EFFECT OF CELL DENSITY ON BINDING AND UPTAKE OF
LOW DENSITY LIPOPROTEIN BY HUMAN FIBROBLASTS

HOWARD S. KRUTH, JOEL AVIGAN, WILBERT GAMBLE, and
MARTHA VAUGHAN

From the Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National
Institutes of Health, Bethesda, Maryland 20205. Dr. Kruth's present address is the Department of
Pathology, Shands Teaching Hospital, University of Florida Medical School, Gainesville, Florida
32610. Dr. Gamble's present address is the Department of Biochemistry and Biophysics, Oregon
State University, Corvallis, Oregon 97331.

ABSTRACT
The effect of cell density on low density lipoprotein (LDL) binding by cultured
human skin fibroblasts was investigated. Bound LDL was visualized by indirect
immunofluorescence. Cellular lipid and cholesterol were monitored by fluores-
cence in cells stained with phosphine 3R and filipin, respectively. LDL binding
and lipid accumulation were compared in cells in stationary and exponentially
growing cultures, in sparsely and densely plated cultures, in wounded and non-
wounded areas of stationary cultures, and in stationary cultures with and without
the addition of lipoprotein-deficient serum. We conclude that LDL binding and
cholesterol accumulation induced by LDL are influenced by cell density. It
appears that, compared to rapidly growing cells, quiescent (noncycling) human
fibroblasts exhibit fewer functional LDL receptors.

KEY WORDS
low density lipoprotein receptor  •
cholesterol  •
human skin fibroblasts  •
atherosclerosis

With an increasing awareness of the importance
of membrane receptors in the regulation of cellular
metabolism has come the recent recognition that
receptors may play a role in the pathogenesis of
certain diseases (14, 15). Brown et al. (3) have
demonstrated that the uptake of cholesterol from
low density lipoprotein (LDL) by cultured fibro-
blasts and smooth muscle cells is mediated by a
specific receptor which may be defective or absent
in some patients with hypercholesterolemia. Dur-
ing the incubation of human fibroblasts with LDL,
we noticed that cells in sparse areas of the culture
developed numerous lipid inclusions, whereas
many cells in more dense areas showed no accu-
mulation of lipid. The studies reported here were
carried out to verify this observation and to deter-
mine whether there is a relationship between cel-
lar density and the presence of LDL receptors in
cultures of human fibroblasts.

MATERIALS AND METHODS

Cell Culture
Cultured fibroblasts from normal human foreskin were grown
in 75-cm² polystyrene flasks (Corning Glass Works, Corning, N.
Y.) in Eagle's minimal essential medium (MEM) supplemented
with 10% (vol/vol) fetal calf serum (FCS) (North American
Biologicals, Inc., Miami, Fla.) at 37°C. After gassing for 30 s with
a mixture of 95% air and 5% CO₂, cultures were incubated.
Medium was changed weekly.

For experiments, a flask of cells was washed twice with 10 ml
of Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline
(DPBS) warmed to 37°C. 1 ml of the same medium containing
a dry heat oven at 180°C for 2 h. Cultures were incubated in 35-mm wells (3004, Falcon Labware, Oxnard, Calif.), which contained a 22 x 22-mm glass coverslip previously sterilized in 0.125% trypsin (Grand Island Biological Co., Grand Island, N. Y.) and then incubated with either MEM, MEM with 10% FCS, or MEM plus 10% lipoprotein-deficient serum (LDS).

Cell cultures were monitored for mycoplasma by culturing and by a fluorescent staining procedure utilizing acridine orange similar to that described by Russell et al. (21).

In those experiments in which growth curves were obtained, cells were plated into 35-mm wells as described above, but without coverslips. Cells were trypsinized at 37°C and dispersed by pipetting before taking an aliquot for cell counts made in a Neubauer chamber.

Preparation of LDL and LDS

LDL (d 1.019-1.063 g/ml) was prepared from human plasma by standard techniques (11) of density flotation with centrifugation at 215,000 g for 18 h at 4-10°C. Solid KBr was used for adjustment of density (18). LDS was prepared by centrifugation of FCS at 215,000 g for 48 h at 4-10°C after density adjustment to 1.250 using solid KBr. The isolated fractions were dialyzed for 48 h against four changes of 4 liters of 0.85% saline (containing 0.1% aqueous phosphine 3R for 3 min. Cells were washed three times with 5 ml of DPBS, followed by a 10-min and a 5-min wash in DPBS, all at room temperature. Coverslips were then mounted in DPBS and observed with a fluorescence microscope as described above, except that a BG 3 filter was used for excitation.

