Co-localization of $^{125}$I-Epidermal Growth Factor and Ferritin-low Density Lipoprotein in Coated Pits: A Quantitative Electron Microscopic Study in Normal and Mutant Human Fibroblasts

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ABSTRACT

Low density lipoprotein (LDL) and epidermal growth factor (EGF) bind to receptors on the surface of human fibroblasts and are internalized in coated vesicles. Each of the ligands has been studied separately by electron microscopy in human fibroblasts using ferritin-LDL as one visual probe and $^{125}$I-EGF as a second visual probe. A mutant strain of human fibroblasts (J.D.) has been described in which LDL does not localize to coated pits and hence is not internalized. Because LDL and EGF do not compete with each other for binding, in the current studies we coincubated the two ligands with normal and mutant cells to visualize their cellular fates. In normal fibroblasts ferritin-LDL and $^{125}$I-EGF both bound preferentially to coated pits at 4°C and both ligands were internalized into endocytotic vesicles and lysosomes. Quantitative studies in normal cells showed that 75% of the coated pits and vesicles that contained $^{125}$I-EGF also contained ferritin-LDL, indicating that both ligands enter the cell through the same endocytotic vesicles. In the LDL internalization-mutant J.D. cells, ferritin-LDL did not localize in coated pits and was not internalized, but $^{125}$I-EGF bound to coated pits and was internalized just as in normal fibroblasts.
sites and the mechanism of internalization of EGF in J.D. fibroblasts, which are unable to internalize LDL? (b) Does the presence of LDL-ferritin alter the surface and intracellular distribution of \( ^{125}\)I-EGF in the normal and J.D. cells, and likewise, does EGF alter the distribution of LDL-ferritin binding? (c) What is the extent of co-localization of LDL-ferritin and \( ^{125}\)I-EGF initially on the cell surface and during internalization?

**MATERIALS AND METHODS**

**Cells, Reagents, and Incubation**

Normal and mutant fibroblasts from a patient with the internalization-defective form of homozygous familial hypercholesterolemia (J.D.) were grown in monolayer culture and processed for study as previously described \((1, 2, 6)\). Ferritin-LDL \((1, 2)\), \( ^{125}\)I-EGF \((3)\), and the incubation medium \((1, 2)\) were prepared as previously described. Cells were incubated with these reagents in dishes as described in the legends to Tables I and II. Incubations were carried out for the normal human fibroblasts (Table I) and for the J.D. cells (Table II).

**Electron Microscopy and Autoradiography**

After successive washings in phosphate buffer, the cell pellet was postfixed in 2% OsO\(_4\) in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature. The pellet was then dehydrated in ethanol and treated with propylene oxide before being embedded in Epon. Sections, cut with a diamond knife on an LKB Ultramicrotome (LKB, Bromma, Sweden), were captured on copper grids. Grids containing the sections were coated with Ilford L4 emulsion by the method of Caro et al. \((11)\) as previously described \((12)\). After 5 wk the grids were developed in Microdol-X (Eastman Kodak Co., Rochester, NY). Sections stained with uranyl acetate and lead citrate were examined in a Philips EM 300 electron microscope (Philips Instruments, Eindhoven, The Netherlands), and grains were photographed only in cells that were judged to be well preserved. Photographs of consecutive autoradiographic grains were taken \((\times 21,600)\) calibrated with a reference grid 2,160 lines/mm (Ernest F. Fullam Inc., Schenectady, NY).

**RESULTS**

**Quantitative Measurements of \( ^{125}\)I-EGF Binding**

To study simultaneously the binding of LDL and EGF, human fibroblasts were subjected to prior incubation in the absence of lipoproteins, so as to induce maximal expression of LDL receptors. The cells were then incubated with ferritin-LDL and with \( ^{125}\)I-EGF, either separately or together. Table I shows the results of quantitative measurements of the amount of \( ^{125}\)I-EGF radioactivity bound to normal cells under the various conditions. Binding was inhibited in the presence of an excess of unlabeled EGF \((10 \mu g/ml)\). The same incubations were performed with cells from patient J.D., which are unable to internalize LDL. At 4°C these cells bound an amount of \( ^{125}\)I-EGF that was comparable to the normal cells (Table II).

