Display of Low Density Lipoprotein Receptors Is Clustered, Not Dispersed, in Fibroblast and Hepatocyte Plasma Membranes

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Although the principal details of low density lipoprotein (LDL) uptake by receptor-mediated endocytosis and its subsequent intracellular fate have been thoroughly investigated, an aspect of this mechanism that continues to provoke controversy concerns the manner of display of LDL receptors upon their initial insertion at the cell surface. While our studies based on electron microscopy of platinum/carbon replicas of gold-labeled cells have previously suggested a clustered display pattern, others have concluded, before and since, that LDL receptors are inserted individually at random widely dispersed sites in the plasma membrane. In this article, we present a series of experiments designed to discriminate between these competing hypotheses. In addition to the use of LDL–colloidal gold complexes, visualized electron microscopically, on cells subjected to a variety of experimental procedures, these experiments include the application of anti–apolipoprotein B-100 antibodies, anti-LDL-receptor antibodies, and direct visualization of native (unlabeled) LDL molecules at the cell surface. All results point to a loose-cluster arrangement, not one involving widely dispersed individual units, as the initial display pattern of newly inserted LDL receptors. A comparison of LDL and β-very low density lipoprotein receptor distribution in fibroblasts and hepatocytes suggests that this cluster pattern is a characteristic of the LDL (apolipoprotein B/E) receptor across cell types, but that the closely related apolipoprotein E receptor differs in that it is inserted individually in a highly dispersed state, in common with a variety of other receptor types. (Arteriosclerosis and Thrombosis 1991;11:261–271)

The gold label–surface replica technique, introduced in the early 1980s, has done much to extend our understanding of the properties of cell surface receptors. Of the new theories that have emerged through the application of this technique, one in particular stands out as being contentious. This concerns the spatial distribution of newly inserted low density lipoprotein (LDL) receptors in cultured fibroblasts. Interest in this issue stems from the pivotal part it plays in determining how LDL receptors are introduced and displayed in the plasma membrane before ligand binding, and how, after receptor–ligand internalization, they are recycled back to the cell surface.

The earlier consensus view was that LDL receptors were initially inserted individually at numerous random sites in the plasma membrane, their aggregation into clathrin-coated pits subsequently occurring by lateral migration over substantial distances. Experiments exploiting the technical improvements afforded by the gold label–surface replication technique revealed a very different picture, however. These have consistently suggested that in the fibroblast, initial insertion and display occur predominantly as "plaques" or groups of loosely associated receptors and not, as previously believed, in the form of individual, widely dispersed receptors. In view of the evidence repeatedly reinforcing this conclusion, it has been something of a surprise to see it disputed and challenged. Notable among the objections that have recently been voiced are the following. Wofsy et al argued that LDL–gold complexes, owing to their highly "multivalent" nature, might be expected to bind preferentially to aggregated receptors rather than to single ones. Put another way, the large, heavy LDL–gold complex could, it was supposed, easily become detached from single receptors during washing, but would far less easily be...
removed if bound to several receptors in a cluster. The net result would be a bias in the observed labeling patterns in favor of aggregates rather than solitary gold particles. A second objection proposed by the same authors was that the multivalent property of the complexes could directly alter the native distribution of receptors, causing them to aggregate artificially upon ligand binding, in a manner akin to capping. These objections notwithstanding, another group, using gold labeling techniques and whole-mounted fibroblasts, has claimed that LDL receptors in fibroblasts are, after all, inserted individually and displayed initially in a dispersed state, as originally envisaged. In this case, the discordancy with our data was attributed to preaggregation of our LDL–gold complexes (giving clusters of gold where only one receptor was present) and/or to the use of a temperature that was insufficiently low to arrest endocytosis.

In view of these conflicting reports, we have designed further experiments using immunocytochemical techniques to test the alternative interpretations of our original results and to take this opportunity to comment on some of the data of others that are purported to contradict them. As part of the study, we compared the labeling patterns of LDL–gold and β-very low density lipoprotein (VLDL)–gold complexes at the surfaces of fibroblasts and hepatocytes, and this investigation has also provided new information on differences in the display patterns of the LDL (apolipoprotein [apo] B/E) and apo E receptors.

Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Boehringer, Mannheim, F.R.G. Fetal calf serum (FCS), penicillin, streptomycin, L-glutamine, and trypsin/EDTA were obtained from Gibco BRL, Eggenstein, F.R.G. Protein A was purchased from Sigma Chemical Co., Munich, F.R.G., and goat anti-mouse immunoglobulin G (IgG, heavy and light chain) from Dianova, Hamburg, F.R.G. Monoclonal mouse anti-human LDL-receptor antibodies (G-C7) were prepared and characterized by Beisiegel et al and kindly provided by G. Schmitz, University of Münster, Münster, F.R.G. Rabbit anti-human apo B-100 antibodies were prepared by V.W. Armstrong, University of Göttingen, Göttingen, F.R.G. Specificity was tested in double gel diffusion experiments. Immunoprecipitation bands were only observed between anti-apo B-100 and LDL. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt, F.R.G., or Sigma.

Lipoproteins

LDL (d = 1.019–1.063 g/ml) was isolated from human serum of individual normolipidemic volunteers, and β-VLDL (d = 1.006 g/ml) from serum of rabbits fed a cholesterol-rich diet (Altromin, Lage, F.R.G.). Lipoproteins were isolated by sequential ultracentrifugation in a Beckman L8-70 ultracentrifuge, Beckman Instruments, Palo Alto, Calif., with a 70 Ti rotor at 69,000 rpm at 4°C for 24 hours. The lipoprotein fractions were dialyzed against 0.15 M NaCl, 5 mM NaEDTA, pH 7.4, at 4°C.

All the lipoprotein concentrations are given in terms of their protein content determined by a modified Lowry method using bovine serum albumin (BSA) as a standard.

Preparation of Gold Conjugates

Monodisperse gold sols of 15-nm-diameter gold particles were prepared by reduction of chloroauric acid with sodium citrate as a reducing agent according to the method of Frens. Conjugation of LDL to the gold particles was performed according to the method described previously. Briefly, the optimal amount of LDL particles necessary to protect a given volume of gold sol against flocculation after addition of 10% NaCl was determined by adding increasing quantities of protein, diluted to a final salt concentration of 0.005 M. For the conjugation procedure, performed at pH 5.3, a 10% excess of LDL was used. Excess unconjugated lipoproteins were removed by two centrifugation steps at 12,000g for 30 minutes.

Binding of gold particles to the β-VLDL particles was accomplished by rapidly mixing 5 ml colloidal gold, pH 5.7, with 100 µg β-VLDL. After conjugation, the β-VLDL–gold conjugates were centrifuged at 10,000g for 30 minutes.

The quality of the lipoprotein–gold conjugates was checked routinely by negative staining. The conjugates were used on the same day of preparation.

Protein A–gold conjugates were prepared, following the protocol of Roth et al, and binding of goat anti-mouse IgG to gold particles was performed according to the protocol of De Mey. These gold conjugates could be stored at 4°C for up to 4 weeks in phosphate-buffered saline (PBS), 1% BSA, and 0.02% azide, with no loss of immunoreactivity. Protein A–gold conjugates were routinely centrifuged in an Eppendorf centrifuge at 10,000 rpm for 5 minutes, and the supernatant was examined by negative staining before use. This ensured removal of any aggregates that might have formed during storage.

Cell Culture

Human skin fibroblasts from healthy donors were grown in DMEM supplemented with 10% FCS, penicillin (100 IU/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C and used between passages 4–10.

