

Video Article

Cholesterol Efflux Assay

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Abstract

Cholesterol content of cells must be maintained within the very tight limits, too much or too little cholesterol in a cell results in disruption of cellular membranes, apoptosis and necrosis¹. Cells can source cholesterol from intracellular synthesis and from plasma lipoproteins, both sources are sufficient to fully satisfy cells' requirements for cholesterol. The processes of cholesterol synthesis and uptake are tightly regulated and deficiencies of cholesterol are rare². Excessive cholesterol is more common problem³. With the exception of hepatocytes and to some degree adrenocortical cells, cells are unable to degrade cholesterol. Cells have two options to reduce their cholesterol content: to convert cholesterol into cholesteryl esters, an option with limited capacity as overloading cells with cholesteryl esters is also toxic, and cholesterol efflux, an option with potentially unlimited capacity. Cholesterol efflux is a specific process that is regulated by a number of intracellular transporters, such as ATP binding cassette transporter proteins A1 (ABCA1) and G1 (ABCG1) and scavenger receptor type B1. The natural acceptor of cholesterol in plasma is high density lipoprotein (HDL) and apolipoprotein A-I.

The cholesterol efflux assay is designed to quantitate the rate of cholesterol efflux from cultured cells. It measures the capacity of cells to maintain cholesterol efflux and/or the capacity of plasma acceptors to accept cholesterol released from cells. The assay consists of the following steps. Step 1: labelling cellular cholesterol by adding labelled cholesterol to serum-containing medium and incubating with cells for 24-48 h. This step may be combined with loading of cells with cholesterol. Step 2: incubation of cells in serum-free medium to equilibrate labelled cholesterol among all intracellular cholesterol pools. This stage may be combined with activation of cellular cholesterol transporters. Step 3: incubation of cells with extracellular acceptor and quantitation of movement of labelled cholesterol from cells to the acceptor. If cholesterol precursors were used to label newly synthesized cholesterol, a fourth step, purification of cholesterol, may be required.

The assay delivers the following information: (i) how a particular treatment (a mutation, a knock-down, an overexpression or a treatment) affects the capacity of cell to efflux cholesterol and (ii) how the capacity of plasma acceptors to accept cholesterol is affected by a disease or a treatment. This method is often used in context of cardiovascular research, metabolic and neurodegenerative disorders, infectious and reproductive diseases.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3810/>

Protocol

1. Preparation of [³H]-cholesterol

1. In the fume hood, dispense the required amount of [³H]cholesterol into a 1.5 ml microfuge tube (0.5 μ Ci (19 kBq) per well is required for a typical assay).
2. If [³H]-cholesterol is suspended in toluene, dry it down completely with N₂ gas and resuspend with 100% ethanol to a final concentration of 1 μ Ci (37 kBq/ μ l). Vortex and mix well.

2. Plating Cells and Labelling Cellular Cholesterol

This protocol has been tested using the following cell types: human monocytes^{4,5}, THP-1 human monocyte-macrophages^{6,7,8}, RAW 264.7 murine macrophages^{5,9,10,11}, HeLa cells¹², human umbilical vein endothelial cells (HUVEC), BHK-21 cells⁹, human and mouse fibroblasts^{13,14}, HepG2 human hepatocarcinoma cells¹⁵ and, in modified form, from platelets¹⁶.

1. Resuspend cells and count them. Plate cells into 12-well plates at the final density of 0.2x10⁶ cells per well in 0.9 ml complete medium. Cells will continue growing and will reach 0.8x10⁶ cells per well by the time of the efflux experiment. For cells that do not divide, such as differentiated THP-1 cells, the seeding density should be 0.8x10⁶ cells per well. If using 6- or 24-well plates, double or half the number of cells per well respectively. The number of wells should be sufficient for quadruplicate determinations for each experimental condition.
2. If differentiation of THP-1 cells is required, add PMA (final concentration 0.1 μ g/ml) to the media for 48-72 h.
3. Add a small aliquot of media into the microfuge tube containing the [³H]cholesterol.
4. Wash sides of tube with media before adding [³H]cholesterol into a separate aliquot of complete media (100 μ l/well).
5. Add the media containing [³H]cholesterol to the wells with cells (final volume per well is 1 ml). If no treatment of cells is required before labelling, cells may be plated in [³H]cholesterol-containing medium.

