Inefficient Internalization of Receptor-bound Low Density Lipoprotein in Human Carcinoma A-431 Cells

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ABSTRACT

Human epithelioid carcinoma A-431 cells are known to express unusually large numbers of receptors for the polypeptide hormone epidermal growth factor. The current studies demonstrate that this cell line also expresses 5- to 10-fold more low density lipoprotein (LDL) receptors per cell than either human fibroblasts or Chinese hamster ovary (CHO) cells. As visualized with an LDL-ferritin conjugate, the LDL receptors in A-431 cells appeared in clusters that were distributed uniformly over the cell surface, occurring over flat regions of the membrane as well as over the abundant surface extensions. Only 4% of the LDL receptors were located in coated pits. The LDL receptors in A-431 cells showed the same affinity and specificity as the LDL receptors in human fibroblasts and other cell types. In addition, they were subject to feedback regulation by sterols in the same manner as the LDL receptors in other cells. However, in contrast to other cell types in which the receptor-bound LDL is internalized with high efficiency, in the A-431 cells only a small fraction of the receptor-bound LDL entered the cell. In CHO cells ~66% of the LDL receptors were located over coated regions of membrane, and the efficiency of LDL internalization was correspondingly 10-fold higher than in A-431 cells. These findings support the concept that the rate of LDL internalization is proportional to the number of LDL receptors in coated pits and that the inefficiency of internalization in the A-431 cells is caused by a limitation in the ability of these cells to incorporate their LDL receptors into coated pits.
involve coated pits that appear identical to those that mediate LDL uptake (reviewed in reference 9).

Haigler et al. (16, 17) have recently introduced the A-431 line of cultured human epithelial carcinoma cells as a tool for the detailed study of receptor-mediated endocytosis. These cells exhibit a marked increase in the number of epidermal growth factor (EGF) receptors per cell as compared with human fibroblasts (8, 16, 17). Moreover, in contrast to human fibroblasts, which show a preferential localization of EGF receptors in coated pits (15), the EGF receptors in the A-431 cells appear to be distributed diffusely over the cell surface (16, 17).

In the current studies, we have carried out a combined biochemical and ultrastructural analysis of receptor-mediated endocytosis of LDL in the A-431 cells. The data indicate that the A-431 cells express an increased number of LDL receptors as compared with human fibroblasts and that, as with EGF receptors, the LDL receptors are diffusely distributed over the cell surface without preferential localization in coated pits. Inasmuch as only a small proportion of the LDL receptors are located in coated pits, the efficiency of LDL internalization is markedly reduced in the A-431 cells as compared with other cell types, such as human fibroblasts and Chinese hamster ovary (CHO) cells, in which the LDL receptors are preferentially clustered in coated pits.

MATERIALS AND METHODS

Materials

Human epidermoid carcinoma A-431 cells (8) were a generous gift from Dr. Stanley Cohen. Chinese hamster ovary cells (CHO cells) were obtained from the American Type Culture Collection, Rockville, Md. (catalogue no. CCL 61). Sodium [125I]iodide ([125I]-iodide (11–16 mCi/g) and [1-14C]oleic acid (56 mCi/mmol) were obtained from New England Nuclear, Boston, Mass. Fetal calf serum was purchased from Flow Laboratories, Inc., Rockville, Md. Dulbecco's Modified Eagle's Medium (DMEM, catalogue no. 320-1285), powderied Eagle's minimum essential medium (catalogue no. 410-1100), powdered F-12 (Ham) medium (catalogue no. 430-1700), and 1 x trypsin/EDTA solution (catalogue no. 610-5300) were obtained from Grand Island Biological Co., Grand Island, N. Y. HEPES was obtained from Sigma Chemical Co., St. Louis, Mo. Other supplies were obtained from previously reported sources (1, 4, 14).

Lipoproteins

Human LDL (d. 1.019–1.063 g/ml), HLDL (d. 1.120–1.215 g/ml), and low-density lipoprotein-deficient serum (d. >1.215 g/ml) were obtained from the plasma of individual healthy subjects and prepared by ultracentrifugation (4). For LDL and HLDL, the mass ratio of total cholesterol/protein was 1.5:1 and 1.4, respectively. LDL was radiolabeled with [125I] and LDL was coupled to ferritin (2), as described in the indicated references. The concentration of LDL-ferritin is expressed in terms of its content of LDL-protein (4).

Cultured Cells

Stock cultures of A-431 cells were maintained in monolayer culture in a humidified incubator (5% CO2) at 37°C in 250-ml flasks containing medium A (DMEM supplemented with penicillin [100 U/ml], streptomycin [100 μg/ml], and 10% fetal calf serum). All experiments were carried out using a similar format. Confluent monolayers of cells from stock cultures were dissociated by incubation at 37°C with trypsin-EDTA solution. Cells were seeded (day 0) at a concentration of 5.0-7.5 x 105 cells/dish in Falcon petri dishes (60 x 15 mm; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) containing 3 ml of medium A. On day 2 or 3, each monolayer was washed with 3 ml of phosphate-buffered saline, after which 3 ml of medium B (DMEM supplemented with penicillin, streptomycin, and 10% human lipoprotein-deficient serum) was added. Experiments were initiated on day 4 or 5 after the cells had incubated 48 h in the absence of lipoproteins.

