, and there was no effect of replacement of RBCs with fresh cells (Fig. 1D). We then analyzed to determine which plasma lipoprotein fraction receives cholesterol from RBCs. The cholesterol level in the LDL fraction decreased for the first 36 h with little change after longer incubations. The cholesterol level in HDL increased for the first 36 h, saturating at longer incubations (Fig. 1D). Notably, changes in total plasma cholesterol were bigger than the sum of changes in LDL-C and HDL-C. One possible explanation is that some of cholesterol in plasma was residing in VLDL or IDL or is not lipoprotein-bound, such as albumin/fatty acid complexes or free exchangeable apolipoproteins. However, there was no cholesterol release from RBCs to PBS or lipoprotein-deficient plasma (see below) indicating that the presence of lipoproteins is essential. Another possibility is that the direct enzymatic method used in this study underestimated the cholesterol level in individual lipoprotein fractions.

To investigate the effect of temperature on cholesterol flux, RBCs and plasma were mixed and incubated at 4°C, room temperature (22°C), and 37°C. The maximum rate of cholesterol flux was observed at 37°C, while no changes in cholesterol level were observed at 4°C (Fig. 1E).

Thus, incubation of RBCs with plasma results in movement of cholesterol from RBCs to HDL and from LDL to RBCs, with a net movement of cholesterol from RBCs to plasma. This process is time-, dose-, and temperature-dependent and is not influenced by the type of anticoagulant.

**Transfer of cholesterol between RBCs and isolated lipoproteins**

To further investigate the differences in cholesterol transport between RBCs and individual lipoprotein fractions, we incubated RBCs with isolated lipoproteins and assessed changes in cholesterol levels (Fig. 2A). Isolated HDLs were incubated with RBCs at two concentrations, one was equal to the individual’s plasma HDL-C level (75.4 mg/dl) and another was twice as high (150.8 mg/dl). After 12 h incubation, the cholesterol level in the LDL was increased by 5.2 ± 0.9 mg/dl (P < 0.001 vs. 0 h) and 10.4 ± 0.3 mg/dl (P < 0.001 vs. 0 h) for low and high HDL concentrations, respectively. There was no further increase in cholesterol level in the HDL after an additional 12 h of incubation. Conversely, when RBCs were incubated with isolated LDL (two concentrations, one was equal to the individual’s plasma LDL-C level, 122.2 mg/dl, and another was twice as high, 244.5 mg/dl), the level of cholesterol in the LDL fraction decreased continuously for up to 24 h incubation, similarly for both LDL concentrations [-8.8 ± 3.1 mg/dl

Red blood cells regulate cholesterol metabolism
Cholesterol levels slightly increased in VLDL and lipoprotein depleted plasma when these fractions were incubated with RBCs for 24 h [(2.8 ± 1.2 mg/dl (P < 0.001 vs. 0 h) and 3.1 ± 0.6 mg/dl (P < 0.001 vs. 0 h), respectively], while there was no flux of cholesterol between RBCs and PBS (Fig. 2A). Next, we incubated RBCs with isolated apoA-I (1.2 and 2.4 mg/ml) and apoA-II (0.4 or 0.8 mg/ml). Unlike the HDL fraction, cholesterol concentration in isolated apolipoproteins increased by less than 1 mg/dl (Fig. 2B). Incubation of RBCs with plasma for 24 h, included as positive control, led to increments in cholesterol of 16.1 ± 2.8 mg/dl (P < 0.001 vs. 0 h) and 18.9 ± 4.5 mg/dl (P < 0.001 vs. 0 h), as shown in Fig. 2A and B, respectively.

To investigate the contribution of continuing esterification to the movement of cholesterol from RBCs to HDL, we repeated the experiments in the presence of the LCAT inhibitor, DTNB. When plasma treated with DTNB was incubated with RBCs, the TC level in the treated plasma increased at a rate similar to that of the untreated plasma (P < 0.01 vs. 0 h for both treated plasma and untreated plasma) (Fig. 2C).