6. Incubate cells for 48 hours in cell culture incubator (37 °C, 5% CO₂).

3. Equilibration Incubation

1. After 48 hour incubation, check cells under microscope. Ensure they are healthy and are at approximately 80% confluence.
2. Remove media containing [³H]cholesterol. Wash cells gently with PBS. Repeat 3 washing times.
3. Prepare serum-free media. If required, activate cells by adding LXR agonist (such as TO-901317 at 1-4 μMol/L) or cAMP (0.3 mMol/L; for cells of mouse origin only) to serum free-media.
4. Add 500 μl serum free media to each well.
5. Incubate for 18 hours in the cell culture incubator (37 °C, 5% CO₂).

4. Cholesterol Efflux Incubation

1. After 18 hour incubation in serum-free medium, check cells under microscope. Ensure they are healthy and confluent (minimum 80% confluency).
2. Prepare solution of cholesterol efflux acceptors in serum-free medium. Examples of acceptors include :
 - Apolipoprotein A-I (apoA-I) (final concentration 10 μg/ml)
 - High density lipoprotein (HDL)(final concentration 20 μg/ml)
 - Cyclodextrin (final concentration 200 μg/ml)
 - Plasma (final concentration 1-2%)
3. Wash cells gently with PBS.
4. Add 250 μl of serum-free media with acceptors to each well.
5. Leave one set of wells to determine background efflux (efflux to serum free media with NO acceptor)
6. Incubate cells for 2 hours in cell culture incubator (37 °C, 5% CO₂). Duration of the efflux incubation may vary from 30 min to 8 h if required.

5. Processing Samples

1. After 2 hours incubation check cells under microscope.
2. Collect media into 1.5 ml microfuge tubes. Spin at 14,000 rpm for 1-10 min at room temperature to remove cellular debris.
3. Transfer 100 μl media into 7 ml scintillation vial. Add 5 mls of Insta-gel Plus (PerkinElmer) and vortex mixture. Store remaining samples at 4 °C.
4. Place plates in freezer for 30 mins. Add 500 μl dH₂O to each well. Check cells under microscope to ensure that all cells have lifted from the bottom of wells. If still adherent, either leave plates with dH₂O at 4 °C overnight or scrape wells.
5. Once all cells have lifted off, pipette up and down to break up cell clumps. Transfer 100 μl into 7 ml scintillation vials. Add 5 mls of Insta-gel Plus (PerkinElmer) and vortex mixture. Store remaining plates at 4 °C.
6. Place scintillation vials on scintillation counter. Configure counter to count for [³H] in dpm units.

6. Analysing the Results

1. The rate of cholesterol efflux is usually expressed as a proportion of cholesterol moved from cells to the acceptor. The following formula is used:

$$\% \text{ Cholesterol Efflux} = \frac{(\text{media counts} \times \text{dilution factor})}{[(\text{media counts} \times \text{dilution factor}) + (\text{cell counts} \times \text{dilution factor})]} \times 100\%$$

2. The specific efflux is calculated as a difference between the efflux in the presence or absence of the acceptor (blank).

$$\% \text{ Final efflux} = \% \text{ Cholesterol Efflux} - \% \text{ Blank Efflux}$$

7. Timeline



8. Representative Results

An example of an outcome of a cholesterol efflux experiment is shown in Fig. 1. In this experiment THP-1 human monocytes were differentiated into macrophages and cholesterol efflux to different acceptors was tested. Cholesterol efflux to medium with no acceptors ("blank") was 0.79% and this value was regarded as a non-specific efflux and was subtracted from other values. Cholesterol efflux to human apoA-I (final concentration 30 μg/ml) was 4.75%. A reference plasma sample was included into this experiment to monitor inter-experimental variability. A reference sample can be used for normalization of data across a large number of experiments, but we found it prudent to repeat the assay if

variability is high. A patient plasma (final concentration 2%) was tested before and after the patient was treated with medication. It was concluded that in this patient medication had a negative impact on the capacity of plasma to support cholesterol efflux.

Another example of an outcome of the efflux experiment is shown in Fig. 2. In this experiment RAW 264.7 macrophages were activated or not activated by overnight incubation with LXR agonist TO-901317 (final concentration 1 $\mu\text{mol/L}$) and cholesterol efflux to the same sample of human plasma (2%) was tested. It was concluded that activation of cellular expression of ABC transporters with LXR agonist increases the capacity of cells to release cholesterol to extracellular acceptor.

