Visualization of the Binding, Endocytosis, and Transcytosis of Low-density Lipoprotein in the Arterial Endothelium In Situ

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ABSTRACT We investigated the interaction and transport of low-density lipoprotein (LDL) through the arterial endothelium in rat aorta and coronary artery, by perfusing in situ native, untagged human, and rat LDL. The latter was rendered electron-opaque after it interacted with the endothelial cell and was subsequently fixed within tissue. We achieved LDL electron-opacity by an improved fixation procedure using 3,3'-diaminobenzidine, and mordanting with tannic acid. The unequivocal identification of LDL was implemented by reacting immunocytochemically the perfused LDL with anti LDL-horseradish peroxidase conjugate. Results indicate that LDL is taken up and internalized through two parallel compartmented routes. (*a*) A relatively small amount of LDL is taken up by endocytosis via: (*i*) a receptor-mediated process (adsorptive endocytosis) that involved coated pits/vesicles, and endosomes, and, probably, (*ii*) a receptor-independent process (fluid endocytosis) carried out by a fraction of plasmalemmal vesicles. Both mechanisms bringing LDL to lysosomes supply cholesterol to the endothelial cell itself. (*b*) Most circulating LDL is transported across the endothelial cell by transcytosis via plasmalemmal vesicles which deliver LDL to the other cells of the vessel wall. Endocytosis is not enhanced by increasing LDL concentration, but the receptor-mediated internalization decreases at low temperature. Transcytosis is less modified by low temperature but is remarkably augmented at high concentration of LDL. While the endocytosis of homologous (rat) LDL is markedly more pronounced than that of heterologous (human) LDL, both types of LDL are similarly transported by transcytosis. These results indicate that the arterial endothelium possesses a dual mechanism for handling circulating LDL: by a high affinity process, endocytosis secures the endothelial cells' need for cholesterol; by a low-affinity nonsaturable uptake process, transcytosis supplies cholesterol to the other cells of the vascular wall, and can monitor an excessive accumulation of plasma LDL. Since in most of our experiments we used LDL concentrations above those found in normal rats, we presume that at low LDL concentrations saturable high-affinity uptake would be enhanced in relation to nonsaturable pathways.

On a variety of systems, it has been demonstrated that cells acquire cholesterol for membrane synthesis primarily by the receptor-mediated uptake of low-density lipoprotein (LDL)[1] which is internalized and delivered to lysosomes. Within the latter, LDL is hydrolyzed and cholesterol is released to be used by the cell (6, 8, 12, 16, 23, 24, 65–67).

To reach various cells, including those of the vessel wall, the plasma LDL (or its components) has to pass through the vascular endothelium. In vivo, the endothelial cell has a special situation since it is faced with the dual problem of taking up plasma cholesterol (or LDL) for its own membrane synthesis and, at the same time, of transporting LDL or its components to the other cells of the surrounding tissue, or of the vessel wall itself. In the case of the latter, disturbances in the capability of endothelium to monitor the transport of a physiological

[1] *Abbreviations used in this paper:* anti-LDL, rabbit anti-human LDL immunoglobulin; anti-LDL-HRP, anti-LDL coupled to horseradish peroxidase; DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase; LDL, low-density lipoprotein; LDL_h, human LDL; LDL_r, rat LDL; LDS, lipoprotein-deficient serum; LDL-F, LDL-ferritin conjugate.

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amount of LDL, or an excessive accumulation of cholesterol within the arterial wall, largely underlie human atherosclerosis. The available information indicates that the lipids and the apoprotein B detected in the atherosclerotic plaque are derived mainly from plasma LDL (29, 30, 59). In both normal and atherosclerotic arteries, LDL enters the intima in relatively large amounts (1, 5, 17–21, 29–31, 43, 45, 52, 54, 59–67, 76).

The LDL interaction with the endothelium has been explored so far on cultured endothelial cells (14, 17, 27, 28, 51, 68, 71, 72). Very little is known about such interactions and the routes and mechanisms of LDL transport across endothelium in vivo (19, 20, 46, 48, 52, 59, 63, 64).

For visualization of LDL uptake by different cells, in the procedures so far designed, the particles were tagged either with fluoresceine (4, 36) (for light microscopy), ferritin (3) or coloidal gold (25) (for electron microscopy), or with ^{125}I, ^{3}H, or ^{14}C (9, 11, 64) (for radioautography). These labeling methods require chemical treatments which can modify some of the LDL properties. Moreover, the final compound to be taken up by the cell is a macromolecular complex the components of which may differently interact with the endothelial cell. In the case of LDL-gold particles, the size of the conjugate (~60 nm) drastically limits the uptake by plasmalemma vesicles, the vesicle neck of which is usually ~30 nm wide (25).

Our study's purpose was to investigate in situ the transendothelial pathway of native untagged LDL particles that, after their interaction with the endothelium, were rendered electron-opaque by enhancing their contrast or by reacting the bound LDL with IgG antiLDL-peroxidase conjugate.

The major goal of this inquiry was to identify the nature of LDL transport across the endothelium in situ. In addition to using rat LDL, we also used human LDL, though the intensity of its binding to rat LDL receptors was expected to be much lower than that of rat LDL, as indicated by the published data on fibroblasts and smooth muscle cells (12, 15, 32, 66).

The findings reported in this paper indicate that, across the arterial endothelium, LDL particles take a dual pathway through two parallel, compartmented routes: (*a*) by receptor-mediated endocytosis via coated pits/coated vesicles they are delivered to lysosomes of the endothelial cell, and (*b*) via plasmalemmal vesicles they are transported across the endothelium to the other components of the vessel wall.

MATERIALS AND METHODS

Isolation of Lipoproteins

Since the experiments were carried out mostly in rats, two kinds of LDL were isolated: (*a*) homologous LDL (from rat plasma), and (*b*) heterologous LDL (from human plasma).

(a) Rat low-density lipoprotein (LDL_r) (density [*d*] = 1.02–1.045 g/ml) was separated from pooled rat serum by differential ultracentrifugation (15, 26, 48).

(b) Human low density lipoprotein (LDL_h (*d* = 1.019–1.063 g/ml) and lipoprotein-deficient serum (LDS) (*d* = 1.225 g/ml) were isolated from fresh plasma of healthy subjects by differential ultracentrifugation. The density of serum fractions was adjusted by addition of solid KBr. Fractions obtained were extensively dialysed against 1 mM Na_2-EDTA in 0.15 NaCl, pH 7.4, at 4°C, for 72 h (50).

Characterization of LDL

NEGATIVE STAINING: LDL particles at 0.1 mg protein/ml, after deposition on carbon-coated grids, were negatively stained with 1% Na phosphotungstate in 0.1 M Na phosphate buffer, pH 7.2, and examined with a Philips 400-HM electron microscope (operated as indicated in the section, Electron Microscopy). Particle shape and size were measured on 200 free-standing particles on randomly collected electron micrographs.

