Transcellular Transport of Low Density Lipoprotein through Cultured Newborn Rat Skin Epidermal Cell Monolayer

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Epidermal cells from newborn rat skin were cultured on type IV collagen-coated Millipore filter, and the transport of low density lipoprotein (LDL) labeled with Rhodamine B isothiocyanate (RB-LDL) through the cultured cell layer was examined. The transport of RB-LDL was dependent on temperature and biological energy. The transport was low at 17°C, but above 20°C, it became high with increase in temperature up to 37°C. The transport was markedly inhibited by the energy inhibitors 2-deoxyglucose and NaN3. Furthermore, the transport was saturable at the RB-LDL concentration of about 300 μg/ml and the activation energy of the transport was determined as 104.6 kJ/mol. No degradation product of LDL (apolipoprotein B) was observed during LDL transport through the cultured cell layer. The transport of RB-LDL through skin epidermal cells in culture is suggested to be mediated by transcytotic vesicles, but not by endocytosis and exocytosis via the lysosomal system, nor through cellular junctions.

Keywords: skin; epidermal cell; cell culture; lipoprotein transport

Introduction

In skin dermis fibroblasts, low density lipoprotein (LDL) binds to its receptor, which is localized in specialized regions of plasma membrane called coated pits.1) On binding with LDL, the receptor rapidly invaginates into the cell and is pinched off to form coated endocytic vesicles, in which the LDL bound to its receptor is involved. The coated vesicles migrate through the cytoplasm until they are fused into cellular lysosomes. In lysosomes, the proteins in LDL are hydrolyzed to amino acids, and the cholesterol esters in the core of LDL are also hydrolyzed and the liberated cholesterol is used to form cell membranes.2) This transport is important for lipid metabolism and accumulation concerning with lipoproteins such as xanthomatisos.3)

Transport of LDL in the cultured cell system is of interest. Cultured porcine hepatocytes contain the LDL binding site that mediates uptake and degradation of LDL.4) LDL is reported to be transported through cultured porcine endothelial cell monolayer via transcytotic vesicles in temperature and energy dependent ways, but not through cellular junctions nor by endocytosis and exocytosis via the lysosomal system.5) Although the transport of LDL was examined in several cultured cells,6,5) it had not been studied with skin epidermal cells.

Skin epidermal cells have been cultured by various methods: on type I collagen film, on extracellular matrices and with 3T3 cells.6) Recently we developed the method for obtaining a confluent monolayer of skin epidermal cells cultured on a type IV collagen-coated Millipore filter.7) Although the viability of cells cultured on type I collagen film was less than that on type IV collagen film, it was improved by addition of ascorbic acid in culture medium to the level of a type IV collagen.7) The cultured cells on type IV collagen film, and those on type I collagen in the presence of ascorbic acid were found to cover confluentely the whole surface of the Millipore filter coated with collagen film, and bioactive compounds such as nitrophensols permeated through the cell monolayer according to their physicochemical properties.8) In this study we examined the transcellular transport of LDL using a cultured epidermal cell monolayer.

Materials and Methods

Reagents Sources of the reagents used in this study were as follows: Eagle’s minimum essential medium (MEM), Flow Laboratories, McLean, Scotland; Fetal bovine serum (FBS), Whittaker M.A. Bioproducts, Inc. Walkersville, MD, U.S.A.; dispase, Godo-Shusei Co., Tokyo, Japan; Millicell-CM (12-mm diameter, pore size, 0.4 μm), Millipore Products Co., Bedford, MA, U.S.A.; anti-type IV collagen serum (rabbit), Medac Co., Hamburg, Germany, human plasma LDL, Chemicon Inc. Temecula, CA, U.S.A.; and Rhodamine B isothiocyanate, Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation and Culture of Epidermal Cells Cell culture was performed as described previously.9) The skin epidermis removed from 3-d old Wistar rats was digested with dispase (1000 u/ml), and washed with Ca2+ - and Mg2+-free phosphate buffered saline (PBS) and incubated in 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution. The epidermal cells were suspended in Eagle’s MEM buffered at pH 7.2 with 25.3 mm sodium bicarbonate and 20 mm Hepes, supplemented with 10% FBS. A volume of 500 μl of this suspension was seeded on type IV collagen-coated Millipore filter at a initial density of 1.2 × 106 cells per well and cells were incubated at 37°C for 20 h in a CO2 incubator.

Coating of Millipore Filter with Collagen Type IV collagen was obtained from bovine kidney cortex as reported previously.10) The purity of collagen, judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting with anti-type IV collagen serum,11) was more than 99%. Type IV collagen dissolved in 0.1 M acetic acid at 3 mg/ml was diluted with ethanol to a final concentration of 0.016% (w/v). The solution was applied to 12-mm Millipore filters (300 μl/well), and the filters were dried at room temperature under ultraviolet (UV)-irradiation.

