Histochemical Characterization of Low Density Lipoprotein Receptors in Internalization-Defective Familial Hypercholesterolemia

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We studied the localization of low density lipoprotein (LDL) bound to the receptors on the cultured fibroblasts from a patient (MN) with homozygous familial hypercholesterolemia and a defect in internalization of LDL and compared the localization with normal fibroblasts and with those from another internalization-defective cell, GM2408A. Monolayers of cells were cultured with lipoprotein-deficient human serum, and the cells were incubated with I125- or ferritin-labeled LDL. The LDL binding was observed by autoradiography or by an electron microscope. Autoradiographs of bound I125-LDL confirmed that MN’s cells could not internalize LDL inside the cell at 37°C. In these cells, ferritin LDL was found mainly in noncoated regions at 4°C; it was not found in endocytic vesicles after incubation at 37°C. Ferritin cores that bound on the surface of the cells from the normal subject, from GM2408A, from MN, and from MN’s parents, were counted and quantitatively analyzed at 4°C. In normal cells, 62% of the ferritin cores were bound in the coated pits; in MN’s cells, only 11% of the ferritin LDL was found in the coated pits; in the GM2408A cells, 12% of the ferritin LDL was found in the coated pits; in the cells from MN’s parents, 40% of the ferritin LDL was found on the coated pits. The results indicate that the internalization defect in MN’s cells is the same as that in the GM2408A cells; neither can localize the LDL and LDL receptor complex in coated pits. (Arteriosclerosis 5:238–243, May/June 1985)

Cholesterol in blood plasma is transported mainly in the form of LDL and is taken up by cells through specific receptors on the cell surface.1 There is a genetic defect in low density lipoprotein (LDL) catabolism termed familial hypercholesterolemia (FH). Cultured skin fibroblasts obtained from patients with FH generally lack functional LDL receptors that can bind LDL.1 Anderson et al.2 reported a type of FH patient whose cultured fibroblasts (GM2408A) can bind normal amounts of LDL, but cannot internalize them inside the cells. These researchers showed that this defect is due to an inability of the LDL receptor to localize in coated pits.

In a previous paper3 we reported another type of internalization-defective mutant, MN, who was a pure homozygote for this mutation. GM2408A is a genetic compound made of the internalization-defective allele and the binding-deficient allele. The present study examined whether the defect in MN’s cells was in the localization of LDL receptors into coated pits as happened in GM2408A cells, or whether the defect was in other steps of the internalization process. Our results show that the mutation in MN’s cells was a defect in localization of LDL receptors into coated pits.

Methods

Lipoproteins

Human LDL (d = 1.019–1.063) and lipoprotein-deficient serum (LPDS; d > 1.215) were isolated from the serum of healthy subjects and prepared by sequential ultracentrifugation.4,5 LDL was iodinated with 125I (125I-LDL) by the iodine monochloride method.6,7 The specific activity of 125I-LDL ranged between 200 and 400 cpm/ng of protein. Ferritin was conjugated to LDL according to the methods described by Kishida et al.8 and Anderson et al.9 The coupled mixture was centrifuged in a Hitachi RPS
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50-2 rotor (Hitachi Koki Company, Limited, Tokyo, Japan). Negative staining electron microscopy showed that the labeled fraction contained LDL particles conjugated usually with one and sometimes with two or three ferritin cores for each LDL particle. The LDL content in the ferritin-LDL conjugate was estimated from the cholesterol content of the fraction. Cholesterol and protein concentrations were determined by the methods of Zlatkis et al.10 and Lowry et al.,11 respectively.

Cells

A human skin fibroblast strain, GM2408A (JD) was obtained from the Human Genetic Mutant Cell Repository (Camden, New Jersey). Other skin fibroblasts were obtained from skin biopsies and were maintained in culture as described previously.2 Cells were used for experiments before the 20th passage. Stock cultures were maintained as monolayers in a humidified incubator (5% CO₂) at 37° C in Eagle’s minimal essential medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% (vol/vol) fetal calf serum. On Day 0, confluent cells were dissociated with 0.2% trypsin and 0.02% EDTA from stock cultures, and samples of 10⁵ cells were seeded into 60 mm Petri dishes containing 3 ml of growth medium with 10% (vol/vol) fetal calf serum. For the assays of 125I-LDL binding and autoradiography of LDL receptor localization, cells had been incubated with LPDS for 48 hours, the cells were dissociated with 0.2% trypsin and 0.02% EDTA from stock cultures, and samples of 10⁵ cells were seeded into 60 mm Petri dishes containing 3 ml of growth medium buffered with 10 mM Hepes (pH 7.3). Some of the monolayers were cooled to 4° C for 30 minutes. The medium was removed and replaced with either 2 ml of iced cold growth medium containing 5% LPDS and 125I-LDL for autoradiography, or with ferritin LDL for electron microscopy. For the assays of 125I-LDL binding, measurements were performed as described by Goldstein et al.12