To evaluate changes in lipid content in RBC membrane, lipids from RBC ghosts were extracted after incubation with plasma or PBS and analyzed by MS (lipidomics). When RBCs were incubated with plasma, the FC levels in RBC plasma significantly decreased compared with RBCs incubated with PBS (P < 0.05 vs. 0 h) (Fig. 2D), while the levels of cholesteryl esters (CEs) did not change (Fig. 2E). Other notable changes in the lipid profile in RBC membrane were considerable increases in lysophosphatidylcholine, alkanyl-lysophosphatidylcholine, alkyl-lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylethanolamine, alkyl-phosphatidylethanolamine, and alkyl-phosphatidylethanolamine, whereas concentration of other lipids (including an important constituent of the membrane, sphingomyelin) did not change when compared with RBCs incubated with PBS (supplemental Table S1). The results for the individual lipid species are shown in supplemental Database S1. Thus, incubation of RBCs with individual lipoproteins results in movement of FC to HDL and from LDL, a process not driven by cholesterol esterification.

Cholesterol depletion of RBCs does not cause hemolysis

Depletion of RBC cholesterol may potentially affect the integrity of the plasma membrane and release of cholesterol from RBCs to plasma due to hemolysis. We noted that after incubation of RBCs with plasma (50% RBCs v/v at 37°C for 24 h), a few spike-like spur cells appeared. The number of these spur cells was increased with incubation time (Fig. 3A), but the total number of intact RBCs did not change during 24 h incubation (Fig. 3B). Hemolysis was assessed by measuring absorbance at 543 nm, and although the absorbance slightly increased over time, the rate of hemolysis was less than 1% after 24 h (Fig. 3C).

It is also possible that long incubation of RBCs may lead to shedding fragments of the plasma membrane or secretion of extracellular vesicles contributing to the transfer of cholesterol from the cells to the medium. To test this possibility, we incubated RBCs with HDL (300 mg/dl) over 24 h, which led to an increase of the cholesterol content of the
Red blood cells regulate cholesterol metabolism

medium by 7 mg/dl. We then compared the amount of GPA, a marker of the RBC plasma membrane, in RBC ghosts loaded at a cholesterol concentration of 7 mg/dl (the amount of GPA expected in the medium if all cholesterol was exported as fragments of the plasma membrane) with that found in the medium after incubation with RBCs (Fig. 3D). The GPA abundance after 24 h incubation was below 10% (n = 3) of that in RBC ghosts (Fig. 3D). Furthermore, no lipid other than cholesterol was reduced in RBCs after incubation with plasma, as compared with incubation with PBS (supplemental Table S1). We then used native polyacrylamide gel electrophoresis to assess the effect of incubation of RBCs with HDL on the size of HDL particles. We found a slight increase in the size of HDL particles after 24 h incubation with or without RBCs (Fig. 3E). These findings, combined with the observation that the efflux was “specific” (i.e., did not occur in the absence of an acceptor and was dependent on the properties of the acceptor, see above), are inconsistent with nonspecific shedding of cholesterol from RBC plasma membrane or secretion of extracellular vesicles making a significant contribution in transfer of cholesterol between RBCs and plasma.

Mechanism of cholesterol flux to and from RBCs

Temperature dependence and selectivity of cholesterol movement to and from RBCs indicates a possibility that this might be an active process involving a transporter. We employed cholesterol efflux and cholesterol uptake assays to mechanistically analyze the flux of cholesterol between plasma and RBCs.

Consistent with the results shown above (Figs. 1D, 2A), isolated HDL supported \(^{3}H\)-cholesterol efflux from RBCs in a dose- and time-dependent manner (Fig. 4A, B). In contrast, efflux to apoA-I was low and not dose- or time-dependent (Fig. 4A, B).

For the cholesterol uptake assay, we used four types of cholesterol carriers, each at low and high concentrations: apoA-I (2.5 or 25 \(\mu\)g/ml), HDL (2.5 or 25 \(\mu\)g/ml), plasma (0.05% or 0.5% and matching the apoA-I concentration in the experiment with apoA-I and HDL), and \(\beta\)-CD (0.1 or 1.0 mM); the duration of uptake incubation was 1 h. When \(^{3}H\)-cholesterol complexed with low concentrations of \(\beta\)-CD and apoA-I were mixed with RBCs, respectively 85.1% and 75.4% of the added cholesterol was incorporated into the RBCs (Fig. 4C). The uptake rates were lower for HDL and plasma (27.9% and 17.2%, respectively). The uptake was significantly lower for high concentrations of \(\beta\)-CD and apoA-I were mixed with RBCs, respectively 85.1% and 75.4% of the added cholesterol was incorporated into the RBCs (Fig. 4C). The uptake was significantly lower for high concentrations of \(\beta\)-CD, HDL, and plasma \((P < 0.001\) vs. high concentrations) but to a lesser extent for apoA-I \((P < 0.05\) vs. high concentrations) (Fig. 4C). The cholesterol uptake was time-dependent and saturable for all carriers, and the differences in cholesterol uptake from different carriers persisted over the time-course (Fig. 4D).

