A new method for measuring cholesterol efflux capacity uses stable isotope-labeled, not radioactive-labeled, cholesterol

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Abstract The incidence of cardiovascular events correlates inversely with cholesterol efflux capacity (CEC) more than with HDL-cholesterol level. The measurement of CEC is used to qualify cardiovascular disease risk and is conventionally performed with radioisotope (RI)-labeled cholesterol. Here, we established a CEC measurement technique using stable isotope-labeled cholesterol as an alternative, and we compared the new method with RI and fluorescence (boron dipyrromethene difluoride-cholesterol) methods in cells and in patient serum. We incubated J774 cells labeled with \([d_7]C\) cholesterol \([d_7]C\) with patient serum depleted of apoB, and \([d_7]C\) extracted from the culture medium was quantified by liquid chromatography/quadrupole time-of-flight mass spectrometry. \([d_7]C\) efflux increased with greater apoB-depleted serum concentration and longer incubation time. The assay coefficient of variation (CV) of five consecutive measurements of three sets of samples ranged from 7.3% to 9.5%, and the intrassay CV determined by measuring three samples four times ranged from 4.1% to 8.5%, both indicating good precision. We then measured CEC levels of 41 outpatients with serum HDL-cholesterol levels between 36 and 94 mg/dl (mean: 61.7 ± 18.0 mg/dl); in the presence of cAMP, we observed a significant, positive correlation between CEC levels determined with the stable isotope and RI methods that was stronger than the correlation between measurements obtained by the fluorescence and RI methods \((r = 0.73, \ P < 0.0001 \text{ vs. } r = 0.55, \ P < 0.001)\). Therefore, our stable isotope method can be considered useful as a non-RI method and thus deserves evaluation in future clinical studies.—Shimizu, T., O. Miyazaki, T. Iwamoto, T. Usui, R. Sato, C. Hiraishi, and H. Yoshida. A new method for measuring cholesterol efflux capacity uses stable isotope-labeled, not radioactive-labeled, cholesterol. J. Lipid Res. 2019. 60: 1959–1967.

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Many epidemiologic studies have reported an inverse relationship between coronary artery disease (CAD) events and HDL-cholesterol (HDL-C) levels (1–3). It is commonly known that the inhibition of cholesteryl ester transfer protein (CETP) leads to an increase in HDL-C levels in blood. Bowman et al. (4) recently reported that the inhibition of CETP by anacetrapib resulted in a lower incidence of major coronary events. In contrast, recent intervention trials have shown that treatment with CETP inhibitors or niacin fails to reduce CAD risk despite increasing HDL-C levels. Moreover, paradoxically, a high mortality rate was reported among individuals in the general population with extremely high HDL-C levels. It has since been suggested that quality is as important as quantity when measuring HDL-C (6–9). HDL is known to mediate reverse cholesterol transport (RCT), act as an antioxidant, suppress inflammation, and improve endothelial function (10). In RCT, accumulated cholesterol is transported from peripheral tissues back to the liver, a process that requires HDL and apoA-I (10–12). The first step in RCT is cholesterol efflux from macrophages; this is thought to be the most important

Abbreviations: APCI, atmospheric pressure chemical ionization; BODIPY-C, BODIPY-cholesterol; CAD, coronary artery disease; CEC, cholesterol efflux capacity; CETP, cholesteryl ester transfer protein; \([d_7]C\), \([d_7]C\)cholesterol; \([d_7]C\), \([d_7]C\)cholesterol; \([d_7]C\), \([d_7]C\)cholesterol; HDL, HDL-cholesterol; RCT, reverse cholesterol transport; RI, radioisotope; TC, total cholesterol; TG, triglyceride; \([^3]H\)C, \([^3]H\)cholesterol.

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function of HDL (10–12). Indeed, studies have reported that CAD events show a stronger inverse correlation with cholesterol efflux capacity (CEC) than with HDL-C levels (13–15).