ENHANCING LDL ELECTRON-OPACITY: Several preparation procedures were tested for their capability to impart to native LDL particles sufficient electron-opacity to render them directly visible with the electron microscope, without additional tagging to a tracer (e.g., ferritin or colloidal gold). For this purpose, LDL particles were embedded in 4% agarose in Dulbecco's phosphate-buffered saline supplemented with 1% glucose, and with 0.01 $CaCl_2$ (PBS). Thin slices (~50 μm) of LDL-containing agarose were exposed to various schemes of fixation, postfixation, mordanting, and staining in block, then further processed for standard Epon embedding, sectioning, and electron microscope examination. Controls consisted of treating in the same way LDL-ferritin conjugated particles. In the procedures employed, the best results in terms of imparting to LDL particles a suitable opacity were obtained by incubating the fixed specimens in 3,3′-diaminobenzidine (DAB) (LDL fixation procedure worked out by R. Mora, A. Nistor, and N. Simionescu, manuscript submitted for publication) followed by mordanting the specimens with 1% galloylglucose (tannic acid) (Mallinkrodt, Inc., St. Louis, MO) (56). This procedure was then applied to tissue specimens collected from in situ experiments (for details, see Electron Microscopy). By both negative and positive staining, LDL size ranged from 18 to 22 nm in diameter.

LDL particles were characterized by electrophoretic mobility (40, 46) and electrofocusing (75).[2]

POLYACRYLAMIDE ELECTROPHORESIS: Human LDL and rat LDL were delipidated by the method of Brown et al. (7). On examination by PAGE in the presence of SDS, the human LDL fraction contained only apoprotein B, whereas rat LDL fraction was actually represented by 85–90% apoprotein B and 10–15% apoprotein E (as estimated by densitometric scanning). A similar pattern was earlier reported in the literature (15, 48, 74).

Protein content was measured by a modified Lowry's method (41). Cholesterol was determined according to Zak and Zlatkis (see reference 34).

Conjugation of LDL with Ferritin

As an additional test for the reliable identification of contrasted LDL particles during their transport across the endothelium, LDL was coupled to ferritin (3, 35). We estimated the extent of coupling and the number of ferritins bound to each LDL particle by negative staining (as indicated for LDL).

Production of Rabbit Anti-human LDL Antibody and Rabbit Anti-rat LDL Antibody and Their Conjugates

PREPARATION: These two antibodies were obtained by injecting 5–10 mg of LDL into female New Zealand rabbits, twice a week for a period 10 weeks (2). The rabbit immune sera were purified by DEAE-cellulose chromatography.

COUPLING OF ANTI-LDL TO HRP: The anti-LDL was conjugated with horseradish peroxidase according to the technique of Nakane and Kawaoi (44; see also reference 70).

TESTS FOR PURITY AND SPECIFICITY: The purity of LDL_h and the specificity of rabbit anti-human LDL antibody were checked by immunoelectrophoresis (2) and crossed immunoelectrophoresis (13).

The human LDL gave a single precipitin line by immunoelectrophoresis (Fig. 1 *a*) and a single peak by crossed immunoelectrophoresis vs. horse anti-human whole serum (Cantacuzino Institute, Bucharest, Romania) (Fig. 1 *b*), or vs. goat monospecific anti human lipoprotein serum (Miles Laboratories, Inc., Elkhart, IN), or rabbit antihuman LDL serum (Fig. 1 *c*).

The rabbit anti-human LDL antibody gave a single precipitin line vs. human serum and none vs. HDL (by immunoelectrophoresis) and a single peak vs. human serum (by crossed immunoelectrophoresis) (Fig. 1 *d*). The LDL-ferritin conjugate (LDL-F) conjugate gave a similar precipitin line versus anti-LDL antibody as native human LDL.

By immunoelectrophoresis, the anti-rat LDL antibody gave a major precipitin line vs. rat LDL (*d* = 1.02–1.045 g/ml), and a faint trace against probably rat HDL_1 present in the fraction isolated at this range of density (Fig. 2).

The immunological activity of each anti LDL-horseradish peroxidase (LDL-HRP) conjugate was checked by double immunodiffusion vs. the appropriate LDL. The peroxidative activity of the conjugates was tested by incubating the immunodiffusion plate (washed with PBS for 72 h) with DAB in the presence of H_2O_2.

Experimental Procedures

GENERAL CONDITIONS: The pathway of LDL particles across the endothelium of the aorta and coronary artery, in situ, was studied by using, in parallel

[2] The pI of native LDL was found to be 5.4, as compared to pI 5.1 - 5.3 of the LDL-ferritin conjugate. The latter may reflect the presence of a dispersed population of LDL particles produced by the coupling of one to three ferritin molecules to one LDL.

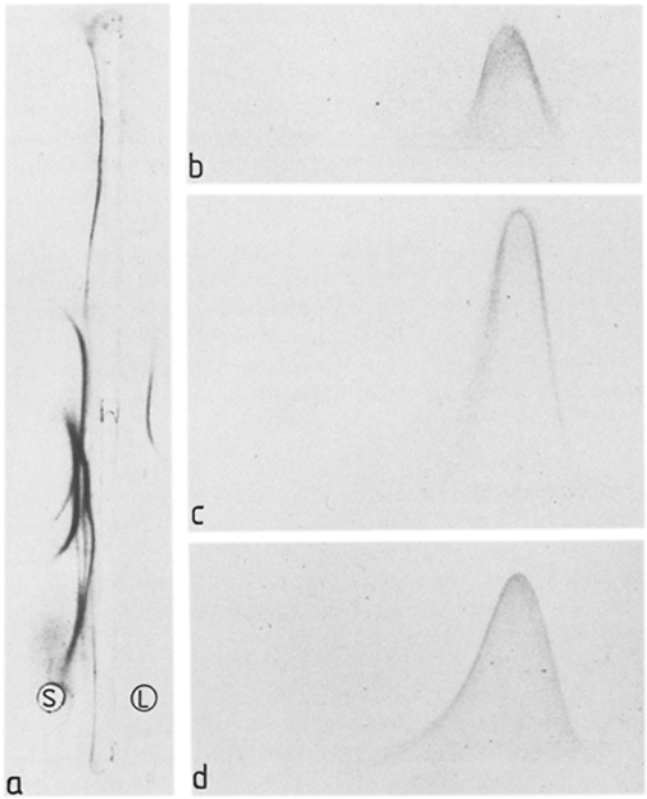


FIGURE 1 (*a*) Immunoelectrophoresis of human serum (*S*) and human LDL (*L*) vs. horse anti-human whole serum. (*b*) Crossed immunoelectrophoresis of human LDL vs. horse anti-human whole serum. (*c*) Crossed immunoelectrophoresis of human LDL vs. rabbit anti-LDL serum. (*d*) Crossed immunoelectrophoresis of human plasma vs. rabbit anti-LDL serum.

experiments, either native untagged particles, untagged LDL subsequently reacted with anti-LDL-HRP or LDL-ferritin conjugate. In male adult R white rats of our colony, the serum cholesterol level was found to be ~100 ± 25 mg/dl, and the electrophoretically β-migrating lipoproteins were estimated to be ~22 ± 5 mg cholesterol/dl. Male adult RAP mice had a serum cholesterol level of ~145 mg/dl, and electrophoretically β-migrating lipoprotein of ~30 mg cholesterol/dl. Since a broad range of plasma LDL level, sometimes threefold ranges in the same rat strain (e.g., Sprague-Dawley animals), has been reported in the literature (3, 10, 12, 20, 39, 42, 49, 53, 55), we calculated the LDL concentrations used in our experiments on the basis of the values detected in this strain of rats. Actually, the concentration found (22 mg LDL cholesterol/dl), falls within the range reported in the literature (from 16 to 50 mg LDL cholesterol/dl serum) (12), and is close to that most frequently reported in the literature (15 mg cholesterol/dl serum [12]). Both kinds of LDL, homologous and heterologous, were used at the following concentrations: (*a*) physiological concentrations: 10 and 30 mg LDL cholesterol/dl; and (*b*) high concentrations: 80, 170, and 220 mg LDL cholesterol/dl. Experiments were carried out at 37°C; in some experiments, the perfusion solutions used were at 4°C. The actual time intervals from LDL administration until fixation in situ ranged from 2 to 10 min. The shortest time interval (2 min) included the time required for LDL injection, and the washing of unbound LDL with perfusion of PBS or lipoprotein-deficient serum (LDS).