To identify cellular mechanisms that may be involved in movement of cholesterol to and from RBCs, we analyzed the mRNA levels of known cholesterol transporters. We
found a relatively high abundance of ABCA1 mRNA, a lower level of ABCG1 mRNA, and negligible levels of LDLR and SCARB1 mRNAs (Fig. 5A). The presence of ABCA1 in RBCs was further confirmed by Western blot (Fig. 5B). Conversely, we were unable to detect the presence of ABCG1 by Western blot (Fig. 5B).

To assess the contribution of ABCA1 to cholesterol flux, we manipulated the abundance of ABCA1 in RBCs using siRNA silencing (Fig. 5C, D). The fact that transfection with siRNA reduced ABCA1 abundance (P < 0.02) indicates that ABCA1 protein is not likely to be a remnant from the megakaryocyte precursor, but instead is actively synthesized.
Red blood cells regulate cholesterol metabolism

in RBCs. However, silencing of ABCA1 in RBCs did not have a significant effect on cholesterol efflux or uptake (Fig. 6E, F). This is consistent with very low specific cholesterol efflux to lipid-free apoA-I (Figs. 2B, 4A, 5E), a preferred acceptor for ABCA1-mediated cholesterol efflux in other models.

To confirm these findings, we tested cholesterol efflux and cholesterol uptake using RBCs from ABCA1 KO mice. Consistent with the results with human RBCs, there was no significant difference in cholesterol flux between wild-type, Abca1<sup>+/−</sup>, and Abca1<sup>−/−</sup> mice (Fig. 5G, H). Thus, although ABCA1 transporter was present in RBCs, it did not contribute to cholesterol fluxes.

To further investigate the involvement of cellular transporters into cholesterol transport in RBCs, we tested the effects of chemical inhibitors of these transporters. For the efflux assay, the compounds were mixed with RBCs after <sup>3</sup>H-cholesterol loading. When RBCs were pretreated with 0.2 mmol/l vanadate, an ATPase inhibitor, specific efflux to HDL increased by 16% compared with untreated RBCs (<i>P < 0.05</i>), while there was no effect on the efflux to apoA-I (Fig. 6A). Similar effects were seen using another ABCA1 inhibitor, Probucol: the efflux from RBCs treated with 25 μmol/l Probucol to HDL was 63% higher compared with untreated RBCs (<i>P < 0.01</i>) (Fig. 6B). However, in contrast to vanadate, Probucol also increased specific efflux to apoA-I (<i>P < 0.01</i>). When cholesterol uptake was investigated, 0.2 mM of vanadate slightly inhibited the cholesterol uptake from MβCD (0.1 mmol/l), apoA-I (2.5 μg/ml), HDL (2.5 μg/ml), or plasma (0.05%) after treatment with siRNA<sub>ABCA1</sub>. G: Cholesterol efflux from RBCs from Abca<sup>+/−</sup>, Abca<sup>−/−</sup>, and Abca<sup>1/−</sup> mice to apoA-I or HDL (2.5 μg/ml). H: Cholesterol uptake by RBCs from Abca<sup>+/−</sup>, Abca<sup>1/−</sup>, and Abca<sup>−/−</sup> mice from MβCD (0.1 μg/ml), apoA-I (2.5 μg/ml), HDL (2.5 μg/ml), or plasma (0.05%). *<i>P < 0.05</i> versus siRNA<sub>scram</sub> by unpaired Student’s t-test, all values represent mean ± SD (n = 3), images are a representative of three separate experiments.
Because the cholesterol transporters experimentally investigated in this study were found not to be responsible for trafficking of cholesterol to and from RBCs, we took advantage of human RBC transcriptomics and proteomics databases (18, 19) to identify possible candidates that may mediate cholesterol transport between RBCs and plasma lipoproteins. We identified genes/proteins expressed in RBCs at both mRNA and protein levels using the following search terms: lipid, lipase, ATP binding cassette, lipoprotein, and scavenger. The latter term provided no hits; the other hits are listed in Table 1. Very few strong candidates were identified using this approach, and only one, ABCA7, had a connection with cholesterol trafficking.