Primary cultures of rat hepatocytes were kindly provided by R. Gebhardt, University of Tübingen, Tübingen, F.R.G. Culture medium consisted of W/AB 77 medium supplemented with 10% FCS, penicillin (50 IU/ml), and streptomycin (50 µg/ml). For the experiments, cells were plated on 35×10-mm tissue culture dishes, incubated for 12
hours (fibroblasts) or 48 hours (hepatocytes) in the above media, followed by a 48-hour incubation in medium supplemented with 10% lipoprotein-deficient serum in place of the FCS for stimulating the activity of the LDL receptors. Cells used in the experiments were grown to near confluency.

**Binding of Lipoprotein—Gold Complexes**

All solutions were prechilled in ice in a refrigerator. During the experiments, the cells were stored on ice in the refrigerator. Binding experiments on fixed cells were performed at temperatures below 4°C. Before incubation with the gold conjugates, cells were given three washings (5 minutes each) with ice-cold PBS. After 1 hour of incubation with lipoprotein—gold conjugates (40 μg/ml PBS), the cells were washed intensively with PBS and fixed with ice-cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hours at less than 4°C. Throughout the entire period of fixation, the cells were kept on ice in the refrigerator. Control experiments were performed by exposing the cells first to 400 μg/ml unlabelled lipoproteins for 1 hour at below 4°C before incubation with the gold conjugates.

In a further set of experiments, cells were fixed with 2.5% PBS-buffered glutaraldehyde for 2 hours at less than 4°C. After extensive washing with PBS containing 0.2% BSA, cells were incubated with lipoprotein—gold conjugates for 1 hour at room temperature, followed by thorough washing.

**Immunocytochemistry**

For the immunocytochemical detection of cell-bound LDL or LDL receptors, cells were prechilled as described above and subsequently incubated with LDL (40 μg/ml), followed by rabbit anti-apo B-100 and protein A-gold, or with mouse anti-LDL-receptor antibody and goat anti-mouse—gold conjugates. Each incubation step was performed for 1 hour at less than 4°C and was terminated by thorough washing with PBS. Appropriate dilutions of antibodies and protein A-gold conjugates were determined by titration. Control experiments were performed by omitting either the primary antibody or LDL. After the last washing procedure, cells were fixed with cacodylate-buffered 2.5% glutaraldehyde for 2 hours.

**Surface Replication**

For surface replication, glutaraldehyde-fixed cells were dehydrated in an ethanol series and subjected to critical-point or air drying. The bottom of the culture dish with the attached cells was cut into 1-cm² pieces with a soldering iron. Platinum/carbon surface replicas of the surface of the cell monolayers were made in a Balzers BA 300 apparatus, Balzers, Liechtenstein, equipped with an electron gun evaporator and a quartz crystal thickness monitor. Replicas were obtained by shadowing the cell surface with platinum/carbon at an angle of 38°, followed by carbon at 90°.

The replicas were cleaned overnight in household bleach (sodium hypochloride, ~12% active chloride, Hedinger, Stuttgart, F.R.G.) and washed in distilled water. They were picked up on 200-mesh copper grids and examined in a Philips transmission electron microscope, Model 410, at 60 kV.

**Results**

**Low Density Lipoprotein—Gold Complexes Examined by Negative Staining**

Electron microscopic examination of the LDL—gold complexes themselves provides the starting point for evaluation of the conflicting interpretations of LDL receptor distributions. Figure 1, upper left panel, illustrates the typical appearance of a preparation of our LDL—gold complexes examined by negative staining, a routine procedure used for screening the probes before experimental use. As described previously, each gold complex is buried within a coating of LDL particles. Experimental use of the complexes depends on the principle that, when presented with them, the cell will “see” the LDL rather than the gold. That this is so in practice comes from the following experimental observations. Cells treated with gold alone are extensively labeled. Cells treated with native LDL followed by LDL—gold complexes, however, show no gold label. Therefore, in cells treated with LDL—gold complexes, all the labeling is due to the binding of LDL to the cell; none is due to the binding of gold.