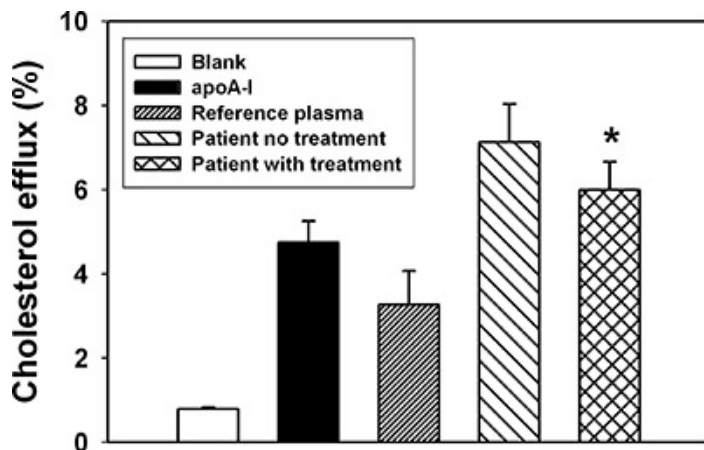


Figure 1. Cholesterol efflux from THP-1 cells to various acceptors. Percentage of cholesterol efflux (i.e. the proportion of labelled cholesterol moved from cells to the specified acceptor) is shown after subtraction of blank values. Mean \pm SD of quadruplicate determinations, * $p < 0.05$.

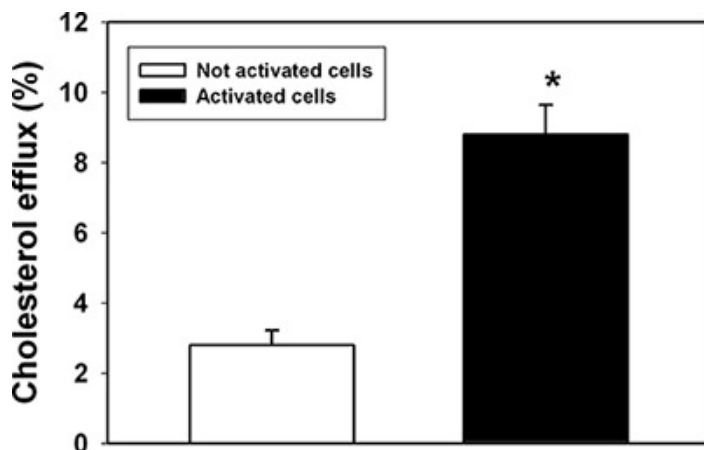


Figure 2. Cholesterol efflux from RAW 264.7 cells activated or not activated with LXR agonist to human plasma. Percentage of cholesterol efflux (i.e. the proportion of labelled cholesterol moved from cells to the specified acceptor) is shown after subtraction of blank values. Mean \pm SD of quadruplicate determinations, * $p < 0.001$.

Discussion

The described method to measure cholesterol efflux is designed to measure movement of cholesterol from cells to an extracellular cholesterol acceptor. There are several critical considerations in understanding this methodology.

Labelling and equilibration

In the described methodology labelled cholesterol is added to serum-containing serum. Although never investigated in detail, it is assumed that cholesterol is incorporated into serum lipoproteins and they are taken up by cells. It is important to allow sufficient time for lipoproteins to be taken up and for cholesterol to move from lipoproteins to cellular membranes. Usually 24 h labelling is sufficient, but labelling for 48 h achieves higher specific activity of intracellular cholesterol. It needs to be taken into account that if [^{14}C]cholesterol is used instead of [^3H]cholesterol (e.g. in double labelling experiments), the specific activity of the former is very low and adding enough labelled cholesterol to achieve intended specific activity may change cell cholesterol content and thus influence the outcome. It is also important that (i) there is no labelled cholesterol or lipoproteins non-specifically trapped on the cell surface as this cholesterol will be rapidly incorporated by an acceptor via non-specific transfer; and (ii) labelled cholesterol is equilibrated between various intracellular pools. Although in our experience 24 h equilibration incubation in serum-free medium is sufficient to achieve these goals, usually 48 h incubation is used. Many cell types do not survive for 48 h or even 24 h in serum-free medium. In this case 0.1% BSA (essentially fatty-acid free) can act as substitute. Lipoprotein-depleted serum or various serum-replacement supplements can be used as a last resort, but it is important to recognize that lipoprotein-deficient serum does and serum-replacement supplements may contain apolipoproteins and that may affect the outcome. Unfortunately manufacturers often do not disclose the composition of the supplements.

