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BINDING OF UNMODIFIED LOW-DENSITY LIPOPROTEINS TO HUMAN FIBROBLASTS

AN INVESTIGATION BY IMMUNOELECTRON MICROSCOPY

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Summary

The binding of unmodified low density lipoproteins to the plasma membrane of fibroblasts was studied at the ultrastructural level. The bound low density lipoprotein was visualized by an indirect immunoperoxidase technique, with the use of an antiserum against apoprotein B. Immunoreactive regions representing bound apoprotein B were found on the plasma membrane, in indented regions with a diameter of 0.15—0.30 μm and a fuzzy coat on the cytoplasmic side. Fibroblasts from a patient homozygous for hyperlipoproteinaemia type IIa showed no immunoreactive material in the indented regions. The specific ^{125}I-labelled low density lipoprotein binding to these homozygous fibroblasts was 7% compared to control fibroblasts.

Using biochemical techniques, Brown et al. [1] showed that cultured normal human fibroblasts possess high-affinity binding sites for low-density lipoproteins (LDL). These binding sites were also demonstrated with the same techniques on cultured smooth-muscle cells [2], lymphocytes [3], and endothelial cells [4], but not on macrophages [5]. Fibroblasts and lymphocytes from patients homozygous for hyperlipoproteinaemia type IIa [6], have defective or no high-affinity LDL binding sites (Refs. 1,3; for review, see Ref.

Abbreviation: LDL, human low density lipoprotein (density range 1.019—1.063 g/ml) prepared by ultracentrifugation [10].

7). Ultrastructural studies with ferritin-conjugated LDL showed the binding of these conjugates to specific regions of the fibroblast plasma membrane [8]. However, the conjugation procedure may influence the biological activity of LDL to such a degree that ferritin-conjugated LDL (in contrast to native LDL) will have an inhibitory effect on the 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in fibroblasts from patients homozygous for hyperlipoproteinaemia IIa [9]. Therefore, we developed an indirect immunoenzymecytochemical method for ultrastructural studies of the binding of unmodified LDL to fibroblasts.

Monolayers of normal human skin fibroblasts were grown to confluency at 37°C on pieces of Melinex plastic in 90-mm plastic petri dishes in 10 ml medium (Hams's F 10 growth medium containing 15% v/v new born calf serum), in an atmosphere of 95% air/5% CO_2. Human low-density lipoproteins (d = 1.03—1.05 g/ml) were isolated from fasting normal human EDTA plasma by preparative ultracentrifugation in a Sorvall OTD 2 ultracentrifuge with Ti 865 rotor (Ivan Sorvall, Inc., Newton, CT, U.S.A.) [10]. The new born calf serum was delipidated by ultracentrifugation at a density of 1.25 g/ml for 36 h at 50 000 rev./min, after which the bottom fraction was dialyzed against isotonic saline and sterilized by millipore filtration.

Antiserum against apolipoprotein B was obtained as described by Vermeer et al. [11]. For the biochemical studies, human LDL were isolated from human serum in an SW 40 Rotor according to Redgrave et al. [12]. ^{125}I-labelled LDL was prepared at pH 10 according to a modification [13] of the ^{125}I chloride labelling method of McFarlane [14]. The iodine/protein ratio was 0.64 atom/mol for human LDL, and 92—94% of the radioactivity was protein bound [15]. Confluent fibroblasts were incubated for 48 h at 37°C in 10 ml medium containing 15% (v/v) delipidated new born calf serum prior to the experimental procedure. For the immunohistochemical procedure, the cells were cooled for 30 min at 4°C and subsequently incubated for 2 h at 4°C in delipidated precooled medium containing freshly isolated human LDL (protein concentration: 0.1 mg/ml medium) [11].