Filipin, a fluorescent histochemical stain for cholesterol (2), was kindly provided by Dr. George B. Whitfield, Upjohn Co., Kalamazoo, Mich. Cells grown on coverslips were washed three times in 2 ml DPBS at room temperature, then fixed in 3 ml of cold 10% lipoprotein-buffered formalin containing 0.1 M sucrose and stored at 4°C in fixative until stained. Cells were washed twice with 2 ml of DPBS, then incubated at room temperature in 1 ml of DPBS containing 0.05% (vol/vol) Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) for 5 min. After washing twice in 5 ml of DPBS for 5 min at room temperature, cells were incubated at 37°C for 2 h in 2 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.4 mg cholesterol esterase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Cells were washed twice with 5 ml of DPBS before staining with 1 ml of DPBS containing 20 μl of filipin stock solution (2.5 mg filipin dissolved in 1 ml of dimethylformamide) for 30 min at room temperature. After washing twice with 5 ml of DPBS, coverslips were mounted in DPBS. Examination of cells by fluorescence microscopy was as described above using a BG 3 filter for excitation.

Photomicrography

Ektachrome 200 daylight color slide film (Eastman Kodak Co., Rochester, N. Y.) “push-processed” to an ASA of 400 was used for fluorescence and phase photography. Where comparisons are made between experimental conditions at a given magnification, the exposure times are the same so that the relative fluorescence intensities are comparable.

RESULTS

In relatively sparse cultures, fibroblasts that had been grown in MEM or in MEM plus 10% LDS bound LDL, which was seen in a punctate distribution over the cell surface (Fig. 1a and c). On many cells, but not all, the bound LDL appeared to be arranged in a linear pattern (Fig. 1a). Cells grown in MEM with 10% FCS bound very little LDL (Fig. 1e and f). When cells grown in MEM or MEM plus 10% LDS were incubated with LDL for 24 h before the binding assay, LDL binding was minimal (Fig. 1h and d). Whereas in sparse cultures grown in MEM or in MEM containing 10% LDS fibroblasts bound relatively large amounts of LDL (Fig. 2c, e, and g), in confluent cultures grown in the same media they bound very little LDL (Fig. 2d, f, and h).
FIGURE 1  Fibroblasts (40,000 cells/well or 4,000/cm²) were incubated in 3 ml of MEM plus 10% FCS at 37°C for 24 h. After washing they were incubated in MEM (a and b), MEM with 10% LDS (c and d), or MEM with 10% FCS (e and f) at 37°C for 48 h. The medium was then replaced with the same fresh medium (a, c, and e) or with MEM containing 50 μg/ml LDL (b, d, and f). After incubation at 37°C for 24 h, cells were prepared for evaluation of LDL binding using fluorescence microscopy as described under Materials and Methods. Bar in f, 20 μm, applies to all (× 280).

The fibroblasts in sparse cultures grown in MEM showed a striking accumulation of lipid after incubation for 23 h with LDL. This was seen as numerous inclusions by phase microscopy (Fig. 3a) and as phosphine 3R-stained lipid by fluorescence microscopy (Fig. 3b). Controls incubated without LDL had no lipid inclusions (Fig. 3c and d). Confluent fibroblasts grown in MEM, with the exception of rate cells (one shown in Fig. 3f), showed essentially no accumulation of lipid when incubated with LDL (Fig. 3e and f) and did not differ in appearance from confluent control cells incubated without LDL (Fig. 3g and h). The microscopic findings were confirmed by measurement of the free and esterified cholesterol content of cells in a parallel experiment (Table I). The cholesterol content of the fibroblasts grown in MEM, like their ability to bind and take up LDL, decreased with increasing cell density (Fig. 4 and Table I).

When stationary fibroblast cultures were “wounded” by scraping cells from an area of the supporting surface, cells from the surrounding layer migrated into this space and, in many cases, divided. Although the dense cells in the undisturbed portions of the stationary culture did not bind significant amounts of LDL (Fig. 5c and e), fibroblasts that had migrated into the wound 24 h after wounding bound LDL (Fig. 5d and f). The cells in the wounded area also accumulated much more lipid (Fig. 5i) and cholesterol (Fig. 5j) during incubation with LDL for 4 h than did cells in the rest of the culture (Fig. 5i and k).

In the experiment shown in Fig. 6, LDL binding in cultures maintained in MEM with 10% LDS was determined during exponential growth and during the stationary phase. Cells in cultures undergoing exponential growth bound LDL (Fig. 6c and e), but in the stationary cultures almost no LDL binding was demonstrated (Fig. 6d and f).