EXPERIMENTS WITH LDL AND LDL-F: 35 male adult R white rats and 15 male adult RAP mice kept in standard housing conditions were fasted for 24 h before experiment. Animals were anesthetized with chloral hydrate 50 mg/100 g body weight (mice) or diethylic ether (rats). The vascular bed was cleared of blood by perfusion with a Harvard pump with PBS supplemented with 0.25% glucose, at 37°C. Depending on the artery under investigation, the perfusion procedure used for both washing out the blood and the administration of LDL was different. In rat, for both the aorta and the coronary artery, perfusion with PBS was done through the thoracic cava, using the abdominal cava as outlet. Then, for perfusing LDL, the thoracic aorta was tightly ligated and a fine needle was introduced into the left ventricle. The latter route was used also for the administration of the subsequent solutions (e.g., PBS, fixative). In mouse, the perfusion system employed the abdominal aorta as inlet, and abdominal cava as outlet.

Through the perfusion systems described above, after blood was washed out, the following solutions prewarmed at 37°C were sequentially infused:

(*a*) 0.2–0.4 ml of LDL_h or LDL_r or 1 ml of LDL-F for time intervals ranging from 2 to 10 min; (*b*) 2 min washing with 3–5 ml of PBS (during which, in the case of rat experiments, the ligature on the thoracic aorta was loosened); (*c*) 2% glutaraldehyde in 0.1 M HCL-Na cacodylate buffer, pH 7.2, for 10 min. Then, small pieces of thoracic and abdominal aorta or coronary artery including their adventitia were removed and immersed in the same fixative for 80 min, and further processed for electron microscopy.

EXPERIMENTS WITH ANTI LDL-HRP: The purpose of these experiments was twofold: to check whether the particles observed in various locations were undoubtedly LDL, and to render LDL particles more electron-opaque. After perfusion of native LDL or LDL-F (in some controls), and washing out of the unbound particles (as indicated above), one of these two protocols was employed: (*a*) perfusion of 0.2–0.4 ml of anti-LDL-HRP (0.1 mg/ml protein content) at 37°C for 2 min followed by washing with PBS, and aldehyde fixation by perfusion; (*b*) after fixation in situ for 10 min by perfusion of 2% glutaraldehyde, small pieces of the aorta and coronary artery were cut and immersed for 20 min in the same fixative. Subsequently, the specimens were processed for immunocytochemistry.

Competition Experiments

To distinguish between high-affinity and low-affinity binding sites, the inhibitory effect of heparin on LDL binding to its receptor (22) was tested in animals which, after receiving LDL or LDL-F at 37°C, were perfused with LDS at 4°C for 3 min, followed by heparin (10 mg/ml in 1 ml of LDS) at 4°C for 2 min. A final wash was done with LDS at 4°C and then fixation with glutaraldehyde.

Controls

(*a*) To check whether some of the visualized LDL is endogenous rat LDL that was being taken up at the time the animal was killed, in some rats, after 5-min perfusion with PBS and not receiving LDL, the specimens were fixed in aldehydes, incubated with DAB · H_2O_2 and further processed according to the same preparation procedure used for electron microscopy and immunocytochemistry.

(*b*) Control experiments consisted in perfusing—instead of LDL or LDL-F—either native ferritin or activated ferritin (0.1–0.6 mg/ml protein content) at 37°C, for periods of 2–10 min.

(*c*) In another series of controls, anti-LDL-HRP (0.1 mg/ml protein content) or HRP alone was infused, the rest of the procedure being followed as described.

(*d*) The possible effect of the presence or absence of proteins in the perfusion media was tested by infusing the vascular bed with either (*a*) PBS followed by LDL, and washing with PBS; (*b*) PBS with LDS followed by LDL, and washing with PBS + LDS, or (*c*) LDS followed by LDL, washed thereafter with LDS.

Electron Microscopy

From experiments with LDL, LDL-F, and some controls, small pieces of the thoracic and abdominal aorta, and coronary artery, fixed in situ with 2% glutaraldehyde, were removed and immersed for 80 min in the same fixative, followed by incubation with 0.15% DAB in 0.06 M HCl-Tris buffer, pH 8.0, for 30 min at 23°C. After brief washing in cacodylate buffer, specimens were postfixed with 2% OsO_4 in 0.1 M HCl-Na cacodylate buffer, pH 7.2, at 4°C, for 90 min, followed by in-block staining with 0.5% aqueous uranyl acetate for 30 min at 2°C, or by mordanting with 1% tannic acid as previously described (56) (see Fig. 3*c*). Tissue processing was completed by standard procedure for dehydration and Epon embedding. Thin sections cut with an American Optical Ultracut (American Optical Scientific Instruments, Buffalo, NY) or Richert ultramicrotome (Richert Spring Corp., Pittsburgh, PA) were stained with lead citrate and examined with a Philips 400-HM electron microscope, operated at 80 kV.

Immunocytochemistry

From experiments with anti LDL-HRP (60), and their pertinent controls, after fixation in situ, small pieces of the aorta and coronary artery were cut and processed through one of the following methods: (*a*) Immersion in 2% glutaraldehyde in 0.1 M HCl-Na cacodylate buffer, pH 7.2, for 80 min, at 23°C; then incubation in 0.15% DAB in 0.06 M Tris-HCl, pH 8.0, and 0.02% H_2O_2, for 60 min at 37°C. After brief washing in buffer, fixation in 2% OsO_4, and further preparation for electron microscopy by the standard procedure. (*b*) Immersion in the aldehyde fixative as in *a* for 10–20 min. After a brief wash with PBS, the specimens were embedded in 5% agarose in PBS, and thin slabs (~50 μm thick) were sectioned with a Smith-Farquhar chopper. Sections were incubated with anti LDL-HRP (0.1 mg/ml protein content) for 40 min, washed with buffer for 15 min, then fixed with 2% glutaraldehyde for 60 min. The peroxidatic reaction

was carried out as described under *a*. Specimens were dehydrated and embedded in Spurr resin (62).