 Autoradiography

Monolayers of normal and MN’s fibroblasts were incubated with 22 μg/ml of 125I-LDL (317 cpm/ng) at 4° C for 2 hours, washed, and then fixed with 6% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). Some of the monolayers were incubated with 2 ml of LPDS-growth medium at 37° C for 60 minutes before fixation. Coverslips on which cells were fixed were washed with distilled water and dried. They were dipped into a 1:1 dilution of Sakura NR-M2; (Konishiroku Photo Industry Company, Limited, Tokyo, Japan) at 40°C in a dark room. The emulsion-coated coverslips were stored at 4° C in a light-proof box containing silica gel for 4 weeks. Then the coverslips were developed with Konidor X, rinsed in distilled water, and fixed in ice with Konifix (Konishiroku Photo Ind. Company, Limited, Tokyo, Japan). Each coverslip was stained with 1% toluidine blue and was covered with a second coverslip. The autoradiographs were examined under a light microscope (Vänox, Olympus Limited, Tokyo, Japan).

Binding of Ferritin LDL to Fibroblasts

Monolayers of normal and FH patients’ fibroblasts were incubated at 4° C for 2 hours with 2 ml of ice-cold growth medium buffered with 10 mM Hepes (pH 7.4), containing 5% LPDS and ferritin LDL at a concentration of 100 μg/ml of LDL protein. For one-half of the monolayer samples, the medium was replaced with 2 ml of warm growth medium containing 5% LPDS; these were incubated at 37° C for 5 minutes. After incubation, each monolayer was washed five times with ice-cold phosphate-buffered saline; then the monolayers were fixed for 1 hour with 2% glutaraldehyde in 0.1 M sodium-phosphate buffer (pH 7.3).13

Electron Microscopy

Fixed monolayers were washed several times with 0.1 M sodium-phosphate buffer (pH 7.3) and then were post-fixed with 2% OsO₄ in 0.1 M sodium-phosphate buffer (pH 7.3) at 4° C for 30 minutes. The post-fixed cells were washed with 0.1% sodium acetate and stained with 2% uranyl acetate at 4° C for 2 hours. The samples were washed again with 0.1% sodium acetate, dehydrated through a graded series of ethanol, and embedded in Spurr’s low viscosity resin. Ultra-thin sections obtained with an MT-2B Ultramicrotome (Sorvall, Dupont, Newtown, Connecticut) were stained with 2% uranyl acetate in 30% ethanol, then treated with Reynolds’s lead citrate solution, and observed under a Hitachi H-600 electron microscope (Hitachi Limited, Tokyo, Japan).

Results

Localization of 125I-LDL Binding on Whole Cells by Autoradiography

The biochemical characterization of MN’s cells showed that the cells can bind LDL in the normal way but cannot internalize it.3 Figure 1 shows autoradiographs of normal fibroblasts (A and B) and MN’s fibroblasts (C and D) that had been exposed to 125I-LDL for 2 hours at 4° C (A and C), and then incubated at 37° C for 60 minutes (B and D). In normal fibroblasts, 125I-LDL was distributed over the cell surface at 4° C (Figure 1 A), and silver grains were not seen over the nucleus, as reported by Anderson et al.13 In the peripheral margins of many cells, the cells were labeled. In MN’s cells, the 125I-LDL binding at 4° C was similar to that of normal cells (Figure 1 C). When normal cells were incubated at 37° C for 60 minutes (Figure 1 B), silver grains were concentrated over the perinuclear area. This may show the internalization of the 125I-LDL inside the cell and its migration to lysosomes.2 On the other hand, when MN’s cells...
Figure 1. Autoradiographs of normal fibroblasts (A and B) and MN’s fibroblasts (C and D). Cell monolayers were grown on glass coverslips (18 x 18 mm) as described in Methods. Monolayers were incubated with 22 µg/ml of 125I-LDL (317 cpm/ng of LDL-protein) at 4°C for 2 hours; then each monolayer was washed six times with albumin-containing buffer at 4°C. Some sets were fixed immediately (A and C), while others were incubated in a growth medium containing 5% LPDS at 37°C for 60 minutes (B and D) before fixation. The monolayers were fixed with 6% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). Coverslips were prepared for autoradiography. The exposure time was 4 weeks at 4°C. Bar = 5 µm.
were incubated at 37 °C for 60 minutes, silver grains were still distributed over the cell surface and did not accumulate near the nucleus (Figure 1 D). There was no difference between the distribution of silver grains in the cell in Figure 1 C at 4 °C and the cell in Figure 1 D at 37 °C. This study with light microscopic autoradiography indicates that MN’s cells could not internalize 125I-LDL, and the study supports the results obtained in our biochemical study.3