CEC is typically measured using radioisotope (RI)-labeled cholesterol (16, 17). For instance, J774, Raw264, or differentiated THP-1 cells are labeled with [3H]cholesterol ([3H]C) and then incubated with serum HDL, and the radioactivity of [3H]C released by cells into the medium is measured. Although this method has been widely used in research laboratories, the use of RIIs presents a challenge in clinical settings. A fluorescence-based high-throughput method using BODIPY-cholesterol (BODIPY-C) has also been developed (18). However, it is unclear whether this can replace the RI method because BODIPY-conjugated and native cholesterols differ in their affinity for HDL and apolipoprotein.

Another alternative method for CEC measurement without RI uses stable isotope-labeled cholesterol (19–23). This method allows native and stable isotope-labeled cholesterol to be measured separately; moreover, it can be applied to molecules other than cholesterol, including phospholipids (24). However, it is not ideal for clinical applications because it involves cumbersome pretreatment steps, including the derivatization of cholesterol for MS analysis and hydroslysis to convert cholesterol esters to free cholesterol. In addition, it is necessary to validate the equivalence of the stable isotope and RI methods by comparing the efflux values of samples measured simultaneously by the two methods, which has not yet been reported.

In this study, we developed a new stable isotope method as an alternative to the conventional RI method. We used highly sensitive LC/quadrupole time-of-flight (QTOF)/MS in atmospheric pressure chemical ionization (APCI) mode to detect a very small amount of cholesterol in culture medium, thereby decreasing the coincubation time of cells with acceptors and simplifying the sample pretreatment process. To validate the established method, we compared CEC of apoB-depleted serum prepared from outpatients with our stable isotope method with that measured with the RI and fluorescence methods. The major component of lipoproteins in apoB-depleted serum is HDL, and thus, apoB-depleted serum is generally used as an acceptor in cholesterol efflux assays. We also evaluated the correlation between CEC values measured by each method and HDL-C or apoA-1 levels.

**Materials and methods**

**Reagents and materials**

J774 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). [25,26,26,26-2H4]Cholesterol ([25,26,26,26-2H4]C), and [25,26,26,26,27,27-2H7]cholesterol ([25,26,26,26,27,27-2H7]C) were from Kanto Kagaku Co. (Tokyo, Japan). BODIPY-C was from Avanti Polar Lipids (Alabaster, AL). [3H]Colesterol ([3H]C; native cholesterol used for calibration in the MS analysis), ACAT inhibitor (Sandoz 58-035), and 8-bromoadenosine cAMP were from Sigma-Aldrich (St. Louis, MO). ApoA-1 purified from human serum was from Alfa Aesar (Hecksham, UK).

**Subjects and preparation of specimens**

This study was approved by the Ethics Committee of the Jikei University School of Medicine. Serum samples were obtained from 22 males and 19 females aged 28–83 years who were outpatients at the Jikei University Kashiwa Hospital. The serum samples had normal total cholesterol (TC) levels of 183.5 ± 24.3 mg/dl (range: 135–217 mg/dl), LDL-C level of 101.1 ± 19.8 mg/dl (range: 68–138 mg/dl), HDL-C level of 61.7 ± 18.0 mg/dl (range: 36–94 mg/dl), and triglyceride (TG) level of 81.5 ± 33.0 mg/dl (range: 38–147 mg/dl). In addition to these specimens, serum samples were also obtained from nine healthy volunteers who were employees of Sekisui Medical Co. (Tokyo, Japan). Written informed consent was obtained from all volunteers at the time of enrollment in accordance with the code of ethics of Sekisui Medical Co. ApoB-depleted serum samples were prepared as previously described (25). Briefly, aliquots of the samples were treated with PEG6000 (Sigma-Aldrich) solution to precipitate apoB-containing lipoproteins by adding 40 parts PEG solution (20% PEG in 200 mM glycine buffer, pH 7.4) to 100 parts serum. After a 20 min incubation, the precipitate was removed by centrifugation. Serum apoA1 and apoB levels were measured to confirm that apoB was removed and apoA1 levels were unchanged by the treatment (data not shown).