**Qualitative Morphologic Observations**

Dishes from the experiments of Table I and II were fixed and processed for electron microscopy and for autoradiogra-

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**TABLE I**

| Incubation conditions | Ferritin-LDL \( \mu g/ml \) | \( ^{125}\)I-EGF \( \mu g/ml \) | Unlabeled LDL \( \mu g/ml \) | EGF \( \mu g/ml \) | \( ^{125}\)I-EGF bound to cells cpm/pellet |
|-----------------------|-----------------|-----------------|-----------------|-------------|-----------------|
| 60 min at 4°C         | 31              | --              | --              | --          | --              |
|                       | --              | 6.9             | --              | --          | --              |
|                       | --              | 6.9             | 510             | 10          | 7,718           |
| 60 min at 4°C, followed by 2 min at 37°C | 31              | 6.9             | --              | --          | --              |
| 60 min at 4°C, followed by 10 min at 37°C | 31              | 6.9             | --              | --          | --              |

Cell monolayers were incubated with LDL-ferritin and \( ^{125}\)I-EGF in the absence or presence of unlabeled LDL or unlabeled EGF as indicated. The incubations were carried out in nonconfluent cells that had been incubated for the previous 48 h in 10% human lipoprotein-deficient serum \((1, 6)\). After incubation with the labeled ligands, the cell monolayers were washed, fixed, and scraped from the dish. The cell pellets from three dishes were combined, counted for their content of \( ^{125}\)I-radioactivity, and then used for electron microscopy and autoradiography. The cell-associated radioactivity is expressed only as cpm/pellet; there is some variability in the number of cells per pellet that can account for some of the differences in radioactivity per pellet.

**TABLE II**

| Incubation conditions | Ferritin-LDL \( \mu g/ml \) | \( ^{125}\)I-EGF \( \mu g/ml \) | Unlabeled LDL \( \mu g/ml \) | EGF \( \mu g/ml \) | \( ^{125}\)I-EGF bound to cells cpm/pellet |
|-----------------------|-----------------|-----------------|-----------------|-------------|-----------------|
| 60 min at 4°C         | 31              | --              | --              | --          | --              |
|                       | --              | 6.9             | --              | --          | --              |
|                       | --              | 6.9             | 510             | 10          | 8,852           |
| 60 min at 4°C, followed by 2 min at 37°C | 31              | 6.9             | --              | --          | --              |
| 60 min at 4°C, followed by 10 min at 37°C | 31              | 6.9             | --              | --          | --              |

Incubations were carried out exactly as described in the legend to Table II.
FIGURE 1 Illustration of various structures that have been quantitatively analyzed. Normal human fibroblasts were coincubated with ferritin-LDL and \(^{125}\)I-EGF as indicated in the legend to Table I. \(\times 64,800\). (a) Coated pit (CP) demonstrating associated autoradiographic grains. The arrows designate ferritin-LDL cores. (b) Coated vesicle (CV). Closed coated vesicle. Though it is not possible to distinguish a coated vesicle from a coated pit without serial sections, we have referred to the apparently closed coated structures as vesicles for the purpose of our quantitation. The arrows designate ferritin-LDL and the grains designate \(^{125}\)I-EGF. (c) Large uncoated clear vesicle (LV). It is not always possible to distinguish this endocytotic vesicle from a multivesicular body, without serial sections. For the purpose of quantitation, we refer to these membrane-bounded structures as large clear vesicles if no small intraluminal vesicles are seen and as lysosomes if small intraluminal vesicles are seen as in d. (d) Multivesicular body (MVB). These structures are referred to as lysosomes, as are several other secondary lysosomal forms previously described (4, 5).

Quantitative Analysis of \(^{125}\)I-EGF Interaction with Normal and Mutant (J.D.) Human Fibroblasts

The distribution of autoradiographic grains was quantified in cells subjected to the incubations described in Tables I and II. When normal human fibroblasts were incubated with \(^{125}\)I-EGF at 4°C for 60 min with or without ferritin-LDL, \(^{125}\)I-EGF localized primarily to the plasma membrane. Only 10% of the grains were observed intracellularly (Table III). ~45-54% of the membrane-associated grains were associated with coated pits (Table IV). This represents a highly preferential localization because we have previously found that coated pits make up <2% of the linear surface of these cells (1, 5). When the cells were warmed to 37°C for 2 min, 42% of the \(^{125}\)I-EGF grains were found within the cell. By 10 min, 72% of the \(^{125}\)I-EGF had been internalized (Table III).

In the mutant J.D. cells, 88% of the \(^{125}\)I-EGF was initially localized to the plasma membrane (Table III) and 35-54% of the membrane-associated grains were located in coated pits.
(Table IV). When the cells were warmed, $^{125}$I-EGF was internalized in a fashion similar to the normal cells (Table IV). This is in marked contrast to the previously reported lack of internalization of ferritin-LDL (5) or $^{125}$I-LDL (4) by these cells.

**TABLE III**

| Incubation conditions       | Normal cells | J.D. cells |
|----------------------------|--------------|------------|
| 60 min at 4°C               | 10           | 12         |
| 60 min at 4°C, plus ferritin-LDL | 19           | 12         |
| 60 min at 4°C, followed by 2 min at 37°C plus ferritin-LDL | 42           | 45         |
| 60 min at 4°C, followed by 10 min at 37°C plus ferritin-LDL | 72           | 50         |

Incubations were carried out as described in the legends to Tables I and II. *Background (i.e., spontaneous events randomly distributed as determined from evaluation of regions of sections devoid of cells) was considered to represent 10% of total grains analyzed. A value of 10% was therefore subtracted from each value.