**Binding and Endocytosis of Receptor-Bound Ferritin LDL Observed under the Electron Microscope**

In normal cells most of the bound ferritin LDL was located in coated pits, when ferritin LDL was incubated with the monolayer at 4° C. Figure 2 A shows a typical invaginated coated pit in which ferritin cores (arrowhead) are clustered. After 5 minutes of incubation at 37° C, ferritin cores were seen in endocytic vesicles (Figure 2 B, arrowhead). In MN’s cells, most of the ferritin cores were found in the noncoated region at 4° C (Figure 2 C-F). Although the size and configuration of coated pits in MN’s cells (Figure 2 C and E) appeared to be the same as that in normal fibroblasts (Figure 2 A), a very small amount of ferritin LDL was found in the coated pits of MN’s cells. In MN’s cells, ferritin cores were bound in various places (Figure 2 C-F) as was true in another internalization-defective type cell, GM2408A.2 When the monolayers of MN’s fibroblasts were incubated at 37° C for 5 minutes, ferritin LDL still remained on the cell surface and endocytic vesicles did not contain a ferritin core (Figure 2 G and H).

**Quantitative Analysis of Ferritin-LDL Binding**

To confirm these qualitative observations, fibroblasts from a normal subject (+/+), from MN (Rb+k/Rb+k), from MN’s parents (+Rb+k+) and from another internalization-defective type of cell, GM2408A (Rb/Rb+k), were incubated with ferritin LDL at 4° C for 2 hours. (See the genotype designations in the footnotes of Table 1.) Sections of the cells were prepared and randomly photographed under an electron microscope. A cell surface membrane of about 0.3 to 0.4 mm in length was examined, and the number of ferritin cores in coated pits or in noncoated regions were counted. Another series of experiments was carried out to measure the 125I-LDL binding to the LDL receptor at 4° C as previously described.12 Table 1 shows that normal fibroblasts accumulated 62% of ferritin LDL into the coated pits. In MN’s cells, only 11% of the ferritin particles was found in coated pits. This value was almost equal to that (12%) in the GM2408A cells. In the cells of MN’s parents, the percentage of ferritin LDL bound in the coated pits was intermediate (approximately 40%) between normal cells and MN’s cells. This observation was consistent with the biochemical data showing that the cells from MN’s parents have the same population of abnormal LDL receptor (the product of the Rb+k allele). Therefore, the cells from MN represent the homozygote containing only one population of the LDL receptor produced by the Rb+k allele. The total number of ferritin cores bound per millimeter of length of cell surface in GM2408A and MN were significantly lower than those of normal cells. This is not consistent with the amount of 125I-LDL bound on the cell surface (Table 1).

The results obtained from our qualitative and quantitative electron microscopic studies show that the defect in internalization of LDL in MN’s cells is due to an inability of the LDL receptor to localize in coated pits, and is thus phenotypically the same as the defect observed in GM2408A cells by Anderson, Goldstein, and Brown.2,14

### Table 1. Quantitative Analysis of Binding of Ferritin LDL to Coated Pits of Cell Surface of Normal and Mutant Fibroblasts

<table>
<thead>
<tr>
<th>Cell</th>
<th>Genotype</th>
<th>Ferritin cores bound (no./mm of cell surface)</th>
<th>125I-LDL bound (ng/mg)</th>
<th>Exp 1</th>
<th>Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In coated pits (1)</td>
<td>In noncoated regions (2)</td>
<td>Total (1 + 2)</td>
<td>Ferritin cores in coated pits (%)</td>
</tr>
<tr>
<td>Normal</td>
<td>+/+</td>
<td>735</td>
<td>445</td>
<td>1180</td>
<td>62</td>
</tr>
<tr>
<td>GM2408A</td>
<td>Rb/Rb+k</td>
<td>99</td>
<td>723</td>
<td>822</td>
<td>12</td>
</tr>
<tr>
<td>M.N.</td>
<td>Rb+k/Rb+k</td>
<td>73</td>
<td>604</td>
<td>677</td>
<td>11</td>
</tr>
<tr>
<td>Father of M.N.</td>
<td>+/Rb+k</td>
<td>552</td>
<td>747</td>
<td>1299</td>
<td>42</td>
</tr>
<tr>
<td>Mother of M.N.</td>
<td>+/Rb+k</td>
<td>434</td>
<td>640</td>
<td>1074</td>
<td>40</td>
</tr>
</tbody>
</table>