Serum LDL-C, HDL-C, TC, and TG levels were measured using commercial assay kits (TC and LDL-C, Sekisui Medical Co.; HDL-C and TG, Kyowa Medics Co., Tokyo, Japan) with a biochemical autoanalyzer (TBA-200FR; Toshiba Medical Systems Corporation, Tokyo, Japan). Serum levels of apoA1 and apoB were measured using a commercial immunological agglutination assay kit (Sekisui Medical Co.) with a biochemical autoanalyzer (Model 7170; Hitachi Science Systems, Tokyo, Japan).

**Cholesterol efflux assay using stable isotope ([d7]C)**

J774 cells were seeded in 0.5 ml DMEM containing 10% FBS, 10 μg/ml [d7]C, and 2 μg/ml ACAT inhibitor in 24-well plates at a density of 300,000 cells/well for 24 h. After washing with D-PBS (pH 7.5 ± 0.1), the cells were equilibrated with 0.5 ml DMEM containing 0.2% BSA and 2 μg/ml ACAT inhibitor, with or without 0.3 mmol/l cAMP, for 18 h. The cells were then washed with D-PBS and incubated in 0.25 ml DMEM containing 0.2% BSA and apoB-depleted serum for 2–4 h.

**Hydroslysis and extraction of cholesterol**

The cell culture medium obtained from the cholesterol efflux assay using [d7]C was centrifuged, and the remaining cells were removed. Cell lysates were prepared by adding 1% sodium cholate solution to cells cultured without extracellular acceptors, followed by incubation for 1 h at room temperature. Next, 230 μl water-methanol (22:78; v/v) or 330 μl water-ethanol (10:90; v/v) were added to 30 μl of each culture medium and cell lysate sample. We prepared hydrolyzed culture medium and cell lysate samples, as well as the abovementioned nonhydrolyzed samples, by adding 8.9 mol/l potassium hydroxide instead of water, followed by incubation for 2 h at 50–60°C. Then, in the case of adding water (potassium hydroxide)-methanol (22:78; v/v), 360 μl chloroform containing [d7]C as an internal standard was added to each sample, while in the case of adding water (potassium hydroxide)-ethanol (10:90; v/v), 600 μl hexane containing [d7]C was added to each sample. After vortexing for 5–15 min, the samples were centrifuged, and aliquots of the chloroform or hexane phase were collected and dried under vacuum using a centrifugal evaporator.

**Measurement of cholesterol by MS**

The dried samples were redissolved in isopropanol, and a portion was injected into the LC/QTOF/MS instrument, which
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included a La Chorom (Hitachi Science Systems), ultra-high-resolution spectroscopy QTOF MS Maxis3G system (Bruker Daltonics, Billerica, MA), and CORTECS ultra-performance LC C18 column (1.6 μm; 2.1 × 50 mm) (Waters Corporation, Milford, MA). LC separation was performed at a flow rate of 0.3 ml/min (at a column temperature of 40°C) by an isotropic elution method using a solvent mixture of acetonitrile-methanol (4:1; v/v) as a mobile phase. MS detection was carried out in APCI mode. \( [d_7]C \), \( [d_{12}]C \), and \( [d_{13}]C \) were quantified from calibration curves generated using diluted cholesterol standards (10, 5, 2, and 1 ng/ml). An internal standard was used to correct for recovery efficiency of \( [d_0]C \) or \( [d_{12}]C \). Finally, the blank value (medium incubated without extracellular acceptors) was subtracted from each medium value, and CEC (percentage efflux) was calculated on the basis of this value divided by the value for cells incubated without acceptors using the following formula:

\[
\% \text{ Efflux} = \frac{\text{medium value of } [d_7]C - \text{blank value}}{\text{cell value of } [d_7]C - \text{blank value}}
\]

Cholesterol efflux assay using RI (\( { }^3\text{H} \text{C} \))