Further, a global comparison of hematopoietic cells was performed using publicly available microarray data (20), specifically focusing on the cholesterol metabolism and transport KEGG pathway. A global overview of cholesterol metabolism and transport showed that three different mature RBC subsets were quite distinct in their cholesterol metabolism and transport from other hematopoietic cells (Fig. 7A). In particular, ABCG5, an enterocyte sterol transporter (21), is expressed in mature RBCs. A comparative analysis between the three RBC populations and monocytes, cells with well-defined cholesterol transport pathways, demonstrated that ABCG5 expression is higher in subsets ERY5 and -4 (Fig. 7B). Further, this analysis identified two highly expressed genes in RBCs with known functions in cholesterol metabolism, lipoprotein lipase and translocator protein, a mitochondrial cholesterol transporter (22).

**DISCUSSION**

Accumulation of cholesterol in the arterial wall is a key pathogenic mechanism of atherosclerosis, a leading cause of cardiovascular disease. Cholesterol is synthesized by all cells but cannot be catabolized by most of them except for hepatocytes and adrenal and gonadal cells. Accumulation of cholesterol is often a result of imbalance between delivery and removal of cholesterol to and from cells by lipoproteins. Delivery of cholesterol occurs through forward cholesterol transport, where apoB-containing lipoproteins, mainly native or modified LDL, play a key role in delivering CE to cells through receptor-mediated endocytosis. Removal of cholesterol from cells occurs via reverse cholesterol transport, where FC is transferred from cells to apoA-I-containing lipoproteins and is esterified and transported to liver or intestine for excretion. The two key elements of this paradigm are that most cholesterol is transported as CE and that plasma lipoproteins are the main vehicle for cholesterol transport. However, several recent studies suggested that transport of FC between different pools in the body is much faster and may be quantitatively more important than transport of esterified cholesterol to liver (23) and intestine (24, 25) for excretion. Furthermore, plasma lipoproteins are in constant direct contact with blood cells and endothelial cells of the vessel wall containing considerable quantities of FC in their membranes. RBCs are by far the largest pool of FC in direct contact with plasma lipoproteins and, therefore, can significantly affect cholesterol metabolism in plasma. In this study, we investigated the transport of cholesterol...
TABLE 1. Possible candidates that may mediate cholesterol transport to and from RBCs

| Name | Keyword | Transcriptomics | Proteomics | Function in Lipid or Lipoprotein Metabolism |
|------|---------|----------------|------------|------------------------------------------|
|      |         | (Normalized Signal) | (Copy Number) |                                           |
| Phospholipase A2 activating protein (PLAA) | Lipase | 421.1 ± 140.2 | 604.4 ± 509.2 | Ubiquitin-mediated membrane proteins trafficking to late endosomes in an ESCRT-dependent manner (40). Regulation of cytosolic and calcium-independent phospholipase A2 activities and prostaglandin E2 biosynthesis (41). The function is not known; the expression pattern suggests a role in lipid homeostasis (42). |
| ABCA7 | ATP binding cassette | 988.6 ± 161.7 | 1,635.8 ± 1,297.1 | ATP-independent bidirectional transbilayer migration of phospholipids upon binding calcium ions, regulation of phospholipid asymmetry in the plasma membrane (43). |
| Phospholipid scramblase 1 (PLSCR1) | Lipid | 677.8 ± 280.2 | 6,339.9 ± 1,723.8 | | |
| Protocerolipid protein 2 (PLP2) | Lipid | 1,112.7 ± 326.0 | 2,048.9 ± 1,260.4 | Unknown |
| LDLR class A domain containing 4 (LDLRAD4) | Lipoprotein | 1112.7 ± 326.0 | 2,048.9 ± 1,260.4 | Unknown |

between RBCs and plasma lipoproteins, the first step in a putative pathway of cholesterol transport involving RBCs.