Fibroblasts were incubated with either ferritin-LDL (100 μg/ml of LDL-protein) or 125I-LDL (10 μg/ml of LDL-protein). All the incubations were at 4° C for 2 hours. The monolayers that received ferritin LDL were washed, fixed, embedded, sectioned, and stained for electron microscopy as described in Methods. Cell surface (0.3–0.4 mm) was surveyed for each monolayer for ferritin cores. In the 125I-LDL binding study, the amounts of dextran sulfate-releasable radioactivities were determined.12 Genotypes were designated according to Goldstein et al.12; normal allele (+); receptor-negative allele (Rb+); internalization-defective allele (Rb+k).
Figure 2. Electron micrographs that demonstrate the localization of ferritin LDL on the cell surface of normal cells (A and B) and MN's cells (C-H). Cells were prepared as described in Methods. The cells were incubated with ferritin LDL (100 μg/ml of LDL-protein) at 4°C for 2 hours. Each monolayer was washed five times with ice-cold phosphate-buffered saline, fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3), embedded, sectioned, and stained for electron microscopy. Some of the monolayers were incubated in LPDS-containing growth medium at 37°C for 5 minutes (B, G, H), and then fixed. The arrowheads indicate ferritin LDL. Bar = 100 nm.
Discussion

In the previous paper we reported a homozygous FH patient whose cultured fibroblasts apparently bound 125I-LDL normally, but could not internalize the LDL inside the cells. In the present study we again showed that MN's cells could not translocate 125I-LDL to the perinuclear region when they were incubated at 37°C (Figure 1 D). This autoradiographic observation confirms that MN's cells cannot internalize LDL inside the cells. The biochemical analysis of 125I-LDL binding also revealed that the patient has a single population of the LDL receptor that could not be internalized. The current studies of quantitative analysis of ferritin LDL binding by electron microscope (Table 1) showed that the cells from MN's parents accumulated intermediate numbers of ferritin LDL to their coated pits (approximately 40%), compared with normal cells (62%) and MN's cells (11%). This also indicates that MN is a homozygote and her cells have only a single type of LDL receptor produced by the R10 allele (Table 1).

Ferritin cores bound on MN's cells were found mainly in noncoated regions at 4°C (Figure 2 C-F). Furthermore, in the quantitative analysis of ferritin-LDL binding, it was shown that only 11% of the total ferritin cores bound on the cell surface were localized in coated pits. This percentage was almost the same as that for the ferritin cores in the coated pits in the GM2408A cells (Table 1). From these results we can conclude that the defect in MN's cells is due to an inability of the LDL receptor to localize in coated pits. This type of internalization defect is the same as that found in the GM2408A cells by Anderson et al., although the latter is a genetic compound made of the internalization-defective allele and the binding-deficient allele, R10/R10.

The total number of ferritin cores on the surface of GM2408A and MN cells was significantly lower than on normal cells. This is consistent with the amount of 125I-LDL bound on each of the monolayers (Table 1). Anderson et al. reported that in GM2408A cells, ferritin cores associated with coated pits were 3% of the total number bound to the cell surface. Our results indicated that in MN's cells, this percentage value was 12% (Table 1). Since our quantitation of the ferritin-LDL binding was performed on stained sections by a method slightly different from that described by Anderson et al., we may have underestimated the ferritin core bound in the noncoated region. It was difficult to identify the ferritin core particles that were closely bound to the membrane bilayer. Considering the possible undercounting of the ferritin cores, the percentage of coated pit-associated ferritin cores in the fibroblasts of GM2408A, MN, and the parents may be much lower than those shown in Table 1.

LDL receptor protein was purified and identified as a 160 kd glycoprotein. Tolleshaug et al. described seven mutations that disrupted synthesis, processing, and transport of the receptor fibroblasts in 77 patients with homozygous familial hypercholesterolemia of the receptor-negative, or defective, type. Analysis of the mutation in peptide structure in the mutant cells will give insights into the physiological role of a segment of the peptide structure on localization of the receptor into coated pits.

References

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