The cholesterol efflux assay was performed using \( { }^3\text{H} \text{C} \) as previously described (26). Briefly, J774 cells were seeded in DMEM containing 1% FBS, 2 μg/ml \( { }^3\text{H} \text{C} \), and 2 μg/ml ACAT inhibitor in 24-well plates for 24 h. After washing with D-PBS, the cells were equilibrated with DMEM containing 0.2% BSA, 2 μg/ml ACAT inhibitor, and 0.3 mM CAMP for 18 h. After this equilibration period, the cells were washed with D-PBS and incubated in DMEM containing 0.2% BSA and 2% apoB-depleted serum for 4 h. The radioactivity (dpm) of the medium was measured after adding liquid scintillator to each sample. Cell lysates were prepared by adding hexane-isopropanol solution (2:1) to cells cultured without extracellular acceptors, and the radioactivity of the cell lysates was also measured. Blank values (medium incubated without extracellular acceptors) were subtracted from each medium value, and CEC (percentage efflux) was calculated on the basis of this value divided by the value for cells incubated without acceptors as described above.

Cholesterol efflux assay using a fluorescent compound (BODIPY-C)

The cholesterol efflux assay was performed using BODIPY-C as previously described (18). Briefly, J774 cells were seeded in DMEM containing 10% FBS in 48-well plates for 24 h. After the preincubation procedure, the cells were incubated in DMEM containing 1% FBS, 0.2% BSA, 25 μM BODIPY-C, and 2 μg/ml ACAT inhibitor for 1 h. After washing with D-PBS, the cells were equilibrated with DMEM containing 0.2% BSA, 2 μg/ml ACAT inhibitor, and 0.3 mM CAMP for 18 h. They were then washed with D-PBS and incubated in DMEM containing 0.2% BSA and 2% apoB-depleted serum for 4 h. The fluorescence intensity of the medium was measured using a fluorescence microplate reader (Infinite F PLEX; Tecan, Männedorf, Switzerland). Cell lysates were prepared by adding 1% sodium cholate solution to cells cultured without extracellular acceptors, and the fluorescence intensity of cell lysates was also measured. The blank value (medium incubated without extracellular acceptors) was subtracted from each medium value, and CEC (percentage efflux) was calculated on the basis of this value divided by the value for cells incubated without acceptors as described above.

Statistical analysis

Statistical analyses were performed using Microsoft Excel. All values are expressed as means ± SDs. Differences between groups were assessed with Student’s paired t-test, and the interassay reproducibility was assessed using Student’s unpaired t-test. Relationships between parameters were evaluated using Pearson’s correlation coefficient. We used XLSTAT software (SARL™, Addinsoft, Paris, France) to evaluate correlations by Passing-Bablok regression analysis. For all analyses, \( P < 0.05 \) was considered statistically significant.

RESULTS

Measurement of cholesterol by MS

To evaluate CEC using stable isotopes, it is essential to separately determine the values for native cholesterol, the cholesterol used as an internal standard, and the cholesterol for cell labeling. \( [d_0]C \), \( [d_{12}]C \), and \( [d_{13}]C \) were measured under the LC/APCI/MS conditions described above. The mass-to-charge ratios (m/z) of \(([d_{12}]C + H - H_2O)^+\), \(([d_{13}]C + H - H_2O)^+\), and \(([d_{12}]C + H - H_2O)^+\) were 369.3520, 373.3767, and 376.3955, respectively (Fig. 1).

