Vol. 84, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 29, 1978 Pages 366-373

RELEASE OF LOW DENSITY LIPOPROTEIN RECEPTORS FROM HUMAN FIBROBLASTS OR HELA CELLS BY TRYPTIC DIGESTION

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Received August 14, 1978

SUMMARY

Trypsin treatment of cultured normal human skin fibroblasts or Hela cells releases material which is retained on a low density lipoprotein (LDL)-Sepharose affinity column, may be eluted from it with 2.5 M KI and, after dialysis, agglutinates LDL or apo-B-coated formocells. Such agglutination is prevented by preincubation of the receptor with LDL in solution or with arginine-rich protamine. Trypsin treatment of "receptor defective" or "receptor negative" mutant fibroblasts releases material which is retained on LDL-Sepharose column but fails to agglutinate LDL-coated formocells. The receptor may be labeled with 6-[^{3}H]-glucosamine·HCl and [^{3}H]-leucine, it is inactivated by heating at 80°C for 10 min and may be obtained from normal fibroblasts or Hela cells, whether they were cultured in presence or in absence of lipoprotein-containing fetal calf serum.

The degradation of LDL by a variety of cells from peripheral tissues consists of a series of metabolic events which begins with the binding of LDL to specific cell surface receptors (1).

The paramount importance of this binding is demonstrated by the clinical and biochemical features of several inherited human diseases characterized by a complete absence of receptors or by the presence of defective ones (1,2).

Using cultured human skin fibroblasts or membranes prepared from them, Brown, Goldstein (3,4) and Basu et al. (5) have suggested that the LDL receptors might be proteins or glycoproteins. In fact, brief treatment of the cells with trypsin, pronase or papain or heating at 80°C for 10 min has been reported to destroy their ability to bind LDL while treatment with a variety of glycosidases, phospholipases and arylsulfatases does not. Moreover, the reappearance

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Abbreviations: LDL, low density lipoproteins; apo-B, apolipoprotein B; LDS, lipoprotein deficient serum; FCS, fetal calf serum.

0006-291X/78/0842-0366$01.00/0

of LDL receptors after pronase treatment is blocked by cycloheximide. Essentially the same results have been obtained by Koschinsky et al. (6). These results have been interpreted as indicating "destruction" or "degradation" of LDL receptors by proteases.

On the assumption that the treatment with proteases might cleave the receptors from the cell surface, without destroying their biological activity, we have examined the proteolytic digests for the presence of ligands capable of binding LDL or apo-B. For this purpose we have used the technique of hemagglutination using LDL or apo-B-coated formocells (7). Our results demonstrate that brief treatment of cultured skin fibroblasts or Hela cells with trypsin allows the isolation of functional LDL receptors or fragments therefrom.

MATERIALS AND METHODS

Lipoprotein-deficient serum was prepared by ultracentrifugation of FCS, brought to 1.21 density with KBr, at 50,000 rpm in a SW 50.1 rotor for 18 hr. The bottom layer was exhaustively dialyzed against pyrogen-free saline before use.

Normal and mutant human skin fibroblasts (obtained from the Human Cell Repository, Camden, N.J.) and human serum adapted Hela cells (Flow Laboratories, Rockville, Md., Catalog No. 0-26310) were cultured in Minimal Essential Medium (Gibco, Grand Island, N.Y.) containing 10% FCS or in medium 199 (Pacific Biological, Berkeley, Ca.) containing 5% LDS.