CHO cells were grown in monolayer culture as previously described (14). Confluent monolayers of cells from stock cultures were dissociated by incubation at 37°C with trypsin-EDTA solution. Cells were seeded (day 0) at a concentration of 1 x 106 cells/dish into Falcon petri dishes (60 x 15 mm) containing 3 ml of medium C (Ham's F-12 medium supplemented with penicillin, streptomycin, and 10% fetal calf serum). On day 2, each monolayer received 3 ml of medium D (Ham's F-12 medium supplemented with penicillin, streptomycin, and 10% human lipoprotein-deficient serum). Experiments were initiated on day 4 after the cells had incubated for 48 h in the absence of lipoproteins.

Although attempts were made to fully induce the LDL receptors before each experiment, there was nevertheless a twofold variability in the number of receptors in each cell line from experiment to experiment.

Biochemical Assays

The amount of surface-bound [125I]-labeled LDL (dextran sulfate-releasable [125I]-LDL), internalized [125I]-LDL, dextran sulfate-resistant [125I]-LDL, and degraded [125I]-LDL were measured in intact cell cultures by described methods (10). Unless otherwise stated, degradation represents the cell-dependent rate of proteolysis and is expressed as nanograms of [125I]-labeled TCA-soluble (non-iodide) material released into the culture medium per milligram of cell protein (10). For experiments performed at 4°C, cell monolayers were incubated with medium E (Eagle's minimum essential medium without bicarbonate supplemented with 20 mM HEPES buffer, pH 7.4, and 10% human lipoprotein-deficient serum). Activity of HMG CoA reductase was measured by determining the rate of conversion of [14C]HMG CoA (11,500 cpm/nmol) to [14C]mevalonate in intact, genti-solubilized extracts (4), and enzyme activity is expressed as picomoles of [14C]mevalonate formed per minute per milligram of soluble protein. The incorporation of [14C]oleate into cholesteryl [14C]oleate by intact cells was measured as described (13), and the data are expressed as nanomoles of cholesteryl [14C]oleate formed per hour per milligram of total cell protein. The content of protein was determined by the method of Lowry et al. (18) with bovine serum albumin as a standard. The values in all figures and tables represent the average of duplicate incubations.

Electron Microscopy Procedures

Monolayers of A-431 cells and CHO cells were prepared and grown as described above. After incubation with LDL-ferritin, cells were washed and fixed as monolayers in situ with 2% glutaraldehyde in 0.1 M sodium phosphate (pH 7.3) as described (1, 2). For quantitative analysis, monolayers of cells were flat embedded in Epon as previously described (1, 2). Two samples from each monolayer were punched out and sandwiched together with Epon such that the two layers of cells faced each other. After the Epon had polymerized, sections were made perpendicular to the plane of the cell monolayers and mounted on 100- x 300-mesh grids. The number of ferritin cores was counted and the proportion associated with specific membrane structures was tabulated directly in the electron microscope (1, 2). After the quantitation, each cell was photographed, prints were made, and measurements of the surface area were taken, using a map-measuring device. Care was taken to include extended surface membrane in all measurements. In each case, at least 0.5 mm of cell surface was analyzed, and the number of ferritin cores and coated structures was normalized to 1 mm of cell surface.

For the photographs shown in Figs. 7-9, monolayers of cells were processed according to the following procedure: the cells were dehydrated in situ by treatment with increasing concentrations of ethanol, released from the surface of the plastic dish by brief treatment with propylene oxide (17), pelleted in a microfuge tube (12,000 rpm, 5 min), and embedded in Araldite.

All samples were sectioned on a Porter-Blum MT2-B ultramicrotome (DuPont Instruments, Newington, Conn.), stained with uranyl acetate and lead citrate, and viewed with a JEOL 100 CX electron microscope.

RESULTS

Binding, Internalization, and Degradation of [125I]-LDL in A-431 Cells

Large amounts of [125I]-LDL bound in a saturable fashion to the surface of A-431 cells at 4°C (Fig. 1A). Half-maximal binding occurred at an [125I]-LDL concentration of ~2 μg protein/ml, which is similar to the concentration previously obtained with human fibroblasts (10, 11). About 80% of the [125I]-LDL that was bound at 4°C could be released by subsequent treatment of the cells with dextran sulfate, a sulfated glycosaminoglycan that is known to displace [125I]-LDL from its receptors on the surface of human fibroblasts (10). In another experiment, the binding of [125I]-LDL was competed by unla-
FIGURE 1  Binding of $^{125}\text{I}-\text{LDL}$ to the cell surface of A-431 cells at $4^\circ\text{C}$. Exp. A: After incubation for 48 h in lipoprotein-deficient serum, each monolayer received 2 ml of ice-cold medium containing the indicated concentration of $^{125}\text{I}-\text{LDL}$ (141 cpm/ng protein). The cells were incubated at $4^\circ\text{C}$ for 2 h, after which the monolayers were washed by the standard procedure (10). The amounts of dextran sulfate-releasable $^{125}\text{I}-\text{LDL}$ (■) and dextran sulfate-resistant $^{125}\text{I}-\text{LDL}$ were determined. The total amount of $^{125}\text{I}-\text{LDL}$ associated with the cells (○) represents the sum of the dextran sulfate-releasable and dextran sulfate-resistant $^{125}\text{I}-\text{LDL}$. Exp. B: Specificity of surface-binding of $^{125}\text{I}-\text{LDL}$ to A-431 cells. After incubation for 48 h in lipoprotein-deficient serum, each monolayer received 2 ml of ice-cold medium containing 5 pg protein/ml of $^{125}\text{I}-\text{LDL}$ (197 cpm/ng protein) and the indicated concentration of either unlabeled LDL (■) or unlabeled HDL (▲). After incubation for 2 h at $4^\circ\text{C}$, the amount of dextran sulfate-releasable $^{125}\text{I}-\text{LDL}$ was determined.