Analytical performance of CEC measurement method using stable isotope

We evaluated the analytical performance of our method, in which \( [d_0]C \) was used as an internal standard and \( [d_{12}]C \) was used for cell labeling. The relationship between the incubation time of cells with acceptors and percentage efflux was examined. A time-dependent increase in percentage efflux was observed with or without cAMP when apoB-depleted serum was the acceptor and with cAMP when apoA-I was the acceptor (supplemental Fig. S1A, B). We next examined the association between apoB-depleted serum concentration and percentage efflux. A concentration-dependent increase in percentage efflux was observed with or without cAMP (Fig. 2A, B). The percentage efflux was greater with the fluorescence than with the stable isotope and RI methods, with no difference between the latter two. The intraassay coefficient of variation of \( [d_{12}]C \) efflux from five consecutive measurements of three sets of samples obtained on the same plate was 10.6% to 18.8% before recovery correction and 7.3% to 9.5% after correcting with an internal standard (supplemental Table S1). The interassay reproducibility was determined by measuring three specimens in duplicate four times (days 1–4). The coefficient of variation was 4.1% to 8.5%, indicating good precision (supplemental Table S2).

Effect of hydrolysis treatment

To determine the levels of free and total cholesterol, including cholesterol esters, in culture medium and cells, \( [d_0]C \) and \( [d_{12}]C \) levels were quantified with and without hydrolysis treatment. The \( [d_{12}]C \) level was higher for hydrolyzed than for nonhydrolyzed samples in the medium, whereas no difference was observed for cells (Fig. 3A). There was also no significant difference between the \( [d_{12}]C \) of hydrolyzed and nonhydrolyzed samples either in the medium or in cells (Fig. 3A) or between the percentage efflux of hydrolyzed and nonhydrolyzed samples from 16 patients (\( r = 1.00x, r = 0.96, P < 0.0001 \)) (Fig. 3B). These results indicate that hydrolysis treatment had no effect on the measurement of \( [d_{12}]C \).
Correlation with RI and fluorescence methods

Correlations among percentage efflux values determined with the stable isotope, RI, and fluorescence methods were calculated using 41 patient specimens. When cAMP was added to the cells, $[^{13}C]C$ efflux showed a significant, positive correlation with $[^{3}H]C$ efflux ($r = 0.73$, $P < 0.0001$) (Fig. 4A). Although $[^{13}C]C$ efflux was also positively correlated with BODIPY-C efflux ($r = 0.47$, $P < 0.01$) (Fig. 4B), it was not as strong as that between $[^{13}C]C$ and $[^{3}H]C$ efflux. $[^{3}H]C$ efflux showed a stronger positive correlation with $[^{13}C]C$ efflux than with BODIPY-C efflux ($r = 0.55$, $P < 0.001$) (Fig. 4A, C). In the absence of cAMP, $[^{13}C]C$ efflux showed a stronger positive correlation with $[^{3}H]C$ efflux than with BODIPY-C efflux ($r = 0.52$, $P < 0.001$), whereas $[^{3}H]C$ efflux showed a stronger positive correlation with $[^{13}C]C$ than with BODIPY-C efflux ($r = 0.52$, $P < 0.001$), as observed upon the addition of cAMP (Fig. 4D–F). When evaluating each correlation in terms of the difference between percentage efflux in the presence and absence of cAMP, correlations were relatively weak, although significant, positive correlations were seen among each method ($[^{13}C]C$ with $[^{3}H]C$: $r = 0.45$, $P < 0.01$; $[^{13}C]C$ with BODIPY-C: $r = 0.39$, $P < 0.05$; and BODIPY-C with $[^{3}H]C$: $r = 0.53$, $P < 0.001$) (Fig. 4G–I). We subsequently performed Passing-Bablok regression analysis with respect to the relationship between RI and the stable isotope methods. In the presence of cAMP, the intercept of the regression line was $-2.318$ (95% CI: $-6.385$, 0.224), and the slope was 1.342 (95% CI: 1.058, 1.770) (supplemental Fig. S2A). In the absence of cAMP, the intercept of the regression line was $-6.270$ (95% CI: $-8.956$, $-4.128$), and the slope was 2.102 (95% CI: 1.753, 2.517), respectively (supplemental Fig. S2B).