From the images of negatively stained preparations, each gold particle appears to be covered with five to nine LDL particles, emphasizing the theoretical potential for multivalent interactions with receptors as discussed by Wofsy et al. The representative preparation in Figure 1, upper left panel, from a concentrated suspension, shows a random distribution of complexes, indicating a monodisperse preparation. Diluted preparations examined by negative staining show individual complexes, widely separated from one another. Strict quality control is routinely imposed to ensure reproducibility of results; any preparation that shows clumping of complexes, incomplete covering of gold with ligand, or excess unbound ligand is rejected.

**Labeling the Fibroblast Low Density Lipoprotein Receptor With Low Density Lipoprotein—Gold Complexes**

Figure 1, upper right and middle panels, are examples of results that have provoked the current controversy. They show the typical patterns of LDL receptor distribution observed in surface replicas and thin sections, respectively, after labeling with LDL—gold complexes like those shown in Figure 1, upper left panel. When fibroblasts are incubated at less than 4°C for 1 hour to arrest endocytosis and are then exposed to the LDL—gold complexes, the gold label is consistently seen in aggregates bound to the cell surface. The number of gold particles within each
Figure 1. Photomicrographs of low density lipoprotein (LDL)–gold complexes and labeling of LDL receptors in cultured human skin fibroblasts. Upper left panel: LDL–gold complexes examined by negative staining. Note that each gold particle (mean diameter, 15 nm) is completely covered by LDL molecules, which appear as white "blobs." ×61,600 Upper right panel: Surface replica of fibroblast treated with LDL–gold complexes like those in upper left panel for 1 hour at <4°C. Note that all the gold particles, marking positions of LDL receptors, occur in clusters. Almost no single gold particles are present. ×28,400 Middle panel: Thin section of fibroblast treated with LDL–gold complexes under the same conditions (1 hour, <4°C) as in upper right panel. Groups and clusters of gold particles occur both in mildly indented clathrin-coated areas and over noncoated areas. The former represent zones in which endocytosis has been initiated, and the latter represent the more recently inserted receptors. Fewer gold particles are seen per cluster in thin section than in replicas because the thinness of the sectional slices normally results in only part of the cluster being included. Nevertheless, the distribution pattern of gold is quite clearly clustered rather than dispersed. ×38,800 Lower left panel: Cluster of gold-labeled LDL receptors in a surface replica of fibroblast fed excess unlabeled LDL for 1 hour at <4°C followed by LDL–gold complexes for 4 minutes at 37°C. Note that gold particles are widely spaced within the cluster. This arrangement is typical of receptors that have just been inserted in the membrane. ×85,600 Lower middle panel: Example of a cluster of gold-labeled LDL receptors from same experiment as in lower left panel but after extending the 37°C incubation with LDL–gold to 8 minutes. By this time, receptors with their bound LDL–gold complexes have become tightly packed in readiness for endocytosis, although in this example, the membrane has not yet become deeply invaginated. In very late stages of endocytosis, gold particles can eventually appear as if on top of one another, due to steep curvature of the membrane. ×85,600 Lower right panel: Thin section from same experiment as in lower middle panel (i.e., 1 hour, <4°C, LDL; 8 minutes, 37°C, LDL–gold). Note deep invagination of coated pits containing clusters of gold. Groups of gold particles that appear to merge are in reality separated from one another and lie at different depths within the thickness of the section. ×58,400
aggregate varies, as does the density of packing of the marker within the aggregate. In replicas, solitary gold particles are seen only occasionally, and as their frequency does not differ over cell versus noncell regions, they evidently represent a low level of non-specific background labeling. In thin section (Figure 1, middle panel), inclusion of just the edge of a cluster in the section plane can also account for the occasional single particle observed in this preparation procedure.

Convincing evidence that the clusters containing loosely packed gold particles represent newly inserted LDL receptors comes from time-course experiments in which fibroblasts are first fed excess unlabeld LDL for 1 hour at less than 4°C and are then exposed for different periods of time to LDL-gold complexes at 37°C. All the receptors displayed at the cell surface at the start of the experiment bind the excess unlabeld LDL, and on warming, the receptor-LDL complexes are internalized by receptor-mediated endocytosis. As receptors are subsequently recycled back to the cell surface, the LDL-gold lies ready to bind to them, marking them immediately on their reinsertion into the plasma membrane. The first receptors to be detected in this way appear after 4 minutes in the form of clusters of loosely packed gold particles, not as individual widely dispersed particles (Figure 1, lower left panel).