After incubation, the cells were washed 4 times with 5 ml 0.2% (v/v) bovine serum albumin in 0.15 M NaCl and then twice with 5 ml 0.15 M NaCl without albumin, in order to remove aspecifically bound LDL [16]. Next, the cells were fixed in 1% paraformaldehyde in phosphate-buffered saline at 4°C for 10 min, and stained by the following indirect immunoenzyme procedure at room temperature:

(a) incubation (20 min) in normal goat serum (diluted 1:8);
(b) incubation (20 min) in rabbit-anti-human apoprotein B (diluted 1:20);
(c) refixation (10 min) in 0.5% glutaraldehyde in phosphate-buffered saline. Glutaraldehyde fixation can only be used after the immunological reaction with apoprotein B has taken place, because glutaraldehyde destroys the immunoreactivity of apoprotein B [17];
(d) incubation (20 min) in goat-anti-rabbit IgG conjugated with horseradish peroxidase (diluted 1:50), (Nordic, Tilburg, The Netherlands);
(e) visualization of cell-bound peroxidase with 3,3′-diaminobenzidine·4 HCl (Merck, Darmstadt, F.R.G.) and H_2O_2 [18];

(f) postfixation (30 min) in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) containing 0.05% $CaCl_2$, at 4°C.

Between each of these steps, the cells were washed twice with phosphate-buffered saline, except after glutaraldehyde fixation, when the cells were washed with 0.1 M Tris-HCl buffer (pH 7.4) to remove free aldehyde groups [19]. After postfixation, the cells were partially dehydrated in an ethanol series to 70%, in order to reduce the lipid loss [20], and embedded in situ according to Mariano and Spector [21].

Because an antiserum against apoprotein B (the main apoprotein of LDL) was used in the first step of the immuno procedure, immunoreactive regions of the plasma membrane can be regarded as showing LDL binding.

As can be seen from Fig. 1, immunoreactive material is present on indented regions of the plasma membrane. In lead-stained sections (Figs. 2, 3) these indented regions have a fuzzy coat on their cytoplasmic sides, and closely resemble the coated regions described by Anderson et al. [8]. The width of the indented regions is fairly constant, ranging between 0.15 and 0.30 μm. Immunoreactive material was only found very occasionally on the plasma membrane outside these indented regions.

The immunoreactive indented regions were unevenly distributed over the cell surface and were sometimes found in 'clusters' at peripheral cellular margins. Moreover, some cells had many peroxidase-positive regions, whereas neighbouring cells did not show such regions. This uneven distribution is in agreement with the autoradiographic findings of Anderson et al. [8].

In the controls (cells incubated with normal rabbit serum instead of rabbit-anti-apoprotein B or cells exposed to peroxidase (50 μg/ml) instead of LDL) no peroxidase-positive indented regions were found.

In additional biological control experiments, use was made of cells from a patient homozygous for hyperlipoproteinaemia IIa who had extensive tuberous xanthomas and serum LDL cholesterol level of 700 mg/dl. Biochemically, these fibroblasts showed an almost complete absence of high-affinity LDL binding (see below). Ultrastructural immunoenzymehistochemical studies performed with these cells showed indented regions which invariably lacked immunoreactive material (Figs. 4, 5).

To quantitate the binding of LDL to the control and homozygous cells, biochemical studies were done with iodine-labelled LDL. According to the method described by Stein et al. [22], the cells were incubated for 2 h at 37°C with ^{125}I-labelled LDL (14 μg protein/ml). The total specific LDL binding was determined by incubation with ^{125}I-labelled LDL in both the absence and presence of 500 μg unlabelled LDL. At 14 μg protein ^{125}I-labelled LDL/ml, the specific binding of the homozygous cells was 7% of the control values. The degradation of ^{125}I-labelled LDL, measured as trichloric acetic acid soluble radioactivity, was, under the conditions applied, virtually zero.

The results of our ultrastructural studies are in accordance with the findings of Anderson et al. [8], who used ferritin-conjugated LDL. In contrast to Anderson's method (under our conditions) the fibroblasts are exposed to unmodified LDL.