In order to increase the synthesis and the labeling of cell membrane structural components, confluent monolayers were harvested by trypsinization (8) and replated (1:2 split) in 150 cm^2 Falcon plastic flasks, with medium containing 20 µCi/ml of [^{3}H]-leucine or 60 mCi/ml of 6-[^{3}H]-glucosamine·HCl. After 48 hr the medium was collected and the cells were washed twice with cold medium 199 and six times with 0.15 M NaCl containing 7 mM $CaCl_2$. Each culture plate was then treated twice at room temperature for 10 min with collagenase (60 units, Worthington, Freehold, N.J.) in 0.5 ml of 0.2 M Tris-HCl buffer pH 7.7 (9). The collagenase digests were pooled and inactivated with two drops of FCS. The cells were then treated twice, 5 min each, with 0.5% trypsin (Gibco, Grand Island, N.Y.) - 0.2% EDTA in 0.15 M NaCl at 37°C. The cells were removed by centrifugation and tryptic digests pooled and inactivated with FCS. Thereafter, the collagenase and trypsin pools were applied to identical 0.6 x 9 cm LDL-affinity columns (see below); after the samples had drained, the columns were washed with 120 ml of 0.1 M phosphate-buffer pH 8.0 containing 0.15 M NaCl and then eluted with 10 ml aliquots of 0.5 M KCl, 2.5 M KCl in phosphate buffer and eventually with 2.5 M KI (10,11). After dialysis and lyophilization, each effluent was dissolved in 2 ml distilled water, analyzed and tested for ability to agglutinate LDL or apo-B-coated formocells (7) suspended in 1:200 normal rabbit serum diluted in saline and adjusted to pH 7.20 with 1 N NaOH.

Preparation of LDL-affinity column. CNBr-activated Sepharose 4B, 3 g, washed with 0.001 M HCl, was combined with 30 mg of human LDL (12) dissolved in 0.1 M $NaHCO_3$, pH 9.0, containing 0.5 M NaCl. The suspension was rotated for 2 hr. Thereafter the material was washed with bicarbonate buffer on a glass filter, suspended in 1.0 M ethanolamine pH 8.0 and mixed for 2 hr. After ex-

TABLE 1. Partial analyses and biological activity of LDL receptor preparations isolated by trypsinization from normal and mutant fibroblasts.

	[^{3}H] dpm/ml (leucine)	Protein µg/ml	Agglutination of LDL-coated formocells
NORMAL FIBROBLASTS			
Material eluted from affinity column by:			
0.5 M KCl	81,100	100	neg
2.5 M KCl	18,800	0	neg
2.5 M KI	62,000	50	2+
MUTANT FIBROBLASTS			
Material eluted from affinity column by:			
0.5 M KCl	4,270	50	neg
2.5 M KCl	3,100	12	neg
2.5 M KI	6,200	123	neg

Each fibroblasts culture, labeled with [^{3}H]-leucine, was treated sequentially with collagenase and trypsin, as described under Methods. The cellular material removed by trypsin was applied to an LDL-affinity column, washed with 0.1 M phosphate buffer pH 8.0 and eluted with 0.5 M KCl, 2.5 M KCl and 2.5 M KI. The effluents were dialyzed, lyophilized and tested for ability to agglutinate LDL-coated formocells suspended in 1:200 normal rabbit serum, diluted in pH 7.20 saline. The sedimentation pattern of the cells was arbitrarily read from negative (uniform button) to 4+ (compact granular mat covering the bottom of the tube) (7).

haustive washings with 0.1 M acetate buffer pH 4.0 and 0.1 M borate buffer pH 8.0 (both containing 1.0 M NaCl), the material was used for affinity chromatography.

Standardization of LDL-affinity column. A 2 ml aliquot of anti-apo-B immunserum (13) was applied to a 0.6 x 9 cm column, which was than washed and eluted with PBS, KCl and KI as described above. After dialysis and lyophilization, the wash and eluates were tested for ability to agglutinate LDL-coated formocells. The finding of all the agglutinating activity of the original immunserum in the KI eluate proves the successful coating of Sepharose with LDL.

The treatment of formocells with tannic acid, their coating with LDL or apo-B, the technique of hemagglutination and hemagglutination inhibition and the evaluation of their results (7), as well as the preparation of apo-B (14) and that of anti-apo-B immunserum (13), have been described in previous publications.