After the initial binding of the LDL-gold, the receptor-ligand complexes become progressively more tightly packed and concentrated in the invaginating coated pits (Figure 1, lower middle and right panels). Neither this sequential change nor the relatively large interparticle distances in the initial clusters (Figure 1, lower left panel) fit convincingly with the “complex preaggregation” or “multivalent ligand-induced capping” hypothesis.

FIGURE 2. Photomicrograph of surface replica of fibroblast fixed in glutaraldehyde before treatment with low density lipoprotein-gold complexes. Cells were maintained at <4°C for 1 hour before fixation. Note that as in Figure 1, upper right panel, the distribution pattern of receptors is overwhelmingly clustered, not widely and individually dispersed. ×65,600

Does Multivalent Binding of Low Density Lipoprotein-Gold Complexes Cause Artificatly Aggregated Labeling Patterns?

Aldehyde-fixed fibroblasts exposed to low density lipoprotein-gold. The first of a series of experiments designed to test the multivalent hypothesis involved fixing fibroblasts with glutaraldehyde before exposing them to LDL-gold complexes. The aim was to stabilize the receptors in their native positions, thus preventing any redistribution that might occur upon binding of LDL-gold to them. As can be seen in Figure 2, the distribution of gold-labeled receptors observed with this procedure was aggregated, just as in the unfixed cell cultures (Figure 1, upper right panel). Although an apparently clear-cut result, it could nevertheless be argued that as the lipid component of membranes may remain fluid after glutaraldehyde fixation, ligand-induced redistribution of receptors cannot be completely excluded. We therefore went on to devise ways of labeling LDL receptors that did not depend on the LDL-gold complex.

Labeling the low density lipoprotein receptor using sequential exposure to low density lipoprotein, anti-apolipoprotein B-100 polyclonal antibodies, and protein A-gold. Our first alternative approach was to present LDL alone (i.e., with no gold) to the fibroblasts at less than 4°C, so that the ligand would bind to its receptors in an entirely natural manner. The idea then was to label this naturally bound LDL. This was done by using an antibody to the apo B-100 moiety of
the LDL molecule, which in turn was localized with protein A-gold complexes.

The results showed multiple clusters of gold particles and the virtual absence of solitary gold particles (Figure 3, left panel). However, the number of gold particles in the clusters was markedly lower than that observed in the experiments using LDL-gold complexes. Why this was so is unclear. The number of separate steps involved in the labeling sequence might be one factor. The binding of an LDL particle to its receptor would be predicted to lead to a reduction in the number of epitopes on the apo B-100 moiety that would otherwise be available to the antibody (by steric hindrance as well as through the direct blocking of sites involved in binding). However, in view of the large size of apo B, any such effect should, in theory, be minimal, and numerous other epitopes would be expected to remain accessible for polyclonal antibody binding. Indeed, it could be argued that if one antibody were to bind to each LDL particle, a superior labeling efficiency to that achieved with the bulkier LDL-gold complexes would result (at least where groups of receptors were closely packed). Yet, the reverse was found in practice. Whatever the explanation for this observed reduction in overall labeling efficiency, one important consequence is that the probability of detecting isolated receptors, if present in the membrane, would be markedly diminished, while that of detecting aggregates of receptors would remain high (although, of course, fewer gold particles would be found within a given cluster). On the other hand, this effect would, in theory, be at least partly compensated for by the tendency of smaller receptor aggregates to show only a few gold particles, and in some instances, just one. As observed, then, the results are fully consistent with those obtained using the LDL-gold complexes.