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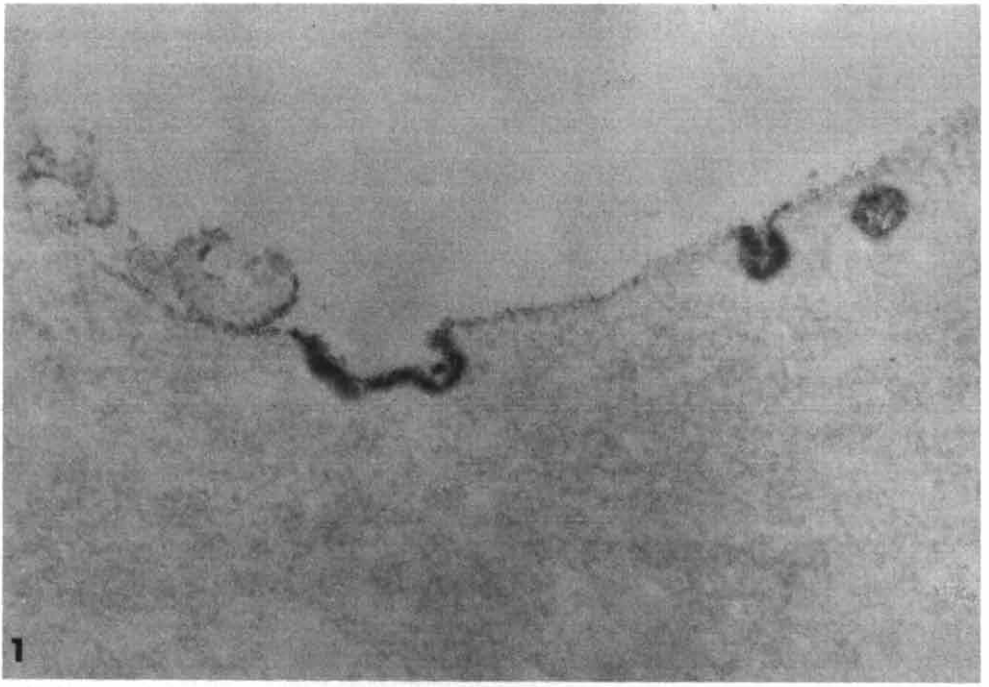
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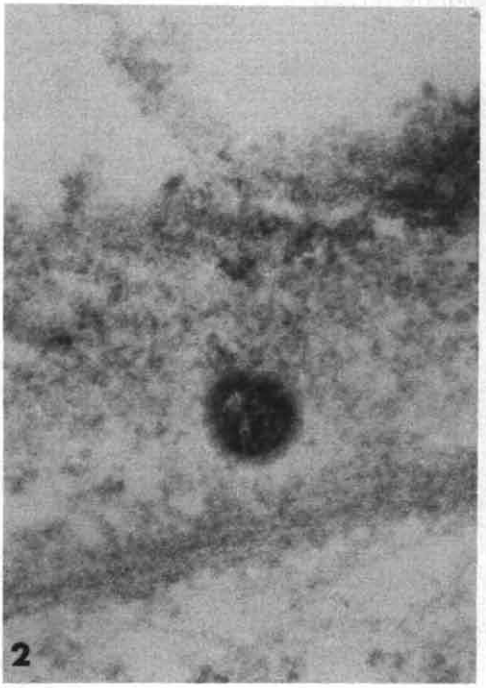
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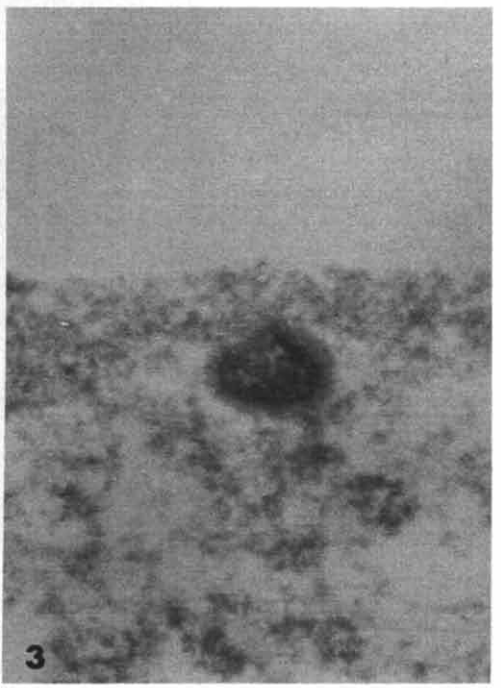
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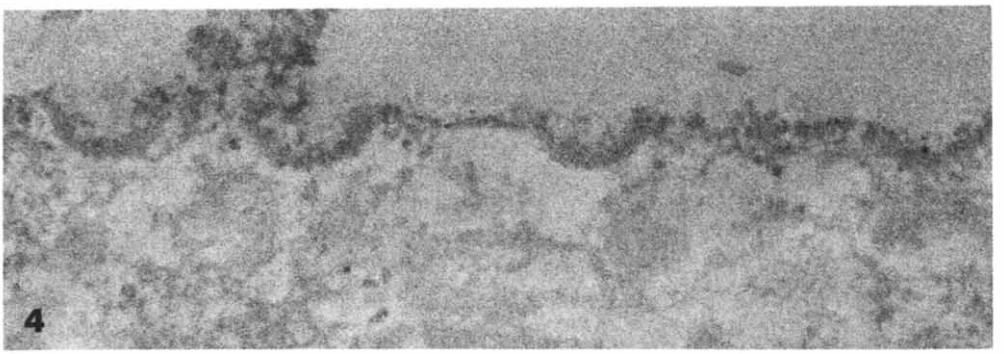

