Analysis of Reversible Lipoprotein-Cell Interactions*

(Received for publication, November 2, 1981, and in revised form, December 22, 1981)

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Quantitation and characterization of freely reversible ligand-receptor interactions in adherent cell lines grown in vitro have been technically difficult to perform. We report the growth of human fibroblasts on a microcarrier bead system which obviates the limitation of cell adherence in such determinations. This system has been applied to the reversible interaction of low density lipoproteins (LDL) with the fibroblast membrane receptor. Of the 125I-LDL specifically bound to trypsinized cells, 52.2 ± 9.8% was reversibly bound. This reversible LDL-cell binding occurred in less than 1 h at 37 °C, was specific for LDL, and occurred without significant 125I-LDL degradation. Using these techniques, it is now possible to obtain direct measurement and characterization of lipoprotein-receptor interactions in adherent cell lines of normal subjects and patients with dyslipoproteinemia.

Low density lipoproteins are bound, internalized, and degraded by a variety of cell types (1–5). The relationship of these events to intracellular cholesterol metabolism has been elegantly characterized (1–7). However, studies on reversible LDL cell interactions in adherent cell lines such as the skin fibroblast and smooth muscle cell have been limited by the inability to quickly separate free and bound LDL. Equilibrium constants, receptor number, and receptor affinity can be directly assessed when one measures steady state equilibrium between bound and free ligand. Therefore, a rapid separation of bound and free ligand is needed for direct evaluation of LDL-cell interactions. Using human fibroblasts grown on a microcarrier bead suspension, we demonstrate the time course and degree of freely reversible LDL-cell interactions. These methods should prove useful in assessing lipoprotein-cell binding kinetics in fibroblasts and with lipoproteins from both normal and dyslipoproteinemic individuals.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free sodium iodide-125I at 50 mCi/ml in 0.1 N sodium hydroxide was purchased from New England Nuclear. Cells were grown in Eagle's minimal essential medium 2 with 0.18% sodium bicarbonate with 10% fetal bovine serum (HyClone; Sterile Systems, Inc.) and 2 mM L-glutamine (Gibco). Eagle's minimal essential medium 2 with 2 mM L-glutamine served as Buffer A in all incubations. Microcarrier beads used were Cytodex-1 by Pharmacia, and Spinco flasks were purchased from Wheaton (Bruxelles, NJ). Bovine albumin fraction V was obtained from Reheis Chemical Co. Tissue culture flasks (150-cm²) were purchased from Costar (Cambridge, MA) and 100-mm Falcon Petri dishes were used for the plated cell experiments. Washes for plated cell experiments were performed with Dulbecco's phosphate-buffered saline obtained from Gibco.

Bead and cell centrifugations were performed in a Sorvall GLC-1. Trypsin (217 units/mg) and Mitex filters (24-mm) were obtained from Millipore Corp. The vacuum filtration support systems were purchased from Hoefer Scientific Instruments. Three-milliliter monopore disposable syringes were used for incubations.

Isolation and Radiolabeling of Plasma Lipoprotein—Human LDL (density 1.030–1.050 g/ml) and lipoprotein-deficient serum (density > 1.215 g/ml) were obtained from the plasma of healthy subjects and prepared by differential sequential ultracentrifugation (8) and used within 4 weeks. After extensive dialysis against phosphate-buffered saline, the concentration of LDL protein was determined by the method of Lowry et al. (9), using a bovine serum albumin standard. LDL was iodinated by a modification (10) of the iodine monochloride method (11). The efficiency of labeling of the LDL was 27.7%, and less than 5.1% of the radioactivity was isolated within the organic phase of a chloroform methanol extraction. LDL was sterilized by passage through a 0.45-mm Millipore filter, stored at 4 °C, and used within 3–4 weeks of iodination.

Plate Binding—Human fibroblasts were grown to preconfluence in 60-mm Petri dishes, following which the media were changed to 10% LPDS and the incubation continued 48 h. The cells were then incubated for 15 min at 4 °C and the media were changed to 3 ml of LPDS media containing 1.07 × 10⁻⁶ mol of 125I-LDL/ml. After 6 h of incubation at 4 °C, the media were removed and the cells washed (6, 4 °C) with phosphate-buffered saline. The radioactivity in 0.5 ml of initial media and each of the six 3-ml 4 °C phosphate-buffered saline washes was quantitated. The total counts for each wash were calculated and divided by the total number of counts obtained from the first wash.