RESULTS

Visualization of LDL Particles

Freshly isolated native LDL could be directly visualized as spherical ~20-nm diameter particles, especially in well-fixed specimens that were treated in-block with galloylglucose (tannic acid) or uranyl acetate. The electron density was particularly enhanced by reacting LDL with anti-LDL-HRP. The reaction product of the latter slightly increased the overall size of the conjugate which frequently appeared less spherical, rather polymorphic (Fig. 3). When anti-LDL-HRP was applied to sections, it could reach accessible LDL particles in various locations. Anti-LDL-HRP perfused in situ had access only to LDL particles found on the cell surface or within open vesicles. Occasionally, the antigen-antibody complex continued to be internalized and eventually detected deep in the endothelium. In similar experimental conditions, the number of LDL particles observed in various locations was comparable, irrespective of the visualization technique used. However, a slightly higher number of LDL was recorded when rabbit anti-LDL antiserum was used, presumably due to a stabilizing effect of the latter on the LDL apoprotein. The findings reported from this point on are based primarily on the experiments in which human and rat LDL were immunocytochemically detected.

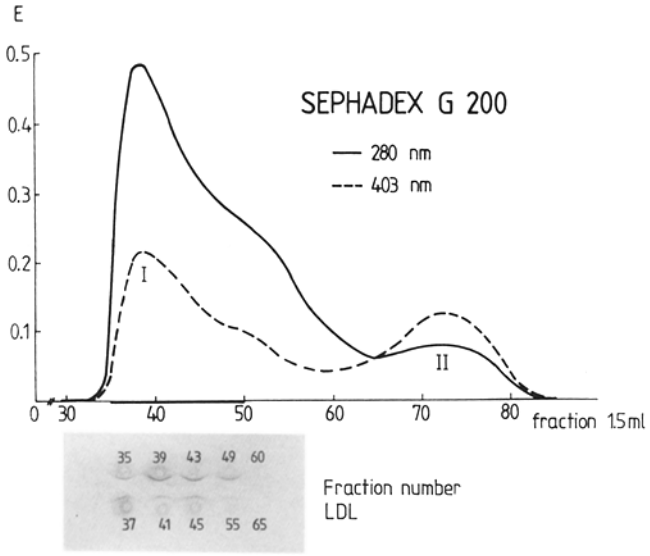


FIGURE 2 Gel chromatography of the crude anti human LDL-HRP conjugate (6 mg anti-LDL IgG conjugated with 5 mg HRP), on a 1.5 × 85 cm column of Sephadex G200 eluted with PBS. Double immunodiffusion of aliquots (40 μm) from peak I vs. LDL_h, indicated that the anti-LDL-HRP conjugate elutes in fractions 35–50 which had peroxidatic activity. These fractions were used for the experiments. All electron micrographs represent aortic endothelium of rats infused with human LDL (10 mg LDL cholesterol/dl).

GENERAL

In animals perfused with PBS and not given LDL, but infused with anti-rat LDL-HRP, no lipoprotein particles were detected on the endothelium or within its vesicles. This indicates that after 5-min perfusion with PBS, the endogenous LDL occurring at the level of endothelium has been practically removed.

The findings showed that, at 37°C, physiological concentrations of homologous or heterologous LDL (10–30 mg LDL cholesterol/dl of perfusate) interact with the arterial endothelium in situ in a stepwise process. This consists of LDL binding to the endothelial surface with subsequent internalization followed either by degradation within the endothelial cell (endocytosis) (Figs. 4–10) or by their transport across the endothelium to the subendothelial space (transcytosis) (Figs. 11–20). This dual pathway involves two separate compartmented routes which have a similar pattern in the endothelium of the arteries examined (aorta, coronary) as well as in their vasa vasorum (69).

As expected, as compared to rat LDL, human LDL bound less extensively to rat LDL receptors. However, because of the known homogeneity of the human LDL fraction (15, 26, 48), the quantitative work was done with human LDL. The emerged figures, though considered underestimates, have been used as relative values which may reflect the nature and some particulars of the process, and not its absolute magnitude. The