Correlation with serum HDL-C/apoA-1

We investigated the correlations between the percentage efflux determined using the stable isotope, RI, or fluorescence methods and the serum HDL-C or apoA-1 levels. The efflux of $[^{13}C]C$ and $[^{3}H]C$ showed significant, positive correlations with the serum levels of HDL-C and apoA-1 ($[^{13}C]C$ with HDL-C: $r = 0.66$, $P < 0.0001$; $[^{13}C]C$ with apoA-1: $r = 0.66$, $P < 0.0001$; $[^{13}C]C$ with apoA-1:

![Fig. 1. Mass spectrometry and chromatograms of $[^{13}C]C$, $[^{3}H]C$, and $[^{3}H]C$.](image)

![Fig. 2. $[^{13}C]C$, $[^{3}H]C$, and BODIPY-C efflux into different concentrations of apoB-depleted serum.](image)
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$\text{r} = 0.65, P < 0.0001$; $[^{3}\text{H}]\text{C}$ with HDL-C: $\text{r} = 0.82, P < 0.0001$; and $[^{3}\text{H}]\text{C}$ with apo-A1: $\text{r} = 0.86, P < 0.0001$ (Fig. 5A, B, D, E). On the other hand, although BODIPY-C efflux was positively correlated with serum HDL-C ($\text{r} = 0.33, P < 0.05$) (Fig. 5C) and apoA-1 levels ($\text{r} = 0.41, P < 0.01$) (Fig. 5F), the correlation was weaker than that between $[d_{7}]\text{C}$ or $[^{3}\text{H}]\text{C}$ efflux and serum HDL-C or apoA-1 levels.

**DISCUSSION**

Cholesterol efflux from macrophages is thought to predict CAD events; as such, CEC measurement methods are becoming increasingly important (13–15). In the present study, we established a method for measuring CEC using a stable isotope and investigated whether it can replace the gold-standard RI method.
There are three issues to consider when establishing a stable isotope method for CEC measurement. The first is measurement precision. In stable isotope methods, cholesterols are ultimately quantified by MS; hence, a process is needed to extract cholesterols from lipoproteins before quantification. Recovery efficiency can be reduced during the process of mixing culture medium with organic solvents, separating and then recovering the organic layer, drying it under vacuum by centrifugal evaporation, redisolving it in organic solvent, and injecting it into the MS instrument, which can affect measurement precision. We added a fixed amount of $[^{14} \text{C}]$ to samples as an internal standard before extraction and corrected the measured value of $[^{7} \text{C}]$ on the basis of the recovery efficiency of $[^{4} \text{C}]$. This markedly improved the coefficient variation, yielding a precise measurement for $[^{7} \text{C}]$ (supplemental Table S1).

The second issue is the measurement of cholesterol esters. Free cholesterols present on cell membranes of peripheral tissues are extracted by HDL and apolipoproteins and are converted to cholesterol esters by LCAT (27). However, it remains unclear whether cholesterols extracted into the medium are converted to cholesterol esters when incubated with diluted apoB-depleted serum in an in vitro cholesterol efflux assay. Assuming that cholesterols extracted into the medium are partly converted to cholesterol esters, both free and ester-type cholesterols must be quantified and their sum must be calculated to determine the total cholesterol extracted from cells into the medium. With apoA-1 as the acceptor, most stable isotope-labeled cholesterols extracted into medium were free and only a very small fraction was esterified (21), but it is unclear whether the same would be true with apoB-depleted serum as the acceptor. In this study, we used medium samples prepared