Labeling of LDL LDL was labeled with Rhodamine B isothiocyanate (RB) as reported previously.12) The LDL solubilized in 0.5 M NaHCO3 buffer (pH 9.3) at 1 mg protein/ml was mixed with 10 mg/ml of RB in dimethylsulfoxide in a ratio of 5:1. The mixture was kept at 4°C for 24 h, and then applied to a Sephadex G-50 column to elminate free RB. The number of RB incorporated to LDL molecules was determined from the optical absorbance according to the relation of A495nm = 80000 M-1 cm-1.12) Protein contents of LDL was determined by the method of Lowry et al.13)

Transport of RB-LDL Transport of RB-LDL was examined with a permeation chamber as described previously.7) Amount of transported RB-LDL through the cultured cell layer was monitored by fluorescence intensity of Rhodamine B at 578 nm excited at 553 nm in a Hitachi Fluorescence Spectrophotometer, model 650-60. We used the serum and phenol red depleted MEM in examination of the transport.

Detection of Approtein by Fluorometry An aliquot of the solution in the receiver in the permeation chamber after transport study of RB-LDL was concentrated up to about 250 fold with a Millipore molec II (Millipore Ltd., Bedford, U.S.A.). The concentrated solution was subjected to SDS-PAGE, and RB-LDL was detected from the fluorescence of RB-LDL with a Transilluminator, model TS-20 (Ultraviolet Inc., Tokyo, Japan).

Results

Effects of Temperature and Inhibitors of Energy-Transport on Transport of RB-LDL Figure 1 shows rat epi-
dermal cells cultured for 20 h on type IV collagen-coated Millipore filter. Cells covered confluently the whole surface of the Millipore filter coated with type IV collagen. The 500 μl RB-LDL solution at 100 μg protein/ml in MEM was applied to the cultured epidermal cell monolayer, and amount of the permeated RB-LDL to the receiver cell that contained 50 ml of MEM was determined. RB-LDL passed freely through a type IV collagen-coated Millipore filter without cell monolayer (cf. Fig. 2A, closed squares). The transport of RB-LDL with time was hyperbolic at 10°C (open triangles), 25°C (open squares) and 37°C (open circles), as shown in Fig. 2. The permeation was greater with increase in temperature. After 3 h, amounts of the transported RB-LDL at 10°C, 25°C and 37°C were 0.92, 4.24 and 8.88 μg protein/cm², respectively, in the ratio of 1.0:4.6:9.7. When the temperature was decreased to 10°C from 37°C at the time indicated by the arrowhead in Fig. 2, the transport was decreased greatly (closed circles). Furthermore, transport of RB-LDL at 37°C was markedly inhibited by the inhibitors of energy-transduction 2-deoxyglucose (50 mm) and NaN₄ (10 mm) (Fig. 2A, closed triangles). These results indicate that the transport of RB-LDL through the cell layer was temperature and energy dependent.

As shown in Fig. 2B, the transport of RB-LDL obeyed first-order rate kinetics and the apparent rate constants $k_{temp}$ (the subscript indicates the experimental temperature) were determined as $k_{10} = 3.1 \times 10^{-3}$ h⁻¹, $k_{25} = 2.2 \times 10^{-2}$ h⁻¹, and $k_{37} = 5.1 \times 10^{-2}$ h⁻¹. When the temperature was decreased from 37°C to 10°C, $k_{37}$ became almost the same as $k_{10}$. These values are summarized in Table I.

To know the dependence of RB-LDL transport on the temperature more precisely, we next determined the amount of RB-LDL that had been transported in 3 h over a wide range of temperature, and the results are shown in Fig. 3A. Transport of RB-LDL was very low, below 17°C, but it was increased greatly above 20°C. The transport seemed to reach a plateau at a temperature slightly higher than 40°C. The Arrhenius-plot was sigmoidal, as shown in Fig. 3B, and activation energy of the transport was determined as 104.6 kJ/mol, from the straight portion of the curve.

### Table I. Rate Constants of RB-LDL Transport across Rat Skin Epidermal Cell Layer at Various Temperatures

<table>
<thead>
<tr>
<th>$k_{temp}$ (h⁻¹)</th>
<th>$k_{temp}/k_{10}$</th>
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<tbody>
<tr>
<td>$k_{10}$</td>
<td>$3.1 \times 10^{-3}$</td>
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<tr>
<td>$k_{25}$</td>
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<td>$k_{37}$</td>
<td>$5.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>$k_{37-10}$</td>
<td>$3.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>$k_{37}$ (inhibitor)</td>
<td>$1.0 \times 10^{-2}$</td>
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The apparent rate constant $k_{temp}$ was determined from the results in Fig. 2. 

a) Rate constant by changing the temperature from 37°C to 10°C. b) Transport at 37°C in the presence of 2-deoxyglucose and NaN₄.
Figure 4. Concentration Dependence of RB-LDL Transport at 37°C (A) and Double-Reciprocal Plot of the Transport (B).

