AN EFFICIENT METHOD FOR MODULATION OF CHOLESTEROL LEVEL IN CELL MEMBRANES

M. SHINITZKY

Department of Membrane Research, The Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

The fluidity of lipid domains can markedly affect the lateral and rotational diffusion [1], as well as the degree of exposure [2–4], of membrane proteins. It is therefore believed that some of the control mechanisms of membrane functions involve the lipid fluidity. Under physiological conditions modulation of the fluidity of the membrane lipid domains is achieved by changes in the mole ratio of cholesterol to phospholipid (C/PL) [5,6], changes in the mole ratio of lecithin to sphingomyelin (L/S) [6,7], or changes in the overall degree of unsaturation of the phospholipid acyl chains.

One of the main mechanisms of changing the C/PL in cell membranes is the passive partitioning of cholesterol between the serum lipoproteins and the cell plasma membrane. Under normal physiological conditions the C/PL levels in the serum and the cell membrane are at an equilibrium which is enforced by a steady-state of cholesterol exchange between these reservoirs [8]. However, alterations of the C/PL of the ambient serum will lead to translocation of cholesterol towards a new equilibrium state which will eventually alter the C/PL of the cell membrane. This mechanism is considered to be the main passive physiological process which modulates membrane fluidity in animal tissues.

Essentially, all the available in vitro techniques for modulation of cellular cholesterol involve treatments with liposomes of abnormally high C/PL for cholesterol enrichment or liposomes free of cholesterol for cholesterol depletion. In most of these procedures the treated cells are incubated in the liposomes in the presence of serum for 12–36 h. The methods using liposomes have been extensively used in studies of blood cells, mostly erythrocytes [5,9], platelets [10] and lymphocytes [11]. Unfortunately, the use of liposomes in tissue culture of most growing cells bears some undesired effects caused by non-specific adsorption and fusion of the liposomes with the cell membrane [12,13] which in the extreme cases may lead to the cell death.

In the following we present a simple and efficient method for cholesterol enrichment or depletion of cell membranes which is especially pertinent to cells in tissue culture. The effectiveness of the method is demonstrated in human red blood cells and in mouse spleen cells.

2. Materials and methods

Cholesterol (CH-S) and egg-lecithin in chloroform–methanol were obtained from Sigma. The lecithin solution was brought to complete dryness under argon before use. Tetrahydrofuran was purchased from Fisher Chemicals. Sera and media for tissue culture were obtained from local sources.

Freshly drawn human blood was obtained from a normal donor. The red blood cells were separated by centrifugation and washed 3 times with phosphate buffered saline (PBS). The removed plasma was incubated at 56°C for 30 min and the heat-inactivated serum was separated by centrifugation and was used in the treating media. Mouse spleen cells were obtained from a 10 weeks old BALB/c mouse.

The methods for cholesterol enrichment or deple-

tion, which are described in the following, are based on introduction of finely dispersed cholesterol or egg lecithin into serum–medium mixtures. The outlined procedures gave optimal results with the various tested cells, though variations in quantities and conditions may be applied for each specific case.

2.1. *Serum–medium mixtures*

Mixtures of 10% heat-inactivated serum in medium supplemented with antibiotics, which are routinely used in tissue culture, are used in the methods for modulating the contents of membrane cholesterol. For most cells a mixture of heat inactivated fetal calf serum in Eagle's medium is appropriate. In treatments of low metabolic activity systems (erythrocytes, platelets, fixed cells and isolated membranes) a mixture of heat inactivated human serum in PBS containing 2 mg/ml glucose is adequate.

2.2. *Lipid solvent*

A mixture of 5:1 (v/v) tetrahydrofuran and 0.6% aqueous KCl ('THF') is used as a dispersing solvent for cholesterol or egg lecithin [14].