When the A-431 cells were incubated continuously with $^{125}\text{I}-\text{LDL}$ at $37^\circ\text{C}$, the cells achieved a steady state within 2 h, at which time the amount of $^{125}\text{I}-\text{LDL}$ bound to the surface and releasable by dextran sulfate remained constant (Fig. 2A). Similarly, the amount of $^{125}\text{I}-\text{LDL}$ that could not be released by dextran sulfate, and hence was internalized, reached a steady state after ~2 h (Fig. 2B). In the steady state, the amount of intracellular $^{125}\text{I}-\text{LDL}$ (~400 ng/mg protein) was about two times greater than the amount bound to the surface (200 ng/mg protein). During this steady state, the surface-bound and internalized LDL were undergoing constant turnover, and the degradation products were released into the medium at a linear rate (Fig. 2C). The addition of the lysosomal inhibitor chloroquine at a concentration of 60 μM completely blocked $^{125}\text{I}-\text{LDL}$ degradation (Fig. 2C). As a result, the intracellular $^{125}\text{I}-\text{LDL}$ no longer reached a steady state value and continued to accumulate linearly throughout the 8-h duration of the experiment (Fig. 2B). The amount of surface-bound $^{125}\text{I}-\text{LDL}$ was reduced only slightly by the chloroquine (Fig. 2A). This combination of events, i.e., a progressive increase in the intracellular content of $^{125}\text{I}-\text{LDL}$ as a result of a complete block in intralysosomal $^{125}\text{I}-\text{LDL}$ degradation, is similar to that seen when chloroquine is added to cultured human fibroblasts (11).

Fig. 3 shows $^{125}\text{I}-\text{LDL}$ concentration curves for A-431 cells studied in the steady state, i.e., after continuous incubation with $^{125}\text{I}-\text{LDL}$ for 5 h at $37^\circ\text{C}$. As in fibroblasts, the half-maximal concentration for $^{125}\text{I}-\text{LDL}$ binding to the cell surface was ~15 μg protein/ml (Fig. 3A), a value that was ~10-fold higher than the half-maximal concentration for $^{125}\text{I}-\text{LDL}$ binding at $4^\circ\text{C}$ (Fig. 1A). At each concentration of $^{125}\text{I}-\text{LDL}$, the steady-state level of intracellular $^{125}\text{I}-\text{LDL}$ was ~1.5- to 2-fold higher than the amount bound to the cell surface (Fig. 3B). This ratio is much lower than in fibroblasts, where the steady-state level of intracellular LDL is about eight times higher than the amount bound to the surface (10, 11). At each concentration of $^{125}\text{I}-\text{LDL}$, the amount of lipoprotein that was degraded was proportional to the amount bound and internalized (Fig. 3C).

FIGURE 2  Metabolism of $^{125}\text{I}-\text{LDL}$ in A-431 cells in the absence and presence of chloroquine. After incubation for 48 h in lipoprotein-deficient serum, each monolayer received 2 ml of medium B containing 10 μg protein/ml of $^{125}\text{I}-\text{LDL}$ (91 cpm/ng protein) and either no chloroquine (○) or 60 μM chloroquine (▲). After incubation at $37^\circ\text{C}$ for the indicated time, the amounts of surface-bound $^{125}\text{I}-\text{LDL}$ (A), internalized $^{125}\text{I}-\text{LDL}$ (B), and degraded $^{125}\text{I}-\text{LDL}$ (C) were measured.

FIGURE 3  Saturation kinetics for the surface binding (A), internalization (B), and degradation (C) of $^{125}\text{I}-\text{LDL}$ in A-431 cells at $37^\circ\text{C}$. After incubation for 48 h in lipoprotein-deficient serum, each monolayer received 2 ml of medium B containing the indicated concentration of $^{125}\text{I}-\text{LDL}$ (47 cpm/ng protein). After 5 h at $37^\circ\text{C}$, the total amounts of surface-bound, internalized, and degraded $^{125}\text{I}-\text{LDL}$ were measured.
Regulatory Actions of LDL in A-431 Cells

Cholesterol released from the receptor-mediated uptake of LDL in A-431 cells had the same regulatory consequences that were observed previously in fibroblasts. Thus, the LDL suppressed the activity of HMG CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (Fig. 4 A). LDL also led to a marked stimulation of cholesterol esterification, as indicated by an increase in the rate at which the cells incorporated $[^{14}C]$oleate into cholesteryl $[^{14}C]$oleate (Fig. 4 B). The concentration range over which LDL exerted its regulatory effects was similar to the concentration range at which LDL bound to its receptor at $37 \, ^\circ C$. HDL, a cholesterol-carrying lipoprotein that is not taken up by the receptor mechanism (Fig. 1 B), did not suppress HMG CoA reductase nor did it stimulate cholesterol esterification in the A-431 cells (Fig. 4).