C: Concentration (μg protein/ml) of RB-LDL in the donor phase. v: Rate of RB-LDL transport (μg protein/ml/h). Results are means (± S.E.) for three determinations.

Figure 5. SDS-PAGE Analysis of Transformed RB-LDL

1 and 2: Marker proteins for molecular mass stained by Coomassie brilliant blue R-250 and their fluorogram, respectively. 3 and 4: Fluorograms of RB-LDL transported at 37°C and 10°C, respectively. 5: Fluorogram of standard RB-LDL. Arrowhead indicates the apoprotein B (about 250kDa), and arrow indicates top of the electrophoresis.

Effect of RB-LDL Concentration

Next, we examined the effect of RB-LDL concentration on its transport at 37°C. The rate of RB-LDL transport (v) was increased with increase in the RB-LDL concentration (C) in a hyperbolic way, reaching a plateau at about 300μg protein/ml (Fig. 4A). The double-reciprocal plots gave a linear relationship between 1/v and 1/C, and values of $K_m$ and $V_{max}$ were determined as 76.9μg protein/ml and 0.56μg/ml/h, respectively (Fig. 4B).

Detection of Apoprotein by Fluorogram

It would be very important to know whether LDL is degraded or not by transport through the cultured epidermal cell layer. We performed SDS-PAGE of the transported LDL, and fluorescence of the labeled Rhodamine B was monitored. As shown in Fig. 5, a fluorescent band at 250kDa was detected for the transported protein at 37°C. This molecular mass was exactly the same as that of apoprotein B of LDL (cf. the arrowhead of lane 3), and no degradation product was observed. In the case of the transport at 10°C, a band corresponding to that of apoprotein B was also observed, but its intensity was much weaker than that at 37°C, as reflected by the lower permeability (cf. lane 4). These results indicate that LDL was transported through the cell layer keeping its original molecular mass without any degradation.

Discussion

In skin dermis fibroblasts, LDL is incorporated into the cells by the receptor-mediated pathway and it is digested to cholesterol and amino acids in lysosome. Fibroblasts of familial hypercholesterolemia (FH) patients can not express LDL receptors. Due to their receptor defect, these cells are unable to take up and degrade LDL, and content of cholesterol in plasma is increased. In hepatocytes, most of the degraded LDL is taken up by an LDL-specific mechanism that is suggested to be similar to the transport in fibroblasts mediated by the receptor.

In this study, we examined the transport of RB-LDL through the confluent skin epidermal cell monolayer in culture. Transport of RB-LDL was greatly dependent on the temperature: less than 17°C, the rate of transport was very small, but it became progressively larger above 20°C with increase in temperature. This transport was completely inhibited by the biological energy inhibitors 2-deoxyglucose and NaN₃. The activation energy of the transport determined from the Arrhenius plot was 104.6 kJ/mol, being in agreement with 102.1 kJ/mol determined with the cultured porcine endothelial cell monolayer. Furthermore, no degradation products of apoprotein B of LDL was observed. This suggests that LDL is not digested by the lysosomal system. Thus, the transport of LDL through the skin epidermal cells is possibly based on the same mechanism as that through a cultured endothelial cell layer, where the transport was temperature and energy dependent and no degradation product of LDL was observed. The marked dependence of the transport on the temperature and the inhibition by the energy inhibitors suggest that the transport is mediated by a carrier system supported by biological energy. The transport of bovine serum albumin and insulin through endothelial cells, and thyroglobulin from the inside to the outside of thyroid follicles and vesicular stomatitis virus G protein from the apical to basal plasma membrane of MDCK cells are reported to be temperature dependent.

We found that the transport of RB-LDL was dependent on its concentration, and the transport was saturated at the RB-LDL concentration of 300μg protein/ml at 37°C. The $K_m$ value was determined as 76.9μg protein/ml (Fig. 4B). LDL (apoprotein B) concentration in rat plasma and interstitial fluid were reported to be 204μg protein/ml and about 20μg protein/ml, respectively. The $K_v$ value of LDL transport through the skin epidermal cell layer is intermediate to the concentrations in plasma and in interstitial fluid, although the physiological meaning of this $K_v$ value is not apparent at present.

It is noteworthy that in the present study the LDL transport through the cultured skin epidermal cell layer is suggested not to be mediated by the lysosomal system as observed in the cultured endothelial cells. However, all mammalian cells require free cholesterol for their plasma membrane. It has recently been shown that mammalian cells in culture cannot survive unless they can acquire cholesterol either from a usable exogenous source or as a result of de novo synthesis in cells. The LDL receptor has not yet been identified in the skin epidermal cell, but LDL receptors in dermal fibroblasts are characterized. Thus, it is expected that skin epidermal cells have LDL receptors and LDL transport is mediated by the lysosomal system. Further study is necessary to examine the existence of LDL.
receptors in intact rat skin epidermal cells. If the receptor is identified, it would be important to know why LDL transport in the cultured cells is not mediated by the lysosomal system.

References