2.3. *Cholesterol-enriching medium*

One volume of solution of 1–1.5 mg/ml cholesterol in THF is introduced into 10 vol. vigorously stirred serum–medium mixture in a lyophilization vessel (practically 10 ml THF solution into 100 ml serum–medium). When applying to mixtures containing human serum the cholesterol in THF can be increased to 2 mg/ml. The mixture is freezed and then lyophilized to complete dryness. The dried material is weighed and standardized for reconstitution. The cholesterol-enriching medium is prepared before each treatment by dissolving the lyophilized material in the proper volume of sterile distilled water.

2.4. *Cholesterol-depleting medium*

A solution of 2–3 mg/ml egg lecithin in THF is mixed with the serum–medium and processed identically to the previous procedure. When human serum is used the lecithin in THF can be increased to 5 mg/ml.

2.5. *Control medium*

One volume of free THF is mixed with 10 vol. serum–medium and the mixture is processed as the above two media.

2.6. *The DMSO modification*

Dimethyl sulfoxide (DMSO) is well tolerated by cells at concentrations below 2%. Cholesterol, 10–15 mg, is dissolved in 1 ml hot DMSO ($\sim$ 80°C. The solution is diluted 1:100 (v/v) while hot by squirting into the vigorously stirred serum–medium. The obtained mixture is used as such for cholesterol enrichment. For cholesterol depletion 20–30 mg egg lecithin are dissolved in hot DMSO and diluted 1:100 (v/v) analogously with serum–medium. The control mixture is of 1% DMSO in the serum–medium mixture.

2.7. *Cholesterol modulation assays*

Red blood cells are incubated in the reconstituted medium at a concentration of 1 ml packed cells/10–25 ml medium. The incubation is carried out at 37°C with shaking in loosely covered vials for up to 36 h. The treated cells are washed 3 times with PBS and are processed for membrane preparation [15] and lipid analysis. In this report free cholesterol was analysed by the $FeCl_3$ method [16] and the phospholipids were determined by organic phosphorus analysis [17].

Treatments of cells in tissue culture are carried out analogously to growth conditions (10^6–10^7 cells/ml). The C/PL can be estimated indirectly by measuring the microviscosity of the cell membrane [6] or by monitoring a certain membrane function.

3. Results and discussion

Cholesterol acquisition by cell membranes occurs when the serum is abnormally rich in unesterified cholesterol. Such a conditions prevails in cirrhosis where the level of free cholesterol is 2–3-fold greater than normal while all other lipid components remain in the normal range [18]. The cirrhotic serum indicates that the capacity of normal sera for free cholesterol may be several-fold greater than the amount present. Since the concentration of free cholesterol in most normal sera is around 0.3–1 mg/ml it may be expected that a normal serum could accomodate additional cholesterol of up to about 2 mg/ml. This assumption furnished the basis for the described method.

The incorporation of the added cholesterol into the serum lipoproteins could be assessed by the trans-

parency of the reconstituted enriched serum–medium (see section 2). After reconstitution the cholesterol enriching or depleting media exhibit only slight translucence as compared with the control medium. The turbidity was estimated by measuring $A_{650\,nm}$ against the control medium. The use of THF–aqueous KCl [14] as a dispersing solvent under the conditions described in sections 2.3 and 2.4 gave optimal results according to the turbidity criteria. In addition, no precipitation could be detected after centrifugation of the reconstituted media at 30 000 × g for 1 h. It is therefore reasonable to assume that before lyophilization the dispersed cholesterol or lecithin were mostly in monomeric or oligomeric forms which during the slow process of freeze-drying integrated into the serum lipoproteins. However, no other evidence as for the location and distribution of the added cholesterol or lecithin is yet available.

Most studies on modulation of membrane cholesterol were carried out with human erythrocytes [3–5,9]. Figure 1 presents the results of a series of treatments of human erythrocytes by the described method. Both the phospholipid and the protein contents per cell remained virtually unaltered throughout the treatments, and the observed changes in C/PL were exclusively due to changes in the absolute amount of cholesterol per cell. Similar results were obtained [5,9] for treatments of erythrocytes with liposomes. However, the total lipid concentration required for the treatments with liposomes is over 10-fold greater than in the present method.