LDL-Microcarrier Binding—Human fibroblasts grown on microcarrier beads at 37 °C were incubated at 4 h in 10% LPDS. The spinco flask stirrer was stopped, the cell-bead suspension settled, and the media were removed. Two hundred milliliters of Buffer A were added to the cell beads, and the suspension was stirred for 3 min. This procedure was repeated four times after which aliquots of cell beads were transferred to 50-mm sterile centrifuge tubes (Corning, NY) and centrifuged at 1000 rpm for 3 min in a Sorvall GLC-1. Incubations with 125I-LDL were carried out in capped 3-ml sterile syringes in which the components were added in the following order: Buffer A with or without 1.66 mg of cold LDL, 125I-LDL, and 1 ml of cell-bead suspension containing 4–6 × 10⁶ cells. Another 1-ml aliquot of cell beads was trypsinized and cells were counted on a hemocytometer.

LDL Degradation Determination—The filtrate from the incubation mixture was collected, and 0.2-ml aliquots were counted directly while an additional 0.5 ml of filtrate was precipitated with 0.5 ml of 50% trichloroacetic acid. The trichloroacetic acid precipitate was incubated for 30 min at 4 °C and centrifuged (15 min, 4 °C, 2000 rpm). To...
1 ml of the tricarboxylic acid supernatant was added 0.01 ml of 40% potassium iodide followed by 0.04 ml of 30% H2O2. This mixture was incubated 5 min and then vortexed with 2 ml of CHCl3. After the phases were separated, the radioactivity of 0.5 ml of the aqueous phase was quantitated. 125I-LDL degraded was expressed as the percentage of radioactivity in the aqueous phase divided by the radioactivity in an equal volume of initial filtrate.

**LDL Competition Assay**—One milliliter of 125I-LDL was diluted with either 1 or 3 ml of cold LDL from the same subject at the same protein concentration.Twenty milliliters of each of these 125I-LDL mixtures were filtered and the filters and filter-bound material were determined.

### Results

**The Effect of Serial Washes on LDL-Cell Interactions**—The effect of serial washes on the dissociation of LDL from adherent fibroblast cultures grown in vitro is shown in Fig. 1. Human fibroblasts were grown to preconfluence, treated 48 h in LPDS, and incubated 6 h at 4°C with 125I-LDL (1). The radioactivity in the media as well as in each subsequent 3-ml wash was determined. Seventy-six percent of the radiolabeled LDL was removed by decanting the incubation media. By the fourth wash, 99% of the LDL had been recovered, and less than 1% of the radioactivity was removed with each of the last three washes. Only 0.8 ± 1.1% of the initial 125I-LDL added to the media remained for solubilization by NaOH. 95.4% of the initial 125I-LDL remaining with the cells after the initial removal of the media was washed with the serial buffer rinses. Therefore, sequential washes of cells previously incubated with 125I-LDL will remove the majority of the 125I-LDL that remains in removal of the initial media.

**Microcarrier LDL Binding**—Human diploid fibroblasts were grown to confluence on microcarrier beads (Fig. 2). With initial microcarrier concentration of 5 g/liter, cells grew to a mean density of 5.39 ± 0.35 × 10^6 cells/ml, and all studies were performed on the same initial culture after confluence was achieved.

**Specificity of LDL Binding**—Competition experiments were conducted on confluent bead-bound cells which were preincubated 48 h with LPDS. As illustrated in Fig. 3, the initial 125I-LDL concentration (100%) resulted in 37.6 ± 4.4 pmol of LDL binding to the 10^6 cells. This initial 125I-LDL sample was serially diluted with unlabeled LDL, resulting in 50 and 25% of the initial specific activity. The quantity of 125I-LDL bound decreased to 26.2 ± 4.8 pmol and 13.2 ± 1.8 pmol/10^6 cells in the 50 and 25% incubation, respectively. This linear decrease in 125I-LDL binding with a decrease in specific activity indicated that the native LDL competed effectively with the cellular binding of 125I-LDL. Therefore, radiolabeled LDL behaved similar to the native LDL in the present studies.