Labeling the fibroblast low density lipoprotein receptor with an anti-receptor monoclonal antibody. The second alternative to the LDL-gold complex was to use an antibody that binds specifically to the LDL receptors; this antibody was then localized using gold-anti-mouse IgG complexes. Again, in fibroblasts maintained for 1 hour at less than 4°C, the gold label was observed in the type of pattern typical of the earlier experiments (Figure 3, right panel). Only occasionally was a single gold particle seen, and then no more frequently over cell surfaces than over noncell regions of the replica.

This and the previous experiment provide strong evidence, but not unassailable proof, that the LDL-gold complexes in the original experiments did not induce clustering. If the LDL-gold complexes do have this action, however, then it must now be postulated that the other immunocytochemical reagents used in these alternative strategies have ex-
FIGURE 4. Photomicrograph of direct visualization of low density lipoprotein (LDL) molecules in surface replicas of fibroblasts (<4°C, 1 hour). By optimizing conditions for replica preparation, it was possible to directly visualize LDL bound to receptors without the aid of gold markers. In this experiment, cells were presented with LDL molecules among which a few LDL-gold complexes were also included. Where gold particles are seen, groups of small, distinct "bumps" are present on the membrane. These bumps, which have a size similar to the white blobs in Figure 1, upper left panel, are therefore identified as LDL molecules. ×36,800

Direct visualization of bound (unlabeled) low density lipoprotein molecules in fibroblasts. When fibroblasts (1 hour, <4°C) were exposed to LDL alone (i.e., no gold) and the conditions for preparing surface replicas were carefully optimized, groups of small particles corresponding in size to LDL molecules were clearly discernible (Figure 4). That each replicated particle represented an individual LDL molecule was confirmed by mixing an excess of unlabeled LDL with LDL-gold complexes and presenting these to the cells. The gold label was always found to be colocalized with the replicated particles, confirming their identity as LDL molecules. The individually visible LDL molecules always occurred bound to the cell surface in groups, closely resembling the distribution patterns seen with the original LDL-gold preparations (Figure 2). Careful inspection of intervening membrane areas failed to disclose single LDL particles.

Comparison of Low Density Lipoprotein and β-Very Low Density Lipoprotein Binding Sites in Fibroblasts and Hepatocytes Using Lipoprotein-Gold Complexes

To determine whether the aggregated pattern of receptor distribution repeatedly observed with the LDL receptor in fibroblasts also characterizes this and related receptors in other cell types, we compared, by gold labeling, the binding sites to LDL and β-VLDL in fibroblasts and hepatocytes. This provided a model for discriminating the specific characteristics of related apo receptor systems.

When fibroblasts (1 hour, <4°C) were treated with β-VLDL-gold complexes (Figure 5, both panels), aggregates of gold label identical in every respect to those observed in the standard LDL-gold experiments were consistently found. Hepatocytes exposed to LDL-gold under the same conditions gave precisely the same results (Figure 6, left panel). However, when hepatocytes were treated with β-VLDL-gold, again under the same conditions, an entirely different labeling pattern was seen (Figure 6, lower panel). Aggregates of gold particles were as abundant as in the fibroblast and liver/LDL-gold experiment, but in addition, a large number of individually dispersed gold particles were present. This difference can be explained by the presence of an additional apo receptor not found in the fibroblast.

Discussion

Our earlier studies on fibroblast LDL receptors led to the conclusion, contrary to the then-prevailing view, that these receptors are not inserted individually at widely dispersed sites on the cell surface. Rather, they appear to be introduced into the plasma membrane as plaques or aggregates of loosely associated receptors. This conclusion, which came
through the application of superior techniques to those that had previously suggested a widely dispersed receptor population, failed to gain general acceptance, however, and a number of objections to it have been raised.9-11

The original view that LDL receptors start their sojourn at the cell surface as individual widely dispersed units came from experiments using ferritin-LDL conjugates and thin-section electron microscopy.2-4,19 Later work with ferritin-anti-receptor antibody labeling, also examined by thin sectioning,20 did not lead to revision of this view. However, it is difficult to picture from two-dimensional views provided by perpendicular sections how the label is distributed as viewed from above the cell. The surface-replica technique entirely overcomes this problem, and the advantages of gold particles over other markers are now widely known and have been discussed in detail elsewhere.8,17,18