Duration of the efflux incubation

Cholesterol efflux leads to loading of cholesterol to an extracellular acceptor and depletion of cholesterol in cells. Changes in cholesterol content of both cells and acceptor may affect the rate and other properties of cholesterol efflux. Furthermore, if the acceptor contains cholesterol (e.g.

HDL) it may move from acceptor to cells and if specific activity of cholesterol in the acceptor becomes significant, that will complicate the interpretation of the results. Thus, it is best to keep efflux incubation as short as possible, allowing however time for sufficient amount of labelled cholesterol to move from cells to the acceptor to enable a reliable detection. Generally 2-6 h is the recommended time. Incubations longer than 24 h would reflect a state of equilibrium and therefore will be indicative of the capacity of acceptor to accumulate cholesterol rather than the rate of cholesterol efflux.

Acceptor

Different acceptors are specific for different pathways of cholesterol efflux. For measuring specific cholesterol efflux, apoA-I is used to reflect ABCA1-dependent efflux whilst HDL or reconstituted HDL for ABCG1 and SR-B1-dependent pathways. Cyclodextrin is often used to assess non-specific efflux. It is important to include in the experiment a "blank" sample, which contains no acceptor at all, to measure the contribution of non-specific dissociation of cholesterol and cholesterol released from disintegrating dead cells.

Concentration of the acceptor

Cholesterol efflux includes (i) release of cholesterol by cells and (ii) its uptake by an acceptor, both steps can be rate-limiting. If the experimental goal is to investigate changes in the capacity of cells to release cholesterol (e.g. after transfection or knock-down), then the concentration of the acceptor should not be rate limiting. For apoA-I and HDL the concentrations of 30-50 µg/ml are usually non rate-limiting. If the capacity of the acceptor to support cholesterol efflux is the goal of investigation (e.g. capacity of isolated HDL or plasma samples from patients or animals to support cholesterol efflux), then the concentration of the acceptor should be rate-limiting. For apoA-I and HDL this is usually 10 µg/ml, for plasma samples this is usually 1-2%, but a preliminary dose-dependence experiment to find a correct concentration is essential to choose the dose.

Activation of cells

The key elements responsible for specific cholesterol efflux are two ABC transporters, ABCA1 and ABCG1. Both are regulated by LXR and pre-incubation of cells with LXR agonist (most popular compound is TO-901317 available from Sigma) used at concentrations 1-4 µmol/L significantly increases the proportion of specific cholesterol efflux. Cells of mouse origin, such as RAW 264.7 or J774, can also be activated by cAMP (0.3 mMol/L).

Loading of cells with cholesterol

Loading of cells with cholesterol by pre-incubating them with acetylated or oxidized LDL or cyclodextrin-cholesterol complex also stimulates cholesterol efflux. It needs to be kept in mind that loading cells, especially macrophages with cholesterol changes many aspects of their metabolism. Choice of model (cholesterol loaded versus not cholesterol loaded) depends on scientific goal of the study.

Presentation of the results

Usually the results of cholesterol efflux experiment are presented as a percentage of cholesterol released from cells: this eliminates variability from cell number in individual wells and in efficiency of labelling. However, this is only valid if treatment did not affect cholesterol uptake and the efflux in the absence of the acceptor (blank). These two conditions need to be experimentally proven.

Theoretically this method can be used to study efflux of any cellular constituency to an extracellular acceptor. If, unlike cholesterol, such compound is metabolised, then an analytical purification step needs to be included. Further, if the compound is synthesized in the cell, synthetic precursors can be used for labelling. For example, [¹⁴C]acetate and [³H]water were used to study the efflux of newly synthesized cholesterol and [¹⁴C]cholate for studying the efflux of phospholipids. In this case newly synthesized cholesterol needs to be separated from precursors and intermediates, usually by thin layer chromatography.

Disclosures

The authors declare no conflicting interests related to this study.

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