Protein was measured with known methods (15,16). QuanTtest (Quantimetrix , Culver City, Ca.) was used as protein standard. Radioactivity was measured by adding from 0.1 ml to 0.5 ml of solution to 5 ml of Aquasol (New England Nuclear, Boston, Ma.) or to 5 ml of Aqueous Counting Scintillant (Amersham/Searle, Arlington Heights, Il.) and counting the clear solution in a Packard Tri-Carb spectrometer. Protamine sulfate (nitrogen 24.0%, arginine 67-70%, sulfate 17.5%) was purchased from Nutritional Biochemical Corporation, Clevelend, Ohio.

RESULTS

Table 1 summarizes the results of analyses of the trypsin pools of normal fibroblasts and of one line (GM-912) of "receptor defective" fibroblasts.

TABLE 2. Biological activity of LDL receptors derived from normal fibroblasts cultured in presence of 6-[^{3}H]-glucosamine.

	[^{3}H] dpm/ml (glucosamine)	Protein µg/ml	Agglutination of LDL-coated formocells			
			100 µl	50 µl	25 µl	10 µl
NORMAL FIBROBLASTS						
Material eluted from affinity column by:						
0.5 M KCl	37,500	10	------neg------			
2.5 M KCl	7,300	26	------neg------			
2.5 M KI	25,000	66	3+	2+	+	±

Normal fibroblasts, labeled with 6-[^{3}H]-glucosamine·HCl, were treated with collagenase and trypsin as described. The trypsin digest was processed as described in Table 1. Decreasing volumes of a solution of LDL receptor were used for agglutination of LDL-coated formocells. Results were read as described in Table 1.

For normal fibroblasts, grown either in presence of 10% FCS or 5% LDS, only the material eluted from the LDL-affinity column by 2.5 M KI agglutinated LDL-coated formocells. This material contained protein and was labeled with [^{3}H]-leucine. Measurement of 3-hydroxy-3-methylglutaryl coenzyme A reductase (17) (EC 1.1.1.34) in parallel cultures of normal fibroblasts demonstrated an activity of 20 pmol product/min/mg protein for those grown in FCS and of 70 pmol product/min/mg protein for those grown in LDS. Similar LDL-agglutinating activities were obtained with Hela cells, cultured either in presence or in absence of lipoproteins (not shown).

For the mutant fibroblasts, the material eluted from the affinity column by 2.5 M KI failed to agglutinate LDL-coated formocells. Two additional lines of "receptor negative" fibroblasts (one being derived from patient M.C., already studied by Basu *et al.* (5)) were studied with the same methodology. In both cases, the material eluted from the affinity column by 2.5 M KI did not agglutinate LDL-coated formocells.

Table 2 shows the results of similar experiments performed with normal fibroblasts cultured in 5% LDS and labeled with 6-[^{3}H]-glucosamine·HCl. The

TABLE 3. Biological activity of LDL receptor toward LDL or apo-B-coated formocells.

	Dilutions of LDL receptor used and agglutination results				
	1:5	1:10	1:20	1:40	1:100
LDL-coated formocells	3+	2+	+	+	not performed
Apo-B-coated formocells	3+	3+	3+	+	±

The material present in a tryptic digest of normal fibroblasts which was retained on an LDL-affinity column and eluted from it with 2.5 M KI was dialyzed, lyophilized and redissolved in 1 ml of H_2O. Decreasing aliquots of this solution were tested for ability to agglutinate coated formocells.

labeled material present in the trypsin pool which was eluted by 2.5 M KI was the only one to show a dose-dependent agglutination of LDL-coated formocells. This agglutinating activity disappeared if the eluate was heated at 80°C for 10 min (5).

When the material derived from a trypsin digest of normal fibroblasts and eluted from the affinity column by 2.5 M KI was tested against formocells coated either with LDL or apo-B, similar agglutination titers were demonstrated (Table 3). This agglutination could be inhibited by preincubation of an adequate volume (5 µl) of LDL-receptor solution with microgram amounts of LDL in solution (Table 4), or with microgram amounts of arginine-rich protamine sulfate. The latter inhibitor, however, does not affect agglutination of LDL or apo-B-coated formocells by anti-apo-B immunserum (Table 5).