FIGURE 2  Fibroblasts were plated at densities of ~420 (a, c, and e), 4,200 (b, d, f, and g), or 38,000 (h) cells/cm² and incubated in 3 ml of MEM with 10% FCS at 37°C for 24 h. After washing they were incubated in 3 ml of MEM containing 10% LDS for 6 d (a-f) or in 3 ml of MEM for 3 d (g and h). Cultures were then stained for LDL immunofluorescence and examined by phase microscopy (a and b) or fluorescence microscopy (c-h). (a and c, and b and d are matching fields.) Bar in d, 40 μm, applies to a-d (× 126). Bar in h, 20 μm, applies to e-h (× 280).
Densely plated cells grown in MEM that did not bind LDL did so after incubation for 48 h with MEM containing 10% LDS (Fig. 7b). Controls incubated for the same duration in MEM alone remained negative (Fig. 7d), although sparsely plated cells maintained in MEM demonstrated LDL binding without the need for serum stimulation (Fig. 7f).

DISCUSSION

In these studies with normal human fibroblasts, alterations in LDL binding associated with changes in the lipoprotein content of the culture medium were monitored with the indirect immunofluorescence technique and appeared to correlate with changes in the numbers of functional LDL receptors as initially described by Brown and Goldstein (4). In relatively sparse cultures, cells grown in medium without serum or with LDS bound LDL, and LDL binding was essentially abolished after incubation of these cells for 24 h with LDL. Cells grown in medium containing FCS bound little or no LDL.

For studies of the LDL receptor in relation to cell density, fibroblasts were grown in medium without serum or with LDS in order to produce conditions under which negative feedback regulation of the receptor by LDL would be minimized and other influences on expression of the LDL receptor might be more readily observed. The virtual absence of LDL binding and of cholesterol accumulation by cells in dense cultures incubated with LDL contrasted strikingly with the extensive LDL binding and cholesterol uptake (observed microscopically and quantified biochemically) exhibited by cells in sparse cultures grown under otherwise similar conditions. The extent of intracellular lipid accumulation following incubation of cells with LDL paralleled LDL binding in all experiments where both were examined, i.e., only cells with demonstrable LDL receptors accumulated cholesterol on incubation with LDL. These observations are in agreement with those of Brown et al. (3), who measured LDL binding and cellular cholesterol using biochemical methods in their investigation of the role of the LDL receptor in the regulation of cholesterol content in human fibroblasts.

Goldstein and Brown (10) reported that high affinity LDL binding to human fibroblasts was much lower on the 7th d of subculture than it was on the 2nd d. Stein and Stein (23) found that uptake of LDL by cultured smooth muscle cells was inversely related to cell density. Vlodavsky et al. (26) failed to observe an effect of cell density on binding or uptake of LDL by cultured smooth muscle cells but did report decreased uptake of LDL by confluent cultured endothelial cells. In our experiments, diminished LDL binding was apparently not entirely the result of an effect of cell density. When cells in dense stationary cultures were induced to divide, they rapidly exhibited evidence of functional LDL receptors. Replacement of the serum-free medium of dense cultures with fresh medium containing LDS, which might be expected to induce a round of cell division (24) in the previously stationary cultures, resulted in the appearance of LDL receptors. After "wounding" of cultures, the cells that migrated into the wound area (many of which underwent

FIGURE 3 Fibroblasts were plated at densities of ~4,200 (a-d) or 3,800 (e-h) cells/cm² and incubated in MEM with 10% FCS at 37°C for 24 h. After washing they were incubated in 3 ml of MEM for 3 d. Medium was then changed to 3 ml of fresh MEM (c, d, g, and h) or MEM containing 50 μg/ml LDL (a, b, e, and f), and cells were incubated for 23 h. Cells were fixed and stained for lipid with phosphine 3R and examined by phase microscopy (a, c, e, and g) or by fluorescence microscopy of the same fields (b, d, f, and h). Bar in h, 40 μm, applies to all (× 126).
TABLE I

| Culture density | LDL added | Cells per cm² | Protein per dish | Cholesterol content | Free | Esterified |
|-----------------|-----------|---------------|------------------|---------------------|------|------------|
|                 |           |               | µg               | µg/mg protein       |      |            |
| Sparse 0        | 1.01 ± 0.20 | 13.6 ± 1.7    | 96 ± 9.1         | 0.9 ± 0.1           |      |            |
| + 1.39 ± 0.09   | 23.9 ± 2.3  | 142 ± 5.5     | 58.2 ± 5.8       |                     |      |            |
| Dense 0         | 17.7 ± 1.1  | 234 ± 4.6     | 62 ± 1.4         | 1.4 ± 0.4           |      |            |
| + 17.1 ± 2.41   | 252 ± 14.9  | 63 ± 2.9      | 4.0 ± 0.3        |                     |      |            |

Fibroblasts were grown and treated as described in the legend for Fig. 3 except that 100-mm dishes (without coverslips) were used and proportionately larger numbers of cells were added to initiate the sparse and dense cultures. Cells were harvested and their protein and cholesterol contents were determined as described (8). Values are the means of data from triplicate cultures ±SEM.