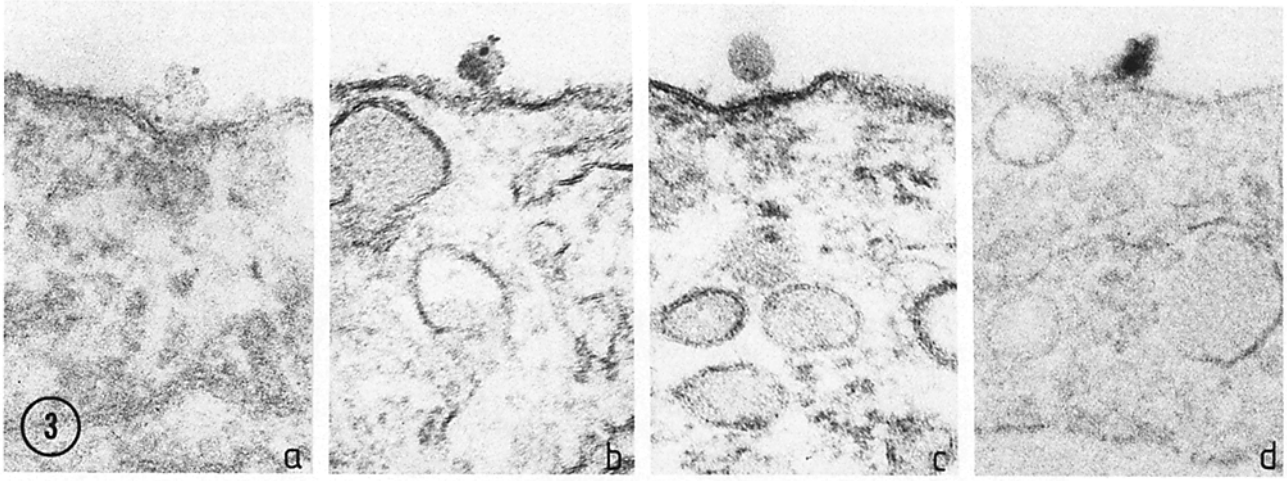
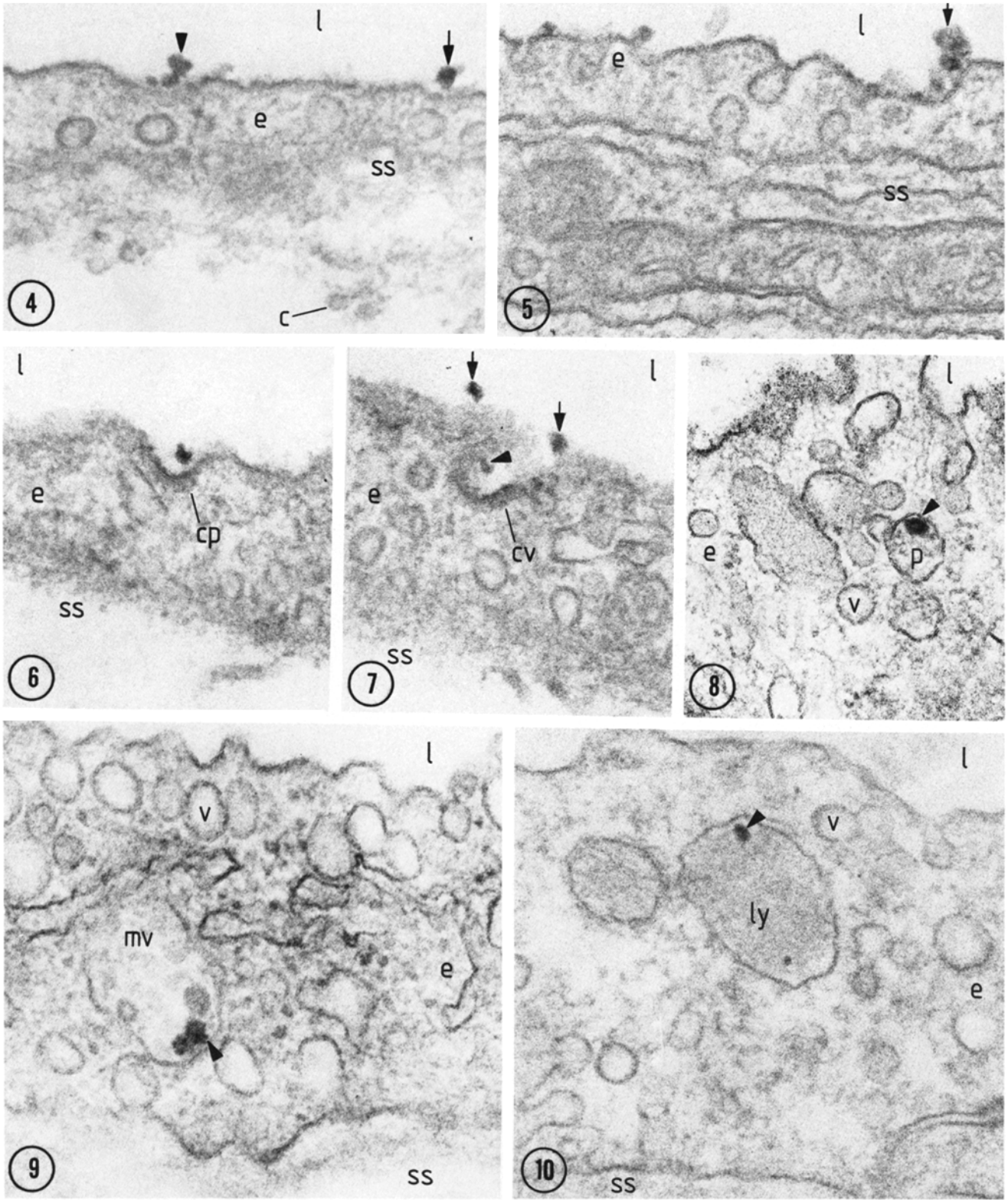
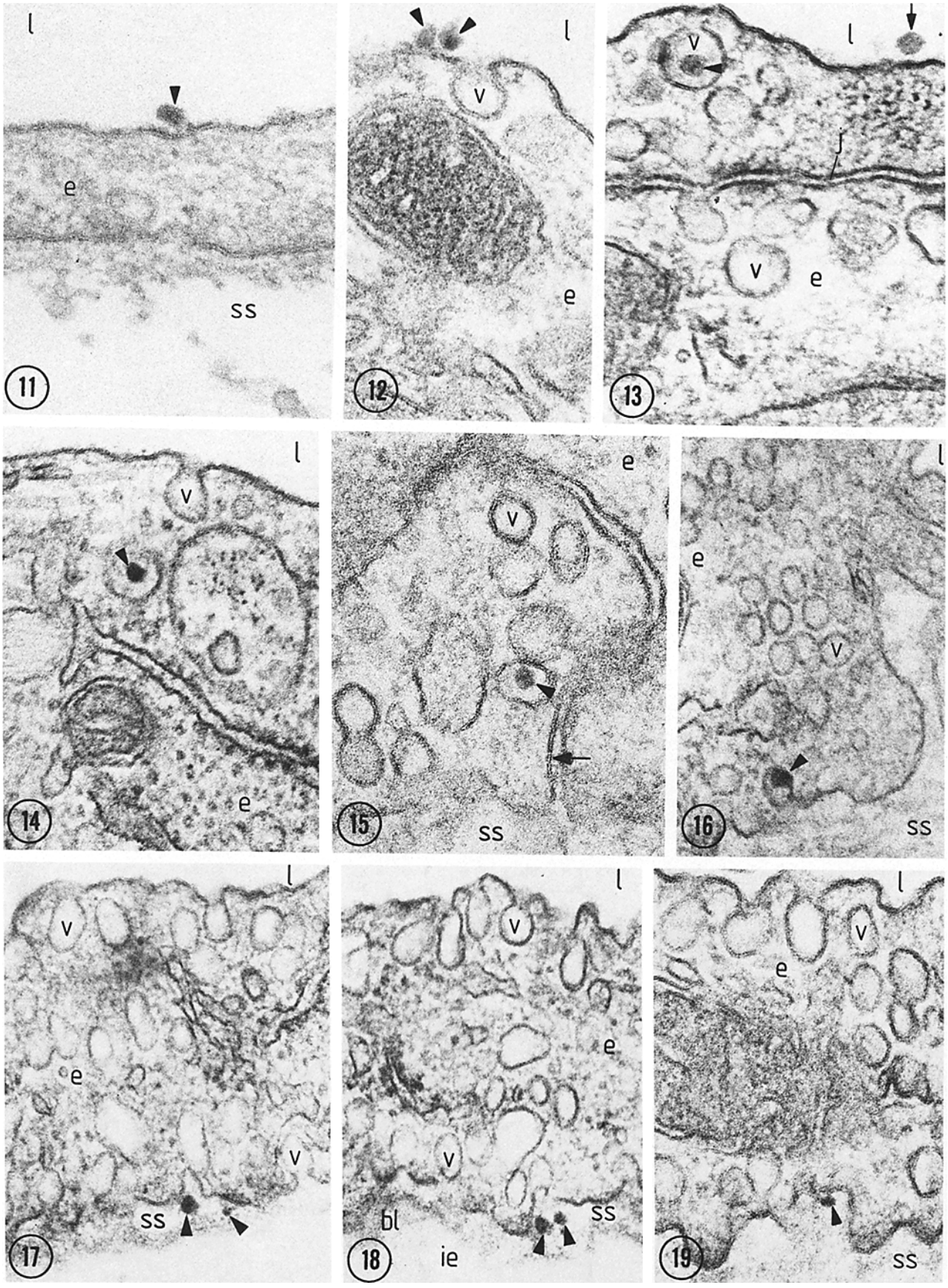


FIGURE 3 Various methods used for in situ visualization of human LDL during its interaction with the arterial endothelium: (*a*) LDL-ferritin conjugate (no additional contrasting); (*b*) LDL-ferritin conjugate, with additional contrasting by mordanting with tannic acid; (*c*) native, untagged LDL fixed with the glutaraldehyde and DAB procedure and contrasted by mordanting with tannic acid; and (*d*)) native, untagged LDL reacted with anti LDL-HRP conjugate. (*a*) × 110,000; (*b*) × 94,000; (*c*) × 86,000; (*d*) × 80,000.