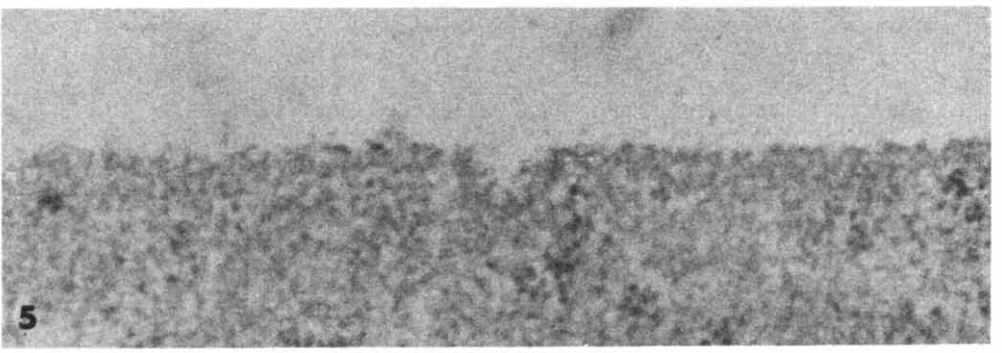

Foreskin fibroblasts, grown to confluency, were cultured in delipidated medium for 48 h, and then exposed for 2 h at 4°C to LDL (0.1 mg protein/ml). After washes with albumin-containing buffer, the cells were fixed for 10 min at 4°C in 1% *p*-formaldehyde, incubated with normal goat serum followed by rabbit-anti-human apoprotein B, refixed for 10 min in 0.5% glutaraldehyde, and re-incubated with goat-anti-rabbit IgG conjugated with horse-radish peroxidase. After cytochemical visualization of the cell-bound peroxidase [18], the cells were postfixed for 30 min in 1% OsO_4, partially dehydrated in ethanol, and embedded and sectioned according to Mariano and Spector [21]. Fig. 1. An unstained ultrathin section of a normal human foreskin fibroblast showing several immunoreactive indented regions with a diameter of 0.15—0.30 μm. × 25 000. Figs. 2 and 3. A lead citrate-stained section of a normal human foreskin fibroblast showing a fuzzy coat on the cytoplasmic side of the plasma membrane in the immunoreactive region. × 60 000. Figs. 4 and 5. An indented region of the plasma membrane of a fibroblast from a patient homozygous for hyperlipoproteinaemia IIa. The indented region shows a fuzzy coat but no immunoreactive material. Lead citrate-stained section. × 60 000.

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