Studies published several decades ago demonstrated that FC from plasma exchanges with that in RBCs (26, 27). Recent studies suggested an active role for RBCs in reverse cholesterol transport (7), and our previous report showed that RBCs participated in cholesterol efflux from foam cells to apoa-I and HDL as a temporary cholesterol storage depot (28). The dynamics and mechanisms of transport of cholesterol between RBCs and plasma lipoproteins are poorly understood. The first important observation from our study was that when RBCs were incubated with autologous plasma, the net movement of cholesterol was from RBCs to plasma lipoproteins. When distribution of cholesterol between lipoprotein fractions was analyzed, we found that this movement is the result of net movement of cholesterol from RBCs to HDL and net movement of cholesterol from LDL to RBCs; this was confirmed by experiments with isolated lipoproteins. Because cholesterol movement was minimal when RBCs were incubated with PBS, VLDL, or LDP, it is unlikely that FL is the result of shedding of cholesterol from plasma membrane or secretion of exosomes from RBCs; this was also confirmed by analysis of transfer of a plasma membrane marker to HDL. The movement was unrelated to cholesterol esterification in plasma; it was rapid, not affected by inhibition of LCAT, and the concentration of plasma was a rate-limiting factor. Cholesterol movement from RBCs to HDL and from LDL to RBCs points to a suggestion that this flux of cholesterol is more likely to be a part of reverse rather than of forward cholesterol transport. Thus, RBCs may take up FC from LDL, and presumably from modified LDL as well, thereby potentially reducing the amount of cholesterol delivered to the cells in the vessel wall. Cholesterol taken up from LDL subsequently can be released to HDL, channelling it into a classical reverse cholesterol transport pathway bypassing non-blood cells. RBCs can also directly transport cholesterol to the intestine and liver by feeding into the transintestinal cholesterol transport pathway or into hepatocytes for excretion or conversion into bile acids. Hung et al. (7) first suggested this possibility in apoA-I-deficient mice, but conceded that, in mice with normal levels of HDL, this pathway may not be quantitatively important. Our studies, however, suggest that, in humans, where plasma levels of HDL are much lower than in mice, this “bypass” reverse cholesterol transport pathway may be physiologically important.

The second important finding of our study relates to the mechanism of cholesterol transport between RBCs and lipoproteins. The movement of cholesterol between RBCs and plasma was saturable and energy-, temperature-, and time-dependent, suggesting active transport involving cholesterol transporters. RBCs are nonnucleated cells, and, given that the half-life of most cholesterol transporters is much shorter than that of RBCs, the presence of these transporters can only be explained by the presence of a pool of translatable stable mRNA. Whether or not RBCs are capable of an appreciable rate of protein synthesis is a contested question; however, a recent study demonstrated that RBCs possess a pleiotropy of proteins participating in protein biosynthesis (19). Another recent study found a spectrum of microRNAs and associated machinery in RBCs (29). Real-time RT-PCR did not identify LDLR mRNA in RBCs, which, together with the observation that it is mainly FC that moves between RBCs and lipoproteins, makes it unlikely that the mechanism of movement of cholesterol from LDL to RBCs involves LDLR. Unexpectedly, however, when we compared uptake by RBCs of cholesterol bound to different carriers, the most effective delivery was observed when cholesterol was complexed with apoa-I, indicating that cholesterol flux may be a more complex process than just an exchange with a carrier. The conformation of apoa-I in the reconstituted HDL used in the uptake experiments is, however, different to both lipid-free apoa-I and the predominantly larger spherical plasma HDL that were used in the efflux experiments, and it is possible that the two forms of HDL interact differently with RBCs.