The LDL receptors in the A-431 cells, as in fibroblasts, were subject to feedback suppression by LDL and by exogenous sterols in a fashion similar to that of fibroblasts (11). When the cells were grown in 10% fetal calf serum, which contains LDL, the amount of surface binding of $^{125I}$-LDL was relatively low (Fig. 5 A). As a result, the rates of $^{125I}$-LDL internalization (Fig. 5 B) and degradation (Fig. 5 C) were also low. When the cells were incubated for 24 h in the absence of lipoproteins, the number of LDL receptors increased after a 3-h lag and this was reflected by a parallel fivefold increase in the amount of $^{125I}$-LDL binding, internalization, and degradation (Fig. 5 A–C). The induction of LDL receptors was blocked by the inclusion of LDL in the culture medium. It was also prevented by the presence of a mixture of 25-hydroxycholesterol and cholesterol.

Efficiency of Internalization of Receptor-bound LDL in A-431 Cells

Quantification of the efficiency of $^{125I}$-LDL internalization in fibroblasts has been achieved in previous studies by calculating an “internalization index” (12). To derive this index, the cells are incubated with $^{125I}$-LDL for 5 h at $37 \, ^\circ C$. At the end of this period, the amount of $^{125I}$-LDL bound to the surface, internalized, and degraded is determined. The sum of the internalized and degraded $^{125I}$-LDL represents the total amount of LDL that has been transferred from the surface receptor into the cell during the experiment. Hence, the sum of degraded plus internalized $^{125I}$-LDL divided by the surface-bound $^{125I}$-LDL is proportional to the efficiency with which the receptors transfer their LDL into the cell. This quotient is designated as the internalization index. In a large number of normal human fibroblast strains, this value averages ~35, ranging from 20 to 45 (reference 12 and unpublished data), suggesting that each LDL receptor mediates the internalization of ~35 particles of LDL in 5 h at $37 \, ^\circ C$.

As shown in Table I, the efficiency of internalization of $^{125I}$-LDL in the A-431 cells, as judged by the internalization index, was much lower than observed previously in fibroblasts. In the A-431 cells, the index averaged ~7 (Table I). The internalization index was the same whether the cells were sparse or confluent, as judged by the amount of protein per dish (Table I).

Comparison of Internalization of Receptor-bound LDL in A-431 Cells and CHO Cells

For comparative purposes, we also studied the internalization of receptor-bound $^{125I}$-LDL in CHO cells, a line of transformed cells with a growth rate that is similar to the rapid growth rate of the A-431 cells. The CHO cells were demonstrated previously to possess functional high-affinity LDL receptors that mediate the uptake of LDL and thereby deliver cholesterol to the cell (14).

Table II shows an experiment that compares the binding, internalization, and degradation of $^{125I}$-LDL in A-431 cells and in CHO cells after incubation with $^{125I}$-LDL for 5 h at $37 \, ^\circ C$. In the CHO cells, the steady-state level of $^{125I}$-LDL binding was only ~10% of the amount observed in the A-431 cells (45 ng/mg protein vs. 394 ng/mg protein). On the other hand, the steady-state level of intracellular $^{125I}$-LDL was only ~50%
lower in the CHO cells. Moreover, the amount of $^{125}$I-LDL degraded during the 5-h period was actually slightly higher in the CHO cells than in the A-431 cells. The increased rate of internalization and degradation relative to binding in the CHO cells was reflected in the internalization index, which was nearly 10-fold higher in the CHO cells than in the A-431 cells.

The low efficiency of LDL internalization in the A-431 cells compared with the CHO cells was not directly related to the larger number of LDL receptors in the A-431 cells. To exclude this possibility, we incubated a group of A-431 cells in the presence of fetal calf serum, which suppressed the number of LDL receptors by ~80%. The rates of internalization and degradation were reduced by a similar percentage, so that the internalization index remained essentially the same as it was when the cells were grown in lipoprotein-deficient serum (Table II). Thus, even when the CHO cells and the A-431 cells were made to express a similar number of LDL receptors, the efficiency of internalization in the A-431 cells was reduced as compared with the CHO cells.

The inefficient internalization of $^{125}$I-LDL in the A-431 cells relative to the CHO cells was also apparent when the cells were subjected to a warmup experiment (Fig. 6). In this type of experiment, the cells are incubated with $^{125}$I-LDL at 0°-4°C to allow the LDL to bind to the receptor, after which the cells are washed and warmed to 37°C. After various times at 37°C, groups of dishes are rapidly chilled to stop the internalization, and the amount of $^{125}$I-LDL remaining on the surface is measured as TCA-precipitable material released into the medium during the warmup period at 37°C.

The amount of degraded $^{125}$I-LDL is measured as the TCA-soluble material released into the medium during this period.

The results of the warmup experiment in the CHO cells (Fig. 6B) differed from those in the A-431 cells (Fig. 6A) in three respects. First, at zero time before warming, a significantly higher percentage (50%) of the total cell-associated radiactiv-
was removed, and its content of duplicate dishes were transferred to a 4°C cold room, the medium and all dishes were incubated at 37°C. After the indicated interval, warm medium B containing 10 pg of protein/ml of unlabeled LDL, to the standard procedure (10). Each dish then received 2 ml of medium E containing either 10 pg of protein/ml of lipoprotein-deficient serum, each prechilled monolayer received 2

**FIGURE 6**

Internalization and degradation at 37°C of 125I-LDL previously bound to the cell surface at 4°C in A-431 cells (exp. A) and at 0°C in CHO cells (exp. B). Exp. A. After incubation for 48 h in lipoprotein-deficient serum, each prechilled monolayer received 2 ml of medium E containing either 10 μg of protein/ml of unlabeled LDL (338 cpm/ng protein). The 125I-LDL was allowed to bind to the cells at 4°C for 2 h, after which each monolayer was washed according to the standard procedure (10). Each dish then received 2 ml of warm medium B containing 10 μg of protein/ml of unlabeled LDL and all dishes were incubated at 37°C. After the indicated interval, duplicate dishes were transferred to a 4°C cold room, the medium was removed, and its content of 125I-labeled TCA-soluble material was measured (1). The amount of 125I-LDL that dissociated from the cell surface was determined by measuring the amount of 125I-labeled TCA-precipitable material (2). The amount of surface-bound 125I-LDL (3) and intracellular 125I-LDL (4) were determined by the dextran sulfate-release technique. Exp. B. The protocol for this warmup experiment was identical to that of exp. A, except that the CHO cells were incubated with 10 μg of protein/ml of 125I-LDL (447 cpm/ng protein) for 2 h in a 0°C ice bath. In both exps. A and B, >90% of the dextran sulfate-releasable and -resistant 125I-LDL at zero time represented high-affinity binding as demonstrated by susceptibility to competition with a 50-fold excess of unlabeled LDL.

By 15 min after warmup, 63% and 54% of the surface-bound 125I-LDL dissociated from the cells in the A-431 and CHO cells, respectively.

**Visualization of LDL Receptors in A-431 Cells**

To understand the reason for the relative inefficiency in the internalization of receptor-bound LDL in the A-431 cells, we performed electron microscopy on cells that had been exposed to LDL covalently coupled to ferritin (LDL-ferritin). So that the plane of section could be oriented perpendicular to the plane of the cell substratum, the cells were fixed in situ in the culture dish and embedded as a monolayer. The A-431 cells viewed at this angle appeared tightly adherent to the substratum and often grew in multiple layers. The surfaces of those cells that faced the culture fluid environment (the apical layer) were distinguished by the presence of numerous surface extensions that in some cases were several micrometers in length. Previous studies in the A-431 cells have shown that the majority of these surface extensions are plicae with a few being microvilli (17).

A-431 cells that had been exposed to LDL-ferritin for 2 h at 4°C and fixed immediately exhibited ferritin particles that were distributed over the apical surface of the cell. Ferritin was not evenly distributed over the surface, but rather occurred in large clusters, measuring up to 35 ferritin particles per cluster (Fig. 7A). Some of the ferritins within a cluster were separated from the membrane by distances up to 120 nm. These clusters of LDL-ferritin were not confined to any specialized regions of the cell surface, occurring on surface extensions, between surface extensions, and over coated pits. Very little LDL-ferritin was seen on the basal surface of these cells (Fig. 8B, b surface). This implies that most of the receptors accessible to LDL were confined to the apical surface of the topmost cell layer. Specificity of the LDL-ferritin binding to the plasma membrane of A-431 cells was demonstrated by showing that a 25-fold excess of native LDL in the culture medium completely prevented cell surface binding of LDL-ferritin, including the large clusters.

To study the route of internalization of LDL, A-431 cells that were labeled with LDL-ferritin for 2 h at 4°C were warmed to 37°C for various periods of time and processed for electron microscopy. After short periods of warming, LDL-ferritin was seen either localized to the cell surface or to coated endocytic vesicles (Figs. 7B and C and 8A and B). Only rarely was ferritin seen within smooth vesicles adjacent to the plasma membrane (Fig. 8A). With further time at 37°C, ferritin accumulated in multivesicular bodies (Fig. 7D). Within the multivesicular bodies, ferritin was preferentially associated with the vesicular elements. Even after 20 min at 37°C, abundant LDL-ferritin was still present in the apical surface. No evidence of further clustering of LDL-ferritin was seen; the average number of LDL-ferritins per cluster was approximately the same after 20 min at 37°C as after zero time at 37°C.

To quantify these ultrastructural observations, segments of the cell surface were analyzed quantitatively for the occurrence of ferritin cores (Table III). When the A-431 cells were incubated with LDL-ferritin at 4°C, and then fixed without warmup, there were 2,760 ferritin cores bound per millimeter of cell surface. Each millimeter of cell surface also contained ~20 coated regions, all of which showed the typical morphology of coated pits. By direct count, 4% of the bound ferritin was associated with coated pits. Inasmuch as 80% of the coated...
FIGURE 7  Electron micrographs showing the distribution of LDL-ferritin in A-431 cells during endocytosis. After incubation for 48 h in lipoprotein-deficient serum, each prechilled monolayer received 2 ml of ice-cold medium E containing 20 µg of LDL-ferritin/ml. After incubation at 4°C for 2 h, the cells were washed extensively (1) and then warmed to 37°C for various times as described in the legend to Fig. 6. (A) The apical surface of an A-431 cell that had been incubated with LDL-ferritin at 4°C and fixed with 2% glutaraldehyde in phosphate buffer without warming to 37°C. LDL-ferritin is seen to be distributed in clusters of up to 35 ferritin particles on surface extensions. The plane of section is tangential through the cortex of the cell and has captured the base of a coated pit (arrow) that contains LDL-ferritin (time at 37°C, 0 min). The inclusion of a 25-fold excess of native LDL in the culture medium completely prevented the cell surface binding of LDL-ferritin, confirming the specificity of the LDL-ferritin binding sites. × 51,500. (B) A coated pit labeled with LDL-ferritin (time at 37°C, 0 min). × 106,000. (C) A coated endocytic vesicle labeled with LDL-ferritin (time at 37°C, 5 min). × 113,000. (D) Two multivesicular bodies containing large amounts of LDL-ferritin. Note that the LDL-ferritin is associated primarily with the vesicular elements of the multivesicular body (time at 37°C, 20 min). × 67,500.
Electron micrographs showing the distribution of LDL-ferritin in A-431 cells during endocytosis. After incubation for 48 h in lipoprotein-deficient serum, each prechilled monolayer received 2 ml of ice-cold medium E containing 20 μg of LDL-ferritin/ml. After incubation at 4°C for 2 h, the cells were washed extensively (1) and then warmed to 37°C for 5 min as described in the legend to Fig. 6. (A) The distribution of LDL-ferritin on the apical surface of A-431 cells. LDL-ferritin is typically found associated with surface extensions, nonextended membrane, and with coated pits (arrows). × 30,000. (B) In some cells, endocytosis has progressed so that multiple-coated endocytic vesicles are visible (arrows). LDL-ferritin in noncoated regions is still distributed as in Fig. 7 A. The upper part of the micrograph (b surface) shows the basal surface of an A-431 cell. Note the relative paucity of LDL-ferritin on this surface compared to the apical surface (a surface) of the adjacent cell. × 57,500.
extensions. As shown in Fig. 9, when the CHO cells were incubated with LDL-ferritin, no ferritin was seen to be bound to surface extensions. Instead, most of the bound ferritin was located in coated pits or coated vesicles (Fig. 9A and B). The presence of LDL-ferritin in coated vesicles after incubation at 4°C suggests that some of the coated pits may have been deeply invaginated into the cell so that in tangential sections they appeared as coated vesicles (Fig. 9C). Alternatively, some of the pits may have pinched off to form coated vesicles, even at 4°C. The coated pits containing LDL-ferritin were found on both the upper and lower surfaces of the cell (Fig. 9E). After brief periods of warmup, LDL-ferritin was seen to be in coated vesicles. After 20 min of warmup, abundant LDL-ferritin particles were seen within lysosomes (Fig. 9D). Unlike those of the A-431 cells, the lysosomes of the CHO cells did not show the morphology of multivesicular bodies.

The bound LDL-ferritin was quantified in the CHO cells in the same manner as in the A-431 cells (Table III). In agreement with the 125I-LDL binding studies, the amount of surface-bound LDL-ferritin in the CHO cells was only 10% of that in the A-431 cells (230 vs. 2,760 ferritin cores per millimeter of cell surface). However, unlike the case of A-431 cells, 66% of the LDL-ferritin bound to the CHO cells at 4°C was associated with coated regions of membrane. Coated regions included both recognizable surface coated pits and apparent coated vesicles. However, because these coated vesicles contained LDL-ferritin after incubation at 4°C, for purposes of quantitation they are considered to be coated pits. We measured ~37 coated regions per millimeter of cell surface and 66% of such regions contained ferritin. As with the A-431 cells, there was an average of six ferritin cores per coated region. When the cells were warmed to 37°C for 20 min, the ferritin cores were nearly completely cleared from the cell surface, and none of the coated pits were labeled with ferritin cores. It is of interest that although the total number of ferritin cores bound per millimeter of cell surface was 10-fold greater in the A-431 cells than in the CHO cells, the number per coated pit was approximately the same in the two cell types. Inasmuch as the absolute rate of internalization of 125I-LDL is similar in the CHO cells and A-431 cells under these conditions (Table II), it is clear that the rate of internalization correlates with the number of LDL-ferritin particles bound in coated pits and not with the total number bound to the cell surface.

It should be noted that the finding of LDL-ferritin in apparent coated vesicles at 4°C in the CHO cells may be unique to this cell type, in that we have never observed LDL-ferritin in coated vesicles at 4°C in human fibroblasts, human lymphocytes, or human A-431 cells. Further studies will be necessary to determine whether endocytosis can occur at 4°C in the CHO cells or whether the apparent coated vesicles represent deeply invaginated coated pits.

In the above ultrastructural experiments, specificity of the LDL-ferritin binding to the plasma membrane of CHO cells was assured, as was done with the A-431 cells, by demonstrating that a 25-fold excess of native LDL completely prevented cell surface binding and uptake of LDL-ferritin.

**DISCUSSION**

In the current studies, the receptor-mediated endocytosis of LDL in human carcinoma A-431 cells differed in three major respects from that in cultured human fibroblasts and in CHO cells. (a) The A-431 cells expressed a larger number of LDL-binding sites when compared with either of the other two cell types. When incubated with LDL-ferritin at 4°C, the fully

| Cell type | Time at 37°C (min) | Coated regions (number/mm (number/mm | Ferritin cores bound | Percent bound ferritin associated with coated regions | Percent coated regions labeled with ferritin cores |
|-----------|------------------|-------------------------------------|---------------------|---------------------------------------------------|--------------------------------------------------|
| A-431 cells | 0 | 20 | 2,760 | 110 | 4 | 80 |
| CHO cells | 0 | 37* | 230 | 152 | 66 | 65‡ |

* Of the 37 coated regions, 22 appeared as typical coated pits on the cell surface and 15 appeared as apparent coated vesicles that contained LDL-ferritin at 4°C (see text).

‡ 55% of the surface coated pits and 77% of the apparent coated vesicles were labeled with LDL-ferritin at 4°C (see text).
Figure 9  Electron micrographs showing the distribution of LDL-ferritin in CHO cells during endocytosis. After incubation for 48 h in lipoprotein-deficient serum, each prechilled monolayer received 2 ml of ice-cold medium containing 20 μg of LDL-ferritin/ml. After incubation at 4°C for 2 h, the cells were washed extensively (1) and then warmed to 37°C for the indicated time as described in the legend to Fig. 6. (A) A typical view of the surface of the CHO cell that was incubated with LDL-ferritin for 2 h at 4°C. Both a coated pit (solid arrow) and an apparent coated vesicle (open arrow) are seen to be labeled with LDL-ferritin. Very little LDL-ferritin is seen on the rest of the cell surface. Apparent coated vesicles labeled with LDL-ferritin are frequently seen in CHO cells that have been maintained for 2 h at 4°C, suggesting that the surface-coated pits are deeply invaginated and can be sectioned such that they appear as coated vesicles (time at 37°C, 0 min). × 35,500. (B) High-magnification view of a coated pit labeled with LDL-ferritin (time at 37°C, 0 min). × 86,500. (C) High magnification view of an apparent coated vesicle containing LDL-ferritin (time at 37°C, 0 min). × 107,000. (D) Typical appearance of LDL-ferritin in the lysosomal compartment (time at 37°C, 20 min). × 91,500. (E) Low-power electron micrograph showing the association of LDL-ferritin with coated pits on the apical (a) and basal (b) surfaces of the same cell. Note the deeply invaginated nature of these two coated pits (time at 37°C, 0 min). × 105,000.
induced A-431 cells bound ~2,700 ferritin cores/mm of cell surface as compared with 90–380 cores/mm in fully induced cultured human fibroblasts (1, 3) and 230 cores/mm in CHO cells (Table III). When the data are expressed as the nanograms of 125I-LDL bound per milligram of cell protein, the A-431 cells bound about fourfold more 125I-LDL than human fibroblasts and ninefold more than CHO cells (3, 10). (b) The distribution of the bound LDL-ferritin in the A-431 cells differed from that in the two other cell types. In human fibroblasts the LDL-ferritin is observed as small clusters of particles, 50–80% of which are located over coated pits at 4°C (1–3, 7). Similar findings were obtained with CHO cells in the current study. On the other hand, in the A-431 cells much of the LDL-ferritin was present as large clusters containing up to 35 ferritin particles, located at various sites on the cell surface, especially on the surface extensions. Small clusters of LDL-ferritin particles, consisting of about seven ferritin particles, were also found in coated pits, but these accounted for only 4% of the total ferritin particles bound to the A-431 cells. (c) The A-431 cells internalized a much smaller percentage of surface-bound LDL than did the CHO cells or fibroblasts.

The receptors that were located both in the coated pits and on the surface extensions of the A-431 cells were bona fide LDL receptors, as evidenced by the observation that both were susceptible to competition by excess unlabeled LDL. Moreover, when the cells were grown with 25-hydroxycholesterol plus cholesterol, the number of LDL receptors declined by 80%. Inasmuch as only 4% of the receptors are in coated pits, the 80% decline indicates that the receptors outside the coated pits must have been suppressed. Thus, these non–coated pit receptors are subject to the same regulation as the receptors of human fibroblasts, most of which are in coated pits.

The large clusters of LDL-ferritin in the A-431 cells were formed during the cell surface binding and were not the result of self-aggregation of the LDL-ferritin in solution, because the same LDL-ferritin preparations did not bind in large clusters to CHO cells or human fibroblasts. Moreover, the occurrence of large clusters of LDL-ferritin correlated with the relatively large amount of 125I-LDL binding in the A-431 cells, suggesting that the LDL-ferritin and the 125I-LDL were behaving similarly.

Kinetic data suggest that the inefficient internalization of LDL in the A-431 cells occurs because the LDL receptors that are outside of the coated pits are not able to transfer their bound ligand into the cell. This conclusion is supported by the following two lines of evidence: (a) The steady-state internalization index, i.e., the nanograms of 125I-LDL taken into the cell at 5 h per nanogram of 125I-LDL bound, was markedly reduced in the A-431 cells as compared with human fibroblasts and with CHO cells, indicating that most of the receptors in the A-431 cells did not promote internalization of their bound LDL. (b) Electron microscopy studies showed that only a small fraction of surface-bound LDL-ferritin entered the A-431 cells after warmup at 37°C and that this entry was associated with a clearing of receptors from the coated pits, but not from the rest of the membrane.

The reason for the failure of the LDL receptors on the surface extension to be internalized with high efficiency in the A-431 cells is apparently related to the failure of these receptors to migrate to coated pits. A possible explanation lies in the observation that much of the LDL-ferritin appeared to be bound to receptor sites that were somehow located several nanometers away from the plasma membrane. These binding sites might represent receptors that have been shed from the surface and then bound to the glyocalyx. Inasmuch as these receptors appear unable to migrate to coated pits, they resemble in a functional sense the genetically altered receptors produced by the mutant J. D. strain of human fibroblasts in which the LDL receptors can bind LDL but are unable to associate with coated pits (3).

Evidence indicates that this inefficient internalization of receptor-bound LDL in the A-431 cells is not attributable to a rapid dissociation of the LDL from the receptor before the ligand can be internalized. For example, as shown in Fig. 3, the steady-state internalization index at 37°C was equally low at all concentrations of 125I-LDL and it persisted even when the concentration of 125I-LDL was fourfold above a saturating level for the receptor and when all the receptor sites were therefore occupied. Moreover, the direct warmup experiment of Fig. 6 showed that the surface-bound 125I-LDL did not dissociate more rapidly from the A-431 cells as compared with the CHO cells.

From close inspection of the A-431 cells, it was our qualitative conclusion that the vast majority of the LDL-ferritin particles that entered the cells did so through coated pits and coated vesicles. This conclusion was supported by the finding that the absolute rate of internalization of LDL in the A-431 cells, CHO cells, and human fibroblasts was similar. This similarity correlated with the similarity in the number of LDL receptors in coated pits among the different cell lines and did not correlate with the total number of receptors on the cell surface.

In general, throughout the current studies, a good correlation was apparent between the 125I-LDL biochemical experiments and the LDL-ferritin ultrastructural studies. For example, in the CHO cells, the biochemical studies showed that 50% of the 125I-LDL that was bound at 4°C was resistant to dextran sulfate release (Fig. 6B). Similarly, the ultrastructural studies showed that 40% of the LDL-ferritin bound at 4°C was present in intracellular structures that appeared to be either coated vesicles or deeply invaginated coated pits where the connection with the cell surface was out of the plane of section (Table III). In the A-431 cells, ~90% of the bound 125I-LDL at 4°C was releasable by dextran sulfate and this correlated with an absence of intracellular LDL-ferritin at 4°C. One discrepancy between the 125I-LDL and LDL-ferritin studies was noted, however. When the A-431 cells were warmed to 37°C for 20 min, 60% of the surface-bound LDL-ferritin remained on the cell surface (Table III), whereas only 20% of the receptor-bound 125I-LDL remained on the cell surface (Fig. 6A). This difference was consistent in several experiments using both techniques. The explanation for the difference is not known, but the most likely possibility is that the LDL-ferritin may not dissociate from the cell surface as readily as does 125I-LDL when the A-431 cells are warmed to 37°C.
EGF entered the cell in coated vesicles that subsequently shed their coats. Another possibility is that the EGF entered the A-431 cells in noncoated vesicles as a result of the EGF-induced stimulation of fluid-phase pinocytosis that occurs in these cells (16).

An important additional point to emerge from the current studies is the similarity in the number of coated membrane regions per millimeter of cell surface in cells as different as A-431 cells (20 coated pits/mm), CHO cells (22 typical coated pits plus 15 apparently invaginated coated pits/mm), and human fibroblasts (25 coated pits/mm). In each of these cell types, the coated pits were counted after the cells had been grown in lipoprotein-deficient serum and incubated for several hours at 4°C. In each case, when the cells were warmed to 37°C, the number of coated pits declined by ~50% (Table III and reference 1). The constancy in the number of coated pits suggests that these structures play a similar and fundamental role in all of these cells and that the total rate of receptor-mediated endocytosis through coated regions may be relatively constant for a wide variety of cells in culture.

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