**TABLE IV**

| Incubation conditions       | Normal cells | J.D. cells |
|----------------------------|--------------|------------|
| 4°C for 60 min              | 54           | 35         |
| 4°C for 60 min + Ferritin-LDL | 45           | 54         |
| 4°C for 60 min, followed by 37°C for 2 min + Ferritin-LDL | 24           | 33         |
| 4°C for 60 min, followed by 37°C for 10 min + Ferritin-LDL | 2           | 8          |

Incubations were carried out as described in the legend to Tables I and II. *A grain was considered to be localized in a coated pit if it was ± 250 nm from a coated pit.

Discussion

The present study complements in several ways previous studies in which cellular binding of $^{125}$I-EGF or ferritin-LDL have been studied separately. First, the current data indicate that the presence of $^{125}$I-EGF or ferritin-LDL on or in the cell does not alter the behavior of the other ligand. This is true not only for binding per se, but also for the plasma membrane and intracellular distributions of the two ligands. Second, the data show that the J.D. cells, which do not have LDL receptors in coated pits and thus do not internalize the ligand, can nevertheless process $^{125}$I-EGF in the same way as normal cells. In the J.D. cells, as in the normal cells, $^{125}$I-EGF incubated at 4°C localized preferentially to coated pits. Upon warming, the $^{125}$I-EGF was internalized into lysosomal structures. Thus, the abnormality in the J.D. cells is specifically related to the LDL receptor and not to more general mechanisms related to the coated pit. These findings are consistent with previous data showing that $^{125}$I-EGF is degraded in a normal fashion in these mutant cells (13).

The current data also show that in normal human fibroblasts $^{125}$I-EGF and ferritin-LDL colocalize to the same morphologic structures. These include coated pits on the cell surface as well as coated and clear vesicles and lysosomes intracellularly. At least three-fourths of the structures that contain EGF also...
contain LDL. This is a minimal estimate since the ferritin-LDL is somewhat difficult to visualize in cells coated with a photographic emulsion. It is perhaps not surprising that both ligands localize to the same coated pits because coated pits are relatively scarce, yet they are known to mediate the uptake of many different types of ligands (7). The two ligands also localize in the same intracellular membrane-bounded structures. As previously reported, these intracellular membrane-bounded structures have the morphologic characteristics of coated or clear uncoated endocytotic vesicles at early times and lysosomal structures somewhat later (1, 2, 4, 5).

When the normal fibroblasts were warmed, we found both 125I-EGF and ferritin-LDL in the same coated as well as noncoated endocytotic vesicles. Even after only 2 min some of the ligand was already associated with lysosomal structures such as multivesicular bodies. Many more lysosomes were labeled by 125I-EGF and ferritin-LDL after 10 min of incubation at 37°C. By studying electron micrographs obtained at different times after warming, it was our impression that both ligands become associated with progressively larger membrane-bounded structures as a function of time. Thus, both ligands may be processed through a series of continuously enlarging membrane-bounded structures. Over the time course studied, we did not find 125I-EGF or ferritin-LDL in Golgi cisternae, nuclei, or endoplasmic reticulum.

These results confirm observations made by Willingham, Pastan, and co-workers (8–10). These investigators concluded that more than one ligand can enter the cell through the same coated pit. The extent of co-localization of 125I-EGF and ferritin-LDL (75–80%) as revealed by quantitative analysis suggests that each coated pit on the cell surface contains multiple receptors for different ligands, and that once internalized these ligands remain together as they traffic to the lysosome. Therefore, all coated pits at the cell surface must be functionally equivalent even though there are important differences in the way each of these receptors is handled by the cell. For example, LDL receptors are known to recycle rapidly to the cell surface after internalization (14) whereas EGF receptors do not (15). The current studies suggest that this difference in recycling behavior may be a function of the receptors, but is independent of the major structures involved in ligand uptake.

In the current studies as well as in our previous work (5), we have found that a substantial portion of receptor-bound 125I-EGF is clustered in coated pits in human fibroblasts even when the cells have been incubated with the ligand at 4°C. In studies of mouse 3T3 cells using the technique of fluorescence microscopy, Schlessinger and co-workers (16) concluded that the EGF receptors were distributed diffusely when the cells were incubated with EGF at 4°C. Clustering was observed only when the cells were warmed to 37°C. We have not studied EGF binding in human fibroblasts by fluorescence microscopy, and thus we do not know whether the observed clustering of 35–50% of receptors at 4°C would be detectable by fluorescence microscopy. Moreover, we cannot be certain that the EGF ligand does not induce clustering to coated pits even at 4°C. However, the current data at least raise the possibility that in human fibroblasts a considerable fraction of the EGF receptors can localize to coated pits and presumably become internalized even in the absence of ligand.

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