**Fig. 5.** Correlations of percentage efflux of $[^{7} \text{C}]$, $[^{3} \text{H}]$, BODIPY-C with HDL-C and apoA-1 levels. After labeling with $[^{7} \text{C}]$, $[^{3} \text{H}]$, or BODIPY-C, J774 cells were equilibrated with cAMP for 18 h and then incubated with 2% apoB-depleted serum prepared from 41 patient specimens for 4 h. For $[^{7} \text{C}]$ efflux, after adding water-methanol (22:78; v/v) to the medium or cell lysate samples, chloroform containing $[^{4} \text{C}]$ was added to each sample, followed by vortexing and cholesterol extraction. The efflux of $[^{7} \text{C}]$, $[^{3} \text{H}]$, and BODIPY-C was measured as described in the Materials and Methods section, and correlations between each percentage efflux and HDL-C (●) or apoA-1 (○) levels were evaluated. Values are presented as mean values from duplicate wells.
after incubating cells with apoB-depleted serum to quantify total and free cholesterol with or without hydrolysis treatment, respectively, to detect cholesterol esters in the medium. The results showed that there was no difference in \([d_{6}]C\) levels between hydrolyzed and nonhydrolyzed samples (Fig. 3). \([d_{6}]C\) increased after hydrolysis treatment, indicating that cholesterol esters derived from HDL in apoB-depleted serum were hydrolyzed. We also confirmed that very little ester-type \([d_{6}]C\) was present in the medium after the cholesterol efflux, demonstrating that the cumbersome hydrolysis treatment is unnecessary when using our method.

Cholesterol efflux assays using stable isotopes have been described by two groups (supplemental Table S3). Brown et al. (21) quantified \([d_{6}]C\) loaded into \(774\) cells and extracted from cells into medium using apoA-I and HDL as acceptors. Wang et al. (23) also measured the efflux of stable isotope-labeled cholesterol; this is the only study using a stable isotope method in which apoB-depleted serum obtained from patients was the acceptor. However, their approach differed from the general RI method in several ways; for instance, there was no activation of ABCA1 (also known as cholesterol efflux regulatory protein), a higher concentration (5%) of apoB-depleted serum, and a longer coincubation time (8 h) with acceptors than in the RI method; moreover, efflux was calculated on the basis of the amount of change in cholesterol level in cells during incubation with acceptors. In contrast, we established a stable isotope method in which the experimental conditions were the same as those in the RI method in all respects, including bioreactor size, ABCA1 activation treatment, specimen type, serum concentration, acceptor coincubation time, and calculation of percentage efflux. A direct comparison of our established method with the RI method shows a similar association between apoB-depleted serum concentration and percentage efflux as well as a significant, positive correlation between the two methods using 41 patient specimens.

Furthermore, there was a difference between the slopes and intercepts obtained in the presence and absence of cAMP, supplementing the correlation between the two methods (Fig. 4A, D). Passing-Bablok regression analysis proved that the intercepts of the regression lines had a tendency to be negative, and the slopes of the regression lines were greater than 1, and this bias was particularly remarkable in the absence of cAMP (supplemental Fig. S2A, B). However, the linearity test showed that the \(P\) values were above the significance level (\(\alpha = 0.05\)), and hence, the possibility that the relationship between the two methods is linear cannot be rejected. In addition, Bland-Altman plots revealed that the difference in percentage efflux between the two methods was getting larger in accordance with the level of CEC, and such a tendency was greater in the absence of cAMP as well (supplemental Fig. S3A, B). To increase the sensitivity in MS analyses, we added a much larger amount of cholesterol to the cells in the stable isotope method than in the RI method, and this may have resulted in the increase in aqueous diffusion, which does not depend on ABCA1. The mechanisms of the differences between these two methods are still unclear, but it is highly possible that they are most likely due to the different amounts of cholesterol added to the cells given that \([d_{6}]\) and \([3H\]C are the same in their chemical structures and that the amount of cholesterol is the only difference in the cell culture conditions between the two methods. In this study, the amount of cholesterol was chosen to achieve enough sensitivity while retaining a good accuracy, and thus, using the same amount of cholesterol used in the RI method will affect the sensitivity and accuracy of our method. Indeed, similar to our method, Brown et al. and Wang et al. added a large amount of cholesterol to cells. Specifically, Brown et al. added 15 \(\mu g/ml\) of cholesterol labeled with a stable isotope (21), and Wang et al. added 20 \(\mu g/ml\) of cholesterol (23). Therefore, it is presently impossible to make all the experimental conditions of the stable isotope method the same as those of the RI method, and this is the major challenge of the stable isotope method.