FIGURES 4–10 Gallery of electron micrographs illustrating the sequential steps of endocytosis of human LDL (10 mg LDL cholesterol/dl) in the rat endothelium as detected by immunocytochemistry. *For all micrographs: 1*, lumen; *e*, endothelium; *ss*, subendothelial space; *v*, plasmalemmal vesicle; *cp*, coated pit; *cv*, coated vesicle; *p*, pinosome (endosome); *mv*, multivesicular body; *ly*, lysosome; and *c*, collagen. Fig. 4: 2 min after perfusion. LDL particles, either isolated or in small clusters, are bound to smooth regions (arrow) or coated regions (arrowhead) of the luminal front of the endothelial cell membrane. × 80,000. Fig. 5: 2 min after perfusion. Occasionally, small clusters of LDL are also bound to indentations of cell surface devoid of conspicuous coating (arrow). × 84,000. Fig. 6: 2 min after perfusion. LDL marks some coated pits × 72,000. Fig. 7: Formation of a coated vesicle labeled by an LDL particle (arrowhead) while two other LDL particles appear bound to the neighboring plasma membrane (arrows). × 70,000. Fig. 8: 5 min after perfusion. LDL (arrowhead) marks some large uncoated vesicles (pinosomes). × 70,000. Fig. 9: 5 min after perfusion. Occasionally, LDL (arrowhead) can be detected within multivesicular bodies. × 94,000. Fig. 10: 5 min (as well as 10 min) after perfusion. LDL (arrowhead) could be detected within lysosomes. × 72,000.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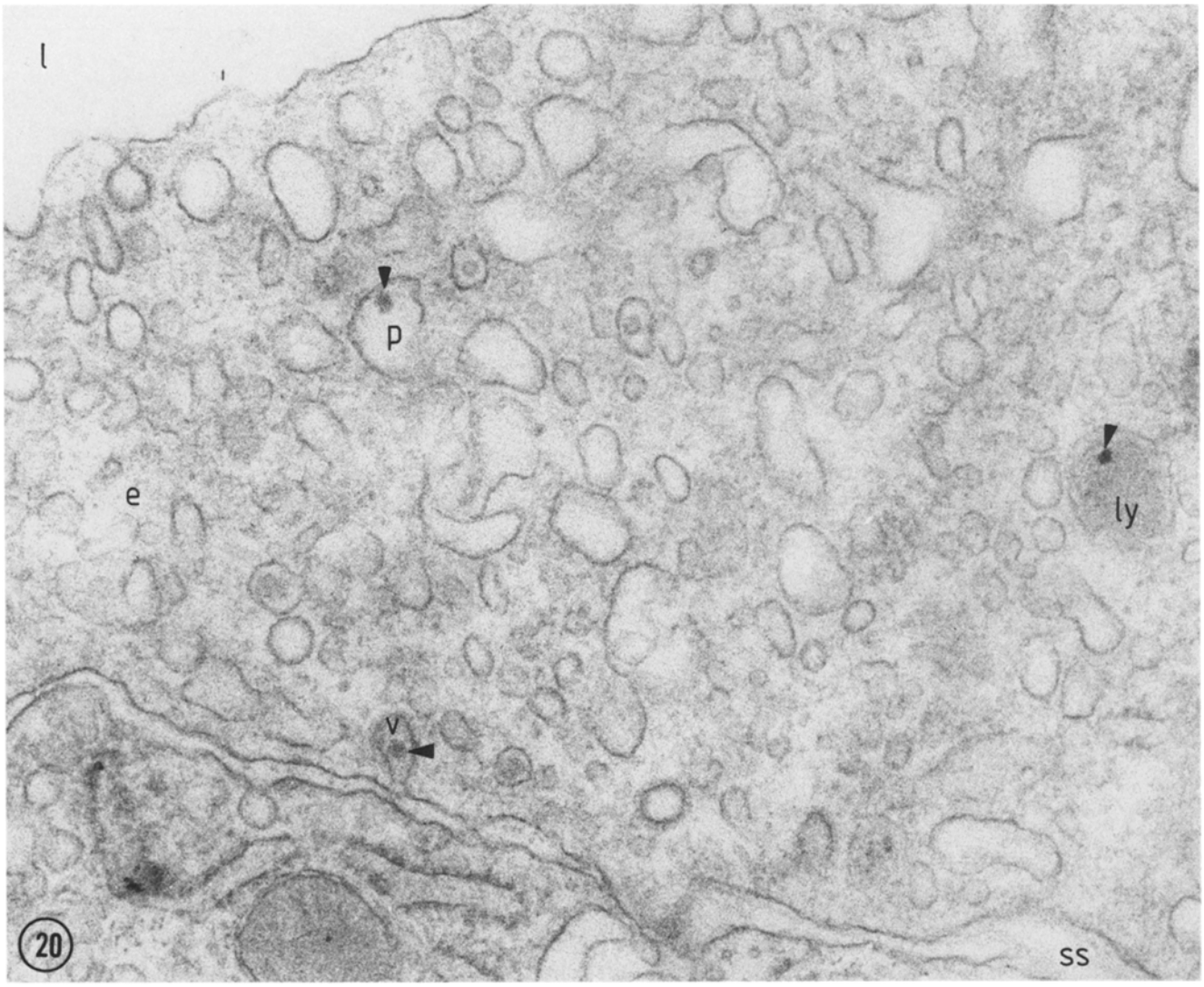


FIGURE 20 Rat aortic endothelium, 5 min after perfusion of human LDL (10 mg LDL cholesterol/dl). LDL (arrowheads) could be concomitantly detected in features involved in endocytosis: e.g., pinosome (endosome) (*p*), and lysosome (*ly*), as well as in features involved in transcytosis: e.g., plasmalemmal vesicles (*v*) open on the abluminal front of the endothelial cell. × 86,000.

observations with homologous LDL were not quantitated because rat LDL fraction contains about 15% HDL_1 particles which cannot be distinguished from the LDL particles. Within these reservations, it can be stated that as a general pattern of the process the binding and uptake of rat LDL by endocytosis appeared qualitatively significantly greater than that of human LDL; this difference was inconspicuous in the case of transcytosis (see the detailed Results).

At high concentrations of either rat or human LDL (80–220 mg LDL cholesterol/dl of perfusate), while endocytosis was not obviously augmented, transcytosis did parallel the increase in LDL concentration in the vascular lumen.

FIGURES 11–19 Gallery of electron micrographs illustrating sequential steps of LDL transcytosis of human LDL (10 mg LDL cholesterol/dl) through rat aortic endothelium, as detected by immunocytochemistry. Fig. 11: 2 min after perfusion. Some LDL particles (arrowhead) are bound to smooth areas of the luminal front of endothelial plasma membrane. × 86,000. Fig. 12: 2 min after perfusion. LDL particles (arrowheads) are also encountered close to the vesicle openings. × 88,000. Fig. 13: 2 min after perfusion. While some LDL particles occur on the endothelial cell surface (arrow), other particles (arrowhead) mark plasmalemmal vesicles (*v*) associated with the luminal front. *j*, intercellular junction. × 98,000. Fig. 14: 5 min after perfusion. LDL (arrowhead) marked plasmalemmal vesicles located inside the cytoplasm. × 85,000. Fig. 15: 5 min after perfusion. LDL (arrowhead) could also be detected within plasmalemmal vesicles associated with the abluminal part of the intercellular spaces (arrow). × 80,000. Fig. 16: 5 min after perfusion. LDL (arrowhead) marked some plasmalemmal vesicles open on the abluminal front of the endothelial cell. × 72,000. Fig. 17: 5 min after perfusion. LDL (arrowheads) occurred as apparently intact particles within the subendothelial space (*ss*). × 78,000. Fig. 18: 5 min after perfusion. LDL (arrowheads) could also be encountered within the subendothelial space, against the basal lamina (*bl*) and internal elastica (*ie*). × 78,000. Fig. 19: 10 min after perfusion. In some locations the immunocytochemical reaction revealed relatively small particles (arrowhead), presumably representing partially degraded LDL or fragments of apoprotein B. × 84,000.

BINDING OF LDL TO ENDOTHELIUM

Physiologic Concentrations of LDL (10–30 mg LDL Cholesterol/dl)

EARLY EVENTS: UP TO 2 MIN: Human LDL: from the earliest time points investigated, LDL_h particles were found bound to the luminal endothelial plasmalemma (apparently smooth areas) (Figs. 4, 5, and 11), on coated pits (Figs. 4 and 6), facing the vesicle stomata (Fig. 12), or occurring within plasmalemmal vesicles open on the luminal front. In incompletely washed specimens, unbound LDL particles could also be observed in the vascular lumen. The number of LDL particles bound to the endothelial cell surface varied largely from one region to another of the endothelium, being relatively higher in the coronary artery than in the aorta. In most cases, one to three LDL particles were observed attached to coated pits (Figs. 4 and 6); only ~50% of coated pits were marked by detectable LDL. The numerical density of LDL bound on coated pits was estimated at 5 to 8 LDL particles/μm^2 of endothelial surface (see legends to Figs. 21 and 22). In the same specimens, the average number of LDL found on smooth regions of plasma membrane, vesicle stomata and luminal plasmalemmal vesicles was 13–14 LDL particles/μm^2 of endothelial surface.

Rat LDL: In general, the binding values for LDL_r particles (which reportedly contains ~15% HDL_1) were about twofold higher than for human LDL. The most characteristic differences observed was a higher number of LDL_r particles (two to five) bound to a coated pit, and an increased number of coated pits (~80–85%) which were marked by LDL_r particles at this early time point.

LATE EVENTS: UP TO 10 MIN: In both LDL_h and LDL_r experiments, the extension of the time interval to 10 min after LDL perfusion (Fig. 21, for LDL_h) resulted in 50–60% decrease in the frequency of coated pits and regions of plasmalemma marked by LDL: concomitantly, augmentation of LDL labeling of endosomes, lysosomes and internalized plasmalemmal vesicles occurred (Fig. 21).

High Concentrations of LDL (80–220 mg LDL Cholesterol/dl)

When high concentrations of either human LDL or rat LDL were employed, the labeling of coated pits did not change significantly. At 5-min time interval, however, the number of LDL_h or LDL_r bound to plasmalemma or associated with vesicle stomata was decreased by ~35–40% (as compared to low LDL concentrations) with concomitant augmentation of the number of LDL particles internalized by plasmalemmal vesicles (Fig. 22). The binding values were not significantly changed by perfusing LDL_h or LDL_r solutions at 4°C.

Heparin was able to displace most of LDL from coated pits and plasma membrane, but only slightly reduced the LDL uptake by plasmalemmal vesicles. These results were interpreted as a suggestion for the existence on the endothelial cell surface of two types of LDL-binding sites: high-affinity binding sites (coated pits) and probably low-affinity binding sites (plasmalemmal vesicles).

LDL INTERNALIZATION

Physiologic Concentrations of LDL

The kinetics of various endothelial features involved in the LDL transport indicated that, over time intervals ranging from

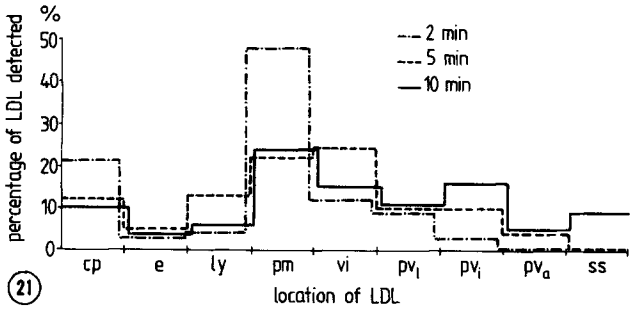


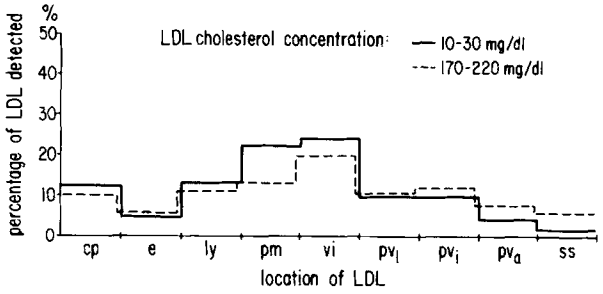
FIGURE 21 Kinetics of human LDL endocytosis and transcytosis through rat arterial endothelium, as a function of time. The LDL concentration employed in these pulse-chase experiments was 10–30 mg LDL cholesterol/100 ml. Features involved in receptor-mediated endocytosis: *cp*, coated pit; *e*, endosome (pinosome); and *ly*, lysosome. Features involved in transcytosis (and possibly in receptor-independent endocytosis): *pm*, plasma membrane; *vi*, vesicle infundibulum; pv_1, plasmalemmal vesicle open on the luminal front; pv_i, plasmalemmal vesicle located inside the cytoplasm; pv_a, plasmalemmal vesicle associated with or open on the abluminal front of the endothelium; and *ss*, subendothelial space. For each time point, 10 to 13 animals were used and from each animal four to six aortic and coronary specimens were collected and processed for electron microscopy. From each specimen, we examined 60 to 80 sections, and carried out the measurements on the following total length of endothelial profiles: 410 μm, 422 μm, and 478 μm for 2, 5, and 10 min, respectively. The number of particles in the various locations, counted on randomly collected specimens, were 707 for 2 min, 682 for 5 min, and 728 for 10 min. From the linear data regarding LDL binding to the cell surface, the endothelial surface was calculated by considering a section thickness of ~60 nm. $\overline{SD}$ of the mean, determined by using large sample theory, ranged from ±23 to ±47.

2 to 10 min, LDL was transported through arterial endothelium via two compartmented pathways by endocytosis and transcytosis. Receptor-mediated endocytosis of rat LDL was significantly more pronounced than that of human LDL, as expressed by a higher number (almost double) of coated vesicles, endosomes and lysosomes marked by LDL_r as compared to LDL_h. Conversely, the extent of LDL internalization by transcytosis (via plasmalemmal vesicles) did not markedly differ between human LDL and rat LDL.

ENDOCYTOSIS: As indicated by the time course experiments ranging from 2 to 10 min, receptor-mediated endocytosis involved successively coated pits (Figs. 4 and 6), coated vesicles (Fig. 7), most frequently large, ~10–15 nm diameter, uncoated vesicles (endosomes or pinosomes) (Fig. 8) (63),[3] multivesicular bodies (Fig. 9), and secondary lysosomes. It could not be distinguished whether, in addition to this characteristic receptor-mediated uptake (3, 6, 23, 24), a certain fraction of LDL-labeled plasmalemmal vesicles were destined for lysosomes as well, by a receptor-independent mechanism. Within lysosomes, totally, or what appeared to be partially, intact LDL particles were detected (Figs. 10 and 20).

TRANSCYTOSIS: A relatively large number of either human LDL or rat LDL particles was taken up by discrete plasmalemmal vesicles open on the luminal aspect of endothelium. Up to 5 min, most of these LDL-labeled vesicles appeared internalized (Figs. 12–15), some of them approaching (Fig. 15)

[3] Because the visualization of the vesicle "coat" is sometimes uncertain, we used also in this paper the term "pinosome" (73) as alternative to "endosome" to define uncoated vesicles or vacuoles of ~10–15 nm diameter, which appear to be instrumental in receptor-mediated endocytosis.



(22)

FIGURE 22 Kinetics of human LDL endocytosis and transcytosis across rat arterial endothelium, as a function of LDL concentration. The time point examined: 5 min. For legend to abbreviations and other data, see Fig. 21. Specimen sampling: for 10–30 mg LDL cholesterol/100 ml, as indicated in Fig. 21; for 170–220 mg LDL cholesterol/100 ml, the total length of endothelial profiles examined was 306 μm, and the number of LDL particles counted was 486.

or discharging their content into the subendothelial space (Figs. 16 and 20). We observed no remarkable difference in the rate of transcytosis between human LDL and rat LDL. In the conditions of our experiments, the number of plasmalemmal vesicles marked by LDL particles was augmented by the increasing time interval (Fig. 23), but was much less or not at all influenced by lowering to 4°C the temperature of the infused solutions.

High Concentrations of LDL

ENDOCYTOSIS: The receptor-mediated uptake of either human LDL or rat LDL seemed not significantly augmented either by increasing several-fold (4- to 10-fold) the LDL concentration in the perfusate or by extending the time interval of the endothelial cell exposure to LDL. The efficiency of endocytic pathway appeared, however, diminished by low temperature.

TRANSCYTOSIS: The transendothelial transport of both human LDL and rat LDL by plasmalemmal vesicles was augmented with both the increasing LDL time interval (Fig. 23) concentration (Fig. 24). This process was virtually unaffected by lowering the temperature of the infused solutions to 4°C.

In none of the experimental conditions used was either LDL_h or LDL_r observed passing through the intercellular junctions of arterial endothelium.

LDL IN THE SUBENDOTHELIAL SPACE

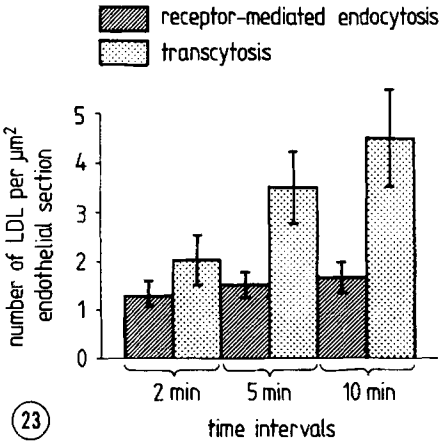
Physiologic Concentrations of LDL

When concentrations of LDL in the range of 10–30 mg LDL cholesterol/dl were perfused, at 2 to 5 min, LDL particles detectable within the subendothelial spaces were extremely rare. At 5–10 min, few LDL particles could be visulized within the subendothelial space against the basal lamina (Figs. 17–19), or beyond the latter. The number of apparently intact LDL observed in the subendothelial space was usually much smaller than the amount of LDL which, at earlier time points, was bound to the endothelial surface or internalized within plasmalemmal vesicles.

High Concentrations of LDL

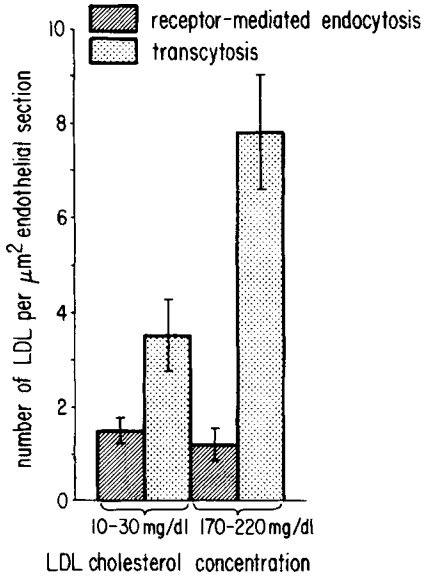
In experiments in which the perfusion solution contained 80–220 mg LDL cholesterol/dl, although the LDL particles visualized in the subendothelial space were more frequent, their average number was much lower than that recorded, at earlier time points, bound to the endothelial cell surface (Figs. 21 and 22). We observed no significant difference in the frequency of human LDL or rat LDL in the subendothelial space.

In areas lacking endothelium (accidentally damaged during perfusion maneuvers), neither LDL_h nor LDL_r accumulated in the local subendothelium. LDL particles were encountered in the subendothelium only in areas covered by normal endothelial cells.



(23)

FIGURE 23 Histogram depicting the numerical density of human LDL/μm^2 of endothelial section as a function of time in rats that have received 10–30 mg of LDL cholesterol per 100 ml of perfusate. Vertical bars represent the SD of the mean estimated by the large sample theory method. Features involved in receptor-mediated endocytosis are: coated pits, coated vesicles, endosomes (pinosomes), lysosomes. Features involved in transcytosis and, possibly to a lesser extent, in receptor-independent endocytosis are: plasma membrane, vesicle infundibulum, plasmalemmal vesicles. Because these structures are located at various depths within the endothelial cell, the values were normalized per μm^2 of cell section.



(24)

FIGURE 24 Histogram showing the numerical density of human LDL per μm^2 of endothelial profile (rat aorta), as a function of LDL concentration. For other data, see legend to Fig. 22.

The interaction of LDL with other components of the arterial wall (smooth muscle cells, elastic lamellae, collagens, ground substance, etc.) will be reported in a forthcoming paper.

Under some peculiarities, the LDL pathway across the endothelium of vasa vasorum occurs in a fashion similar to that observed in the arterial endothelium (69) (manuscript in preparation).

Controls

In animals perfused with either native ferritin (pI 4.5) or glutaraldehyde-activated ferritin (pI 4.4 - 4.6), none of these tracers was found in a significant amount on coated pits or in coated vesicles. Negative results were also recorded in the experiments in which either HRP or DAB alone was intravascularly injected.

In the presence of proteins (brought in by LDS) in the perfusion media, the LDL uptake by the endothelium appeared significantly reduced as compared to animals perfused with LDL in PBS only.

DISCUSSION

The points to be discussed are related to the methodology used, and the results obtained and their possible interpretation.

Visualization of Untagged LDL

The investigation of LDL interaction with the vascular endothelium by using the system presented in this paper has the advantage of an in situ study employing fresh, negative untagged LDL, rendered visible after its interaction with the cell and subsequent fixation within tissues. The techniques previously developed by other investigators to make LDL ultrastructural probes by tagging an electron-opaque tracer (ferritin, colloidal gold) require extensive chemical treatment, repurification of the labeled product, etc., procedures which can modify LDL reactivity. Moreover, in the case of LDL-ferritin conjugate (3), only the ferritin core can be visualized and one has to assume that each core is backed by an LDL. Colloidal gold-LDL conjugate, due to its large size (~60 nm), severely limits its uptake by pinocytic or plasmalemmal vesicles (25), thus becoming unsuitable for studies on LDL transport by such structures. In addition, the LDL-ferritin and LDL-gold conjugates interact with target cells as combined macromolecular complexes, and not strictly as LDL particles.

The omission