DISCUSSION

Although limited proteolysis has been used to isolate a variety of macromolecular components of cell membrane (9,18-24), attempts to recover LDL receptor activity in proteolytic digests have not been made. In fact, it has been assumed that the specific LDL receptors are destroyed or degraded by proteolytic treatment (3,4,6). In this work we have demonstrated that trypsin treatment of cultured fibroblasts or Hela cells releases components capable of binding to an LDL-affinity column and, upon chaotropic dissociation with KI, of agglutinating LDL or apo-B-coated formocells.

TABLE 4. Inhibition of the agglutination of LDL-coated formocells by pre-incubation of LDL receptor with decreasing amounts of LDL in solution.

	Amount of LDL (as µg protein) added to 5 µl of LDL-receptor solution (0.4 µg protein)				
	80	40	20	10	5
Agglutination of LDL-coated formocells	neg	neg	±	+	2+

A constant volume of LDL-receptor solution (5 µl, containing 0.4 µg protein) was preincubated with decreasing amounts of human LDL and then challenged with LDL-coated formocells.

TABLE 5. Inhibition of the agglutination of apo-B-coated formocells by pre-incubation of LDL receptor with decreasing amounts of arginine-rich protamine sulfate.

Agglutination of apo-B-coated formocells by	Amount of protamine (as µg dry weight) added to each tube			
	5	2.5	1.25	0.62
LDL-receptor (4.0 µg protein)	neg	neg	3+	3+
Anti-apo-B immunserum (1:20)	3+	3+	3+	3+

Constant amounts of LDL-receptor (4.0 µg protein/ml) or of anti-apo-B immunserum (50 µl/ml) were incubated with decreasing amounts of arginine-rich protamine sulfate, prior to the addition of apo-B-coated formocells.

The material separated by affinity chromatography demonstrates LDL-binding activity, may be labeled with 6-[^{3}H]-glucosamine and [^{3}H]-leucine, loses its activity upon heating at 80°C for 10 min, and is obviously resistant to limited proteolysis. Thus, it behaves like a glycoprotein, which would be consistent with previous findings concerning the inhibition of receptor synthesis by cycloheximide (3,4,6).

At variance with current views is the finding of receptor activity in a proteolytic digest of fibroblasts or Hela cells cultured in presence of lipoprotein-containing FCS; these conditions should have suppressed the synthesis of LDL receptors (1). However, it is possible that the presence of intracellular free cholesterol inhibits the function of the LDL receptors rather than

their synthesis. Such inhibition would not be evident once the receptors, or part of them, are cleaved by proteolysis and tested in vitro.

Proteolytic treatment of "receptor defective" and "receptor negative" fibroblasts removes material which binds to LDL on a solid phase but does not cause agglutination of LDL-coated formocells. These results support the previously made suggestions (3,6,25) that mutant fibroblasts have structurally altered LDL receptors, but - at the moment - fail to differentiate between receptor defective and receptor negative fibroblasts.

The receptor activity derived from normal fibroblasts or Hela cells agglutinates equally well LDL-coated and apo-B-coated formocells, in agreement with the demonstration that the apoprotein is the critical factor in the binding of LDL to its specific receptors (26,27). Moreover, the inhibition by arginine-rich protamine sulfate of this agglutination is consistent with the demonstrated role that arginyl residues have in the binding of LDL to the specific cell surface receptors (27). These same residues of apo-B apparently are not essential for its interaction with anti-apo-B immunserum.

In conclusion, limited proteolysis of normal and mutant cultured fibroblasts, combined with affinity chromatography, seems to afford a simple method for harvesting the specific LDL receptors in amounts adequate for further physicochemical and functional characterization.

ACKNOWLEDGEMENTS: This investigation was supported by grants HL-18692-02, HL-20447-02 and HL-17269-04 from the National Institutes of Health, and by a grant-in-aid from the American Heart Association, Texas Affiliate, Inc. We are grateful to Drs. Brown and Goldstein for providing cultures of fibroblasts of patient M.C.

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