In contrast to the apparent absence of LDLR, we found appreciable levels of ABCA1 and ABCG1 (but not SCARB1) mRNA and also detected ABCA1 but not ABCG1 protein in RBCs. Moreover, the level of ABCA1 was reduced upon transfection of cells with siRNA ABCA1, consistent with the possibility of a continuing synthesis of ABCA1 in RBCs. This is the first time the existence of a cholesterol transporter in RBCs has been shown, and this finding requires independent confirmation using unrelated methodology. It is, however, unclear whether ABCA1 in RBCs is functional. Specific efflux to apoa-I, the main cholesterol
acceptor of ABCA1-dependent cholesterol efflux, was very low; silencing ABCA1 had no effect on the efflux; there was no difference in cholesterol efflux from RBCs from $\text{Abca1}^{+/+}$ and $\text{Abca1}^{-/-}$ mice; and two ABCA1 inhibitors, vanadate and Probucol, failed to inhibit cholesterol efflux. These findings indicate that although ABCA1 is present in RBCs, it may not mediate cholesterol efflux to apoA-I, and mature HDL is the predominant acceptor of cholesterol.

Fig. 7. Transcriptomic analysis for the identification of potential cholesterol transporters in RBCs. A: Heatmap comparing the expression of genes involved in cholesterol metabolism (KEGG ID: hsa04979) across different blood cells. The dataset contains three different mature RBC subsets denoted as ERY3 (CD34$^-$, CD71$^+$, GpyA$^+$), ERY4 (CD34$^-$, CD71$^{lo}$, GpyA$^+$), and ERY5 (CD34$^-$, CD71$^-$, GpyA$^+$), with ERY5 representing the most mature RBCs. Data was log$_2$ normalized and z-score transformed. B: Volcano plots comparing cholesterol metabolism gene profiles of mature RBC subsets against monocytes. Significant transcripts after multiple comparisons (FDR of 10%) correction are in red.
from RBCs. In the absence of ABCG1 and SR-B1, the nature of the transporter used by HDL is unclear, but it may involve exchangeable apolipoproteins other than apoA1, e.g., apoE, which plays an important role in cholesterol efflux to HDL-like particles in the brain (30). It is, however, possible that ABCA1 has a role in the uptake of FC from plasma, as evidenced by the high rate of uptake of cholesterol complexed to apoA1, which was affected by vanadate and Probucol. The involvement may be indirect: ABCA1 may promote cholesterol uptake by redistributing cholesterol from the plasma membrane to inside the cell, as suggested by Yamauchi et al. (31). Previous studies showed that Tangier disease patients have low cholesterol contents in RBC membrane (32, 33).

We then used a bioinformatics approach to identify other possible candidates that might mediate cholesterol trafficking between lipoproteins and RBCs. ABCA7, ABCG5, lipoprotein lipase, and translocator protein were flagged as possible candidates. The involvement of these transporters, however, remains a speculation, and specific mechanisms of cholesterol uptake and efflux to and from these cells remain to be investigated.

Our study has established that RBCs actively participate in systemic lipoprotein metabolism and can potentially influence trafficking of cholesterol in blood and between blood and vascular cells. Interestingly, anemia, despite being accompanied by a low serum cholesterol level (34, 35), is an independent risk factor for cardiovascular disease (36, 37). Sickle cell disease is also a cardiovascular risk factor and is accompanied by significant impairment of HDL metabolism (38, 39). These findings are consistent with the suggestion of a physiological role of RBC in cholesterol metabolism in blood.

Data availability

The data used to support the findings of this study are available from the corresponding author [Ryunosuke Ohkawa, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), ohkawa. alc@tmd.ac.jp] upon request.

Author contributions

R.O., P.N., A.M., A.T.R., and Dm.S., conceptualization; R.O., H.L., N.M., and P.J.M., methodology; R.O., H.L., and Dm.S., formal analysis; R.O., H.L., N.M., Y.F., S.J.L., M.S., A.H., A.Y., T.K., Y.H., G.P., G.L., M.D., B.V., and De.S., investigation; R.O., P.J.M., A.M., A.T.R., and Dm.S., resources; R.O., H.L., A.M., A.T.R., and Dm.S., data curation; R.O. and Dm.S., writing-original draft; R.O., P.N., A.M., A.T.R., Dm.S., and M.T., writing-review and editing; H.L., Y.F., and M.D., visualization; R.O. and Dm.S., supervision; R.O. and Dm.S., project administration.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

CE, cholesteryl ester; FC, free cholesterol; GPA, glycoporphin A; LDP, lipoprotein-depleted plasma; MBCD, methyl-β-cyclodextrin; RBC, red blood cell; TC, total cholesterol; WB, whole blood.

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