On the other hand, some divergences were observed when evaluating the correlation between the stable isotope and the RI methods from the point of view of the difference between percentage efflux in the presence and absence of cAMP (Fig. 4G). This difference means ABCA1-specific efflux. In the stable isotope method, these differences were exactly or almost zero in the case of the serum specimens that caused the divergence between the two methods, and most of them had high HDL-C levels. Moreover, as mentioned above, the difference in percentage efflux between the two methods was directly proportional to the level of CEC, and the tendency was particularly evident in the absence of cAMP; higher CEC levels were observed with the stable isotope method than the RI method. Accordingly, the difference between percentage efflux in the presence and absence of cAMP was relatively small, particularly in the specimens of high CEC levels with the stable isotope method. There was no definite evidence that the aqueous diffusion described above was promoted and that cholesterol extraction via the ABCA1 pathway was inhibited in such specimens. Even though different amounts of cholesterol added to cells between the two methods may have resulted in the divergence, the mechanism is still unclear. Another determinant of the divergence can be an issue with ABCA1 induction by cAMP owing to various factors such as cell culture conditions. Generally, about 40% of total efflux is attributable to ABCA1; however, the majority of specimens showed a lower rate than 30% in our study. Moreover, the interday variability could be another determinant of the divergence because we performed each assay on different days. These examinations are preliminary studies, and hence, further studies are needed to clarify the causal relationship.

The fluorescence method for CEC measurement using BODIPY-C is useful for high-throughput analysis, can be carried out in different facilities, and does not involve the extraction procedure required for the stable isotope method (14, 28, 29). However, the affinity of BODIPY-C for cell membranes or acceptors is thought to differ from that of native cholesterol due to differences in structure. In fact, efflux of BODIPY-C was much higher than that of \([3H\]C
when cholesterol-labeled cells were incubated with apoB-depleted serum for 4 h (Fig. 2). Additionally, whereas [3H]C efflux showed relatively strong correlations with serum HDL-C and apoA-1 levels, BODIPY-C efflux was only weakly correlated (Fig. 5). Thus, CEC evaluated by the fluorescence method does not correspond well to the value determined by the RI method.

A variety of HDL subpopulations are present in the blood that differ in terms of size, apolipoprotein composition, and specific gravity; moreover, the CEC of macrophages depends on the HDL composition of subpopulations (11, 30). In contrast to the RI method, cholesterol efflux determined using BODIPY-C was highly correlated with pre-β1-HDL but not with serum HDL-C levels (18). Thus, BODIPY-C efflux has a larger proportion of ABCA1-mediated efflux to total efflux than does native cholesterol efflux. In addition, the correlations were relatively weak both in the presence and absence of cAMP between the fluorescence and the other two methods (Fig. 4B, C, E, F), in addition to the difference between percentage efflux in the presence and absence of cAMP (Fig. 4H, I). This result is probably because of the difference in the structure between BODIPY-C and native cholesterol and indicates the mechanism of BODIPY-C efflux may be different from other two methods regarding ABCA1-mediated pathway, including other pathways.

In summary, we established a new method for measuring cholesterol efflux using a stable isotope. This method does not involve cumbersome operations such as cholesterol derivatization and hydrolysis treatment and yields a precise measurement. It can be performed under almost the same cell culture conditions as the gold-standard RI method and shows a significant, positive correlation with it. Thus, the stable isotope method established in this study can be considered useful as a nonradioisotope method and expected to contribute to progress in research on HDL metabolism and functions. However, it is hardly possible to evaluate all the specimens in the same way as the RI method, for the reason that some discrepancies were observed between RI and the stable isotope methods. There seems to be a limitation to the establishment of a perfect alternative to the RI method because of the differences in characteristics between each method. Although several cholesterol efflux assays have also been developed in the past, slight differences, such as cell types and acceptor species, in each assay protocol may also lead to different results (31). Therefore, standardization needs to be considered, and it would be necessary to compare the assays in future clinical studies.

REFERENCES