Although the cells may be expected to recognize and react to LDL-gold as if it were native LDL, the negative-stain photomicrographs emphasize important differences between the probes and their natural counterparts. In particular, LDL-gold complexes differ from LDL by being much heavier and bulkier and by having multiple potential sites for interaction with receptors. These features became the focus of alternative interpretations proposed by Wofsy et al,9 and it was in response to these objections that we devised the series of different approaches to labeling and viewing LDL receptors described in this report.

As a first step, we compared the labeling patterns revealed by treating fixed and unfixed fibroblasts with LDL-gold complexes. Aldehyde fixation would be expected, through its cross-linking action, to stabilize the LDL receptors in their "correct" position in the membrane, so that the risk of any redistribution due to multivalent ligand binding would be minimized. Our demonstration, that similar clustered distributions with negligible individual dispersed label occur whether or not prior fixation is performed, suggests that no redistribution is induced by LDL-gold complexes. However, redistribution after fixation cannot be completely excluded, and this experiment does not address the question of preferential labeling of receptors that are situated in clusters.

If, as proposed by Wofsy et al,9 the nature of the LDL-gold complex is responsible for the cluster patterns—either by directly inducing cluster formation or by binding more firmly to clusters than to isolated receptors—then strategies for labeling the receptor that do not depend on this complex should reveal the "true" distribution. Therefore, we devised two such strategies, one involving sequential treatment with LDL, anti-apo B-100 antibodies, and protein A-gold, and one using anti-receptor monoclonal antibodies followed by secondary antibody-gold complexes. Both gave labeling patterns equivalent to those observed with the LDL-gold complexes. The anti-receptor antibody experiment in particular excludes any LDL-induced redistribution, as this strategy did not involve the use of LDL, even in its natural (uncomplexed) form, and the antibody would
Figure 6. Photomicrograph of surface replica in upper panel shows a hepatocyte treated with low density lipoprotein (LDL)-gold complexes (1 hour, <4°C). Note that distribution pattern observed in the hepatocyte is similar to that of the fibroblast. ×28,800 Photomicrograph in lower panel shows surface replica of a hepatocyte treated with β-very low density lipoprotein-gold (1 hour, <4°C). The distribution pattern differs markedly from that of fibroblasts exposed to the same lipoprotein-gold (Figure 5, upper panel) and from that of both hepatocytes and fibroblasts treated with LDL-gold (Figure 6, upper panel, and Figure 1, upper right panel). Both widely dispersed and clustered patterns of gold label are apparent. This result may be explained by the presence in the hepatocyte of both B/E receptors (giving a cluster pattern) and E receptors (giving a dispersed pattern). ×20,800

be expected to bind to all the LDL receptors with equal affinity, whether they are situated in clusters or in isolation.

To explain these results by the multivalent hypothesis of Wofsy et al,9 it would have to be postulated that 1) not only does the LDL-gold complex induce receptor distribution, but so, too, do the two different antibodies used in these alternative protocols or 2) preferential labeling of clusters occurs by different mechanisms in each of the very different labeling protocols. Such possibilities look implausible but not entirely impossible. The only way to settle the matter unequivocally is direct visualization of native (untagged) LDL bound to the cell surface. Accordingly, we refined the surface-replica procedure to improve preservation of structural detail and demonstrated colocalization of replicated particles, which had provisionally been identified as LDL, with LDL-gold complexes. In this way, it was established that native LDL could indeed be directly visualized in favorable replicas as particles. Using this approach, we found that the same cluster-type distribution pattern with a lack of individually bound LDL particles was present as before. Admittedly, if one looks hard enough, deficiencies can be found in any individual experimental procedure. However, these experiments, when taken together, provide a substantial body of evidence in favor of our original conclusions.