**Characterization of Reversible Binding**—Cells attached to beads were incubated with 125I-LDL after 40 h of LPDS treatment. To assess the quantity of LDL-cell binding that was freely reversible, samples were either counted directly after filtration or after washing six times with 3-ml aliquots of Buffer A. The time course of reversible LDL-cell binding is shown in Fig. 4. For samples incubated both with and without cold LDL, unwashed cells bound significantly more LDL (p < 0.05; paired two-tailed t test) than did the washed cells for each time interval. Samples incubated without cold LDL bound significantly more 125I-LDL than samples incubated with cold LDL for all the time intervals (p < 0.05; paired two-
on cell cholesterol metabolism have been elegantly conceptualized and characterized in a variety of cell types (1-7). The interaction of LDL with the cell membranes has been demonstrated by electron microscopy (14, 15). Although attempts at characterizing this lipoprotein cell receptor interaction have been made in adherent cell lines, the importance of reversible interaction has only recently been addressed in the literature (16). The techniques outlined in this report have been used to evaluate and characterize freely reversible LDL-cell interactions in an adherent cell line, the human fibroblast. In the routinely employed assay for LDL binding to cells, 5 to 7 washes are employed to remove nonspecifically adsorbed material (1). Although this leads to useful information about uptake, internalization, and degradation of ligand by the cell, it obviates the direct measurement of reversible ligand binding to the cell. As illustrated in Fig. 1, 17.5% of original 125I-LDL was associated with fibroblasts following removal of the media and with subsequent washes 95.4% of the lipoprotein was dissociated from the cells. Thus, with the washing procedure, LDL, which interacted reversibly with the cell, dissociated from the receptor.

The limitation in studying reversible binding in fibroblasts has been the adherence of these cells to their culture vessel. Unlike cell lines grown in suspension such as lymphocytes, adherent cell lines cannot be rapidly separated from their incubation media by centrifugation or filtration. The growth of fibroblasts on a microcarrier system obviates this limitation. The fibroblasts continue to adhere to a solid matrix, yet they can be suspended in culture without inducing significant membrane perturbation. The advantage of the cell suspension system is that the cells can be rapidly separated from the media which can then be utilized to quantify reversible binding. The microcarrier technique with human fibroblasts described in this report was used to assess reversible LDL-cell interaction. 125I-LDL binding to the fibroblast bead system increased after incubation with lipoprotein-deficient serum (Fig. 4) and decreased linearly with reduction in specific activity of radiolabeled LDL (Fig. 3). LDL reversibly bound to cells represented 0.4 to 0.7% of the total LDL added to the media. Bound LDL comprised 52.2% of the LDL that was specifically bound to the filtered cells. As shown in Fig. 4, the quantity of LDL, which represented bound and internalized LDL steadily increased over the first 60 min and remained constant thereafter. The radiolabeled lipoprotein which eluted off with serial washes, designated as reversibly bound LDL, remained much more constant during the time course of the study. The LDL-cell interaction was specific (Fig. 4), and no significant degradation of the radiolabeled LDL occurred during the first hour of lipoprotein-cell interaction (Fig. 5). Using the cell suspension system, reversible binding can be separated from irreversible lipoprotein binding at 37°C. Using the new techniques, direct quantitation and characterization of lipoprotein-receptor interactions in adherent cell lines of normal and dyslipidemic man are now feasible.

REFERENCES


FIG. 4. Time course of LDL binding to bead-adherent fibroblasts. Bead-bound fibroblasts were incubated with LPDS for 40 h followed by incubation with 42 μg of 125I-LDL (1207 dpm/μg) in Buffer A and with (O) or without (●) 1.660 mg of cold LDL. At the indicated time intervals, the incubation mixtures were filtered. The radioactivity associated with unwashed filters (●) (top curve) was then counted and washed filters (O) (bottom curve) following six 3-ml rinses with Buffer A were determined. Values for the 125I-LDL bound are the means ± S.E. of three determinations.

FIG. 5. Time course of 125I-LDL degradation. At the indicated time intervals, the 125I released from degraded LDL was measured as described under “Experimental Procedures” and these data are expressed as the percentage of 125I-LDL that was degraded. Values represent the mean of duplicate samples.

tailed t test). Specific 125I-LDL binding was determined by subtracting the 125I-LDL bound in samples incubated with excess cold LDL from samples not exposed to cold LDL. The specific reversible binding was then derived by obtaining the difference of specific 125I-LDL binding between the washed and unwashed samples. The total specific 125I-LDL binding steadily increased with time and ranged from 50.8 μg/10⁶ cells at 5 min to 90.1 μg/10⁶ cells at 120 min. The specific reversible 125I-LDL binding represented 52.2 ± 9.8% of the total 125I-LDL bound.

Fig. 5 demonstrates the percentage of 125I-LDL that was degraded in the present studies. In 1 h, less than 1% of the 125I-LDL in the filtrates was degraded; however, by 2 h, 15.8% of the 125I-LDL was degraded, indicating that these cells were actively internalizing and catabolizing the 125I-LDL. Of more importance was the observation that little, if any, of the filtrate 125I was from catabolized 125I-LDL in the first hour. Therefore, serial washing of the filtered cells represents dissociation of reversibly bound 125I-LDL rather than elution of degradation products in the first hour of incubation.

DISCUSSION

LDL uptake internalization and degradation and its effect...
Evaluation of Reversible LDL-Cell Interactions

Analysis of reversible lipoprotein-cell interactions.
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