Evidence has been presented (1, 17) that confluent cultured fibroblasts enter a noncycling or G₀ state. Such cells can be stimulated to leave the G₀ state and cycle by the addition of serum (1). It is possible that the noncycling (G₀) state is associated with a loss of LDL binding, whereas non-G₀ cells including sparsely cultured fibroblasts maintained in MEM, which may be arrested in the G₁ phase of the cell cycle, rather than in the G₀ phase, retain their ability to bind LDL and accumulate cholesterol. All of our findings are consistent with the possibility that LDL receptors are functional only on cells that are cycling (i.e., in the G₁, S, G₂, or M phase of cell cycle) and are not present or accessible on the surface of noncycling (G₀) cells. There is evidence that certain other kinds of cell surface receptors, e.g., those for lectins (6, 22), hormones (25), growth factors (12), and antibodies (13), may be exposed or functional only at specific times in the cell cycle. The mechanisms underlying this type of receptor regulation are unknown. In the case of the LDL receptor, it is possible that in cycling cells dependent on endogenously synthesized cholesterol for assembly of cell structures, the cholesterol content of some regulatory pool¹ which determines LDL binding capacity is kept relatively low, and LDL receptors are maintained, whereas when cells enter a G₀ state the cholesterol content of this regulatory pool tends to increase, leading to a decrease in LDL receptors through a mechanism similar to the neg-

¹ In our experiments, the total cholesterol content of the fibroblasts decreased with increasing cell density.
FIGURE 5 Fibroblasts were plated at a density of \(-4,200 \text{ cells/cm}^2\) and incubated in 3 ml of MEM with 10% FCS at 37°C for 24 h. After washing, cultures were incubated in 3 ml of MEM containing 10% LDS for 6 d. The stationary cultures were then wounded with the tip of a 10-ml, sterile plastic pipette, washed three times with 2 ml of MEM, and incubated in 3 ml of MEM for 24 h. Cultures were then stained for LDL immunofluorescence (\(a-f\)) or fixed and stained for lipid with phosphine 3R (\(g-j\)) or for cholesterol with filipin (\(k\) and \(l\)) and examined by phase microscopy (\(a, b, g, h\)) and fluorescence microscopy (\(c-f\) and \(i-l\)). Cells in the wounded area are shown in \(b, d, f, h, j, \) and \(l\); those in the undisturbed area in \(a, c, e, g, i, \) and \(k\). (\(a\) and \(c, b\) and \(d, g\) and \(i,\) and \(h\) and \(j\) are matching fields.) Bar in \(f, 20 \mu m,\) also applies to \(e (\times 280).\) Bar in \(l, 40 \mu m,\) applies to all others (\(\times 126).\) Counts of cells in replicate wells in this experiment established that growth was exponential for 4 d and there was essentially no increase in cell number after day 5.

FIGURE 6 Fibroblasts were plated at a density of \(-4,200 \text{ cells/cm}^2\) and incubated in 3 ml of MEM with 10% FCS at 37°C for 24 h. After washing, cultures were incubated in 3 ml of MEM with 10% LDS. Cultures were stained for LDL immunofluorescence 4 d after plating during exponential growth (\(a, c, \) and \(e\)) or 8 d after plating during the stationary phase (\(b, d, \) and \(f\) and \(i\)) and examined by phase microscopy (\(a\) and \(b\)) or by fluorescence microscopy (\(c-f\). (\(a\) and \(c, b\) and \(d, g\) and \(j\)) and \(h\) and \(j\) are matching fields.) Bar in \(d, 40 \mu m,\) also applies to \(a-e (\times 126).\) Bar in \(f, 20 \mu m,\) also applies to \(e (\times 280).\) Counts of cells in replicate wells established that growth was exponential for 4 d and that there was little increase in cell number after day 5.

Effective feedback regulation demonstrated with exogenous cholesterol or LDL. If expression of the LDL receptor is dependent on whether cells are cycling, it might be difficult to make meaningful comparisons between cultures with different growth rates because of the possibility of differences in percentages of cycling cells. In any case, however, the experiments reported here emphasize the importance of cell density as a variable in studies of the LDL receptor. They may also have relevance to an understanding of the pathogenesis of the atherosclerotic lesion in which cells stimulated to migrate and proliferate in response to vascular injury accumulate large amounts of cholesterol (19, 20).
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