Superficial screening of a series of normal and transformed cell lines has indicated that the described media did not cause any significant alterations in cell viability in treatments which lasted less than 24 h. Because of technical difficulties, we have not isolated the membranes of the treated cells for determination of C/PL, and the efficiency of the method was inferred indirectly by parameters like morphology, agglutination and membrane microviscosity [6,11]. In lymphocytes determination of microviscosity ($\bar{\eta}$) by fluorescence polarization of 1,6-diphenyl 1,3,5-hexatriene (DPH) is believed to relate almost exclusively to the plasma membrane [6,11]. Figure 2 describes the change in the degree of fluorescence polarization (P) of mouse spleen cells labelled with

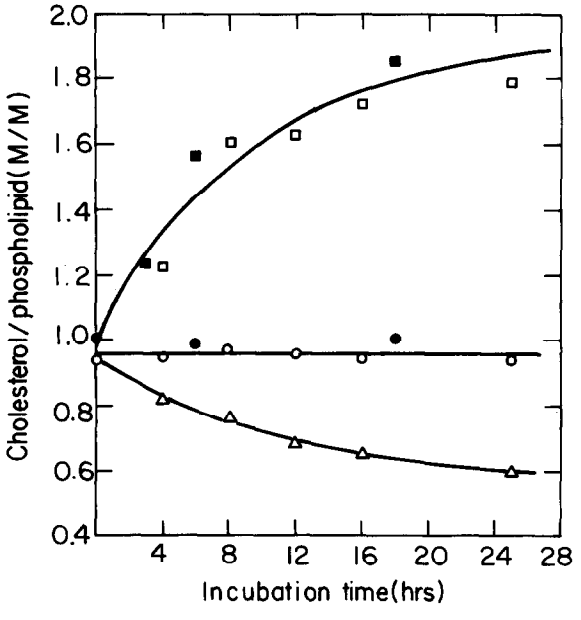

Fig.1. The change in cholesterol to phospholipid mole ratio of human erythrocytes upon incubation with the cholesterol-enriching (□), the cholesterol-depleting (△) and the control media (○). The filled marks present experimental data obtained independently by Dr S. Ip from the University of Pennsylvania.

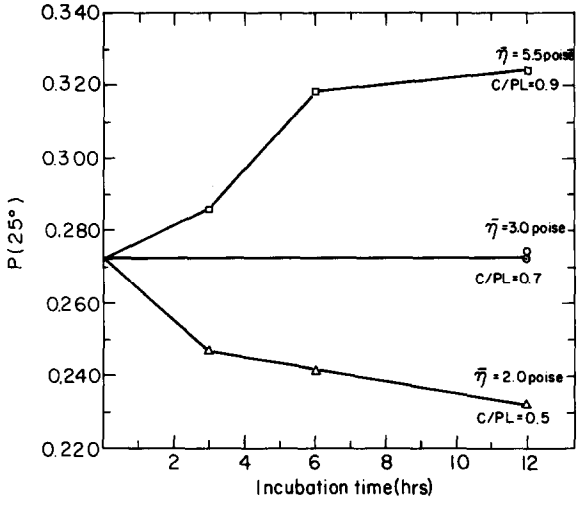

Fig.2. The change in the degree of fluorescence polarization (P) of mouse spleen cells labelled with DPH after incubation with the cholesterol enriching (□), the cholesterol depleting (△) and the control media (○). The figure also presents the estimated $\bar{\eta}$ and C/PL values after 12 h incubation.

DPH after treatments with the cholesterol modulating media. The figure also presents the estimated values of $\bar{\eta}$ and C/PL after 12 h treatment.

The described method offers important advantages over the commonly used methods for cholesterol modulation. It is carried out under conditions which are close to physiological, and it is highly efficient and reproducible. In principle, this method could be also applied for enrichment with other lipophilic materials like steroids, fatty acids, carcinogens and drugs.

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