Sanan et al10,11 have recently presented results in which LDL-gold complexes were used to localize the LDL receptor in whole-mounted fibroblasts. From these results, it was claimed that individual widely
dispersed receptors are present, as well as clustered ones, exactly as originally envisaged. To account for the discrepancy between their conclusion and ours, Sanan et al. suggested that our probes were preaggretaged and/or that we used an inadequately low temperature to arrest endocytosis. The former explanation is discounted by the monodisperse appearance of our probes as seen by negative staining. That our LDL-gold complexes do not aggregate during the experiment, before binding to the cell, is apparent from the relatively large distance between individual gold particles in our putative newly inserted receptor clusters, a feature that is, incidentally, equally inconsistent with the notion of multivalent-induced clustering. Even more persuasive is our observation that directly visualized LDL binds in similar loosely packed clusters. On the question of the temperature required to arrest endocytosis, we took particular precautions during the present study to ensure that the temperature was maintained below 4°C. A temperature of 4°C is widely used and accepted by other research groups, including that of Sanan et al., as being adequate for this purpose.

What other factors might contribute to the differences between our results and those of Sanan et al.? One potentially important factor concerns the widely reported affinity of LDL for proteoglycans, collagen, and other extracellular components synthesized by fibroblasts. Our experience has shown that if cells that are actively synthesizing and releasing these components at their surfaces are treated with LDL-gold, spurious labeling can occur. This may happen if the cells are maintained for excessive periods in culture, and it has not escaped our attention that some of the photomicrographs published by Sanan et al. show evidence of such LDL-attracting material at the cell surface. We would add that differential labeling between cells and noncell regions of the replica is no proof of specificity; apart from the confounding effect of extracellular components, other natural differences may exist in the affinity of different substrates for LDL. Additionally, it has to be borne in mind that there are many other technical factors that can artifactually influence the final labeling patterns observed. Much has also been made of data on mutant fibroblast lines that are defective in LDL receptor internalization. If, as Sanan et al. report, these mutant cells do have only a dispersed population of receptors—and this is yet to be confirmed—it would be unwise to assume that the normal fibroblast with normal LDL receptors must necessarily show this distribution, too.

If, as we maintain, LDL receptors are normally initially inserted and displayed as aggregates, how general is this phenomenon? Does it apply to all receptors, is it a feature peculiar to the fibroblast, or is it characteristic of the LDL receptor regardless of cell type? Our comparative study on LDL and β-VLDL binding patterns in fibroblasts and hepatocytes sheds new light on these issues. As shown in Figure 5, the fibroblast LDL receptor recognizes apolipoprotein B and E; thus, it binds LDL (which contains apo B) and β-VLDL (which contains both apolipoproteins B and E). As would be predicted, identical cluster-type labeling patterns are observed in fibroblasts treated with gold complexes made from each of these lipoprotein types. Liver cells contain the same LDL/B/E receptor as the fibroblasts but are reported to have an additional receptor, the E receptor, that recognizes only lipoproteins containing apo E. Thus, when hepatocytes are treated with LDL—gold, only the B/E receptor is labeled, and this shows the same cluster-type pattern with no dispersed label as that characteristic of the fibroblast. When hepatocytes are treated with β-VLDL-gold complexes, however, both a clustered pattern corresponding to the B/E receptor and a dispersed pattern corresponding to the E receptor are revealed. This suggests that 1) the LDL B/E receptor is characteristically inserted and displayed in cluster pattern, irrespective of cell type, and 2) the E receptor differs by being displayed in a more dispersed form. Elsewhere, we have reported widely dispersed receptors/binding sites to IgA receptors in hepatocytes and to acetylated LDL in macrophages. Apart from demonstrating that gold-labeled ligands, in our experience, appear to have the ability to discriminate between different patterns of receptor distribution, these findings suggest that the cluster-type arrangement of the LDL B/E receptor may represent an exception to what may be the more usual initial display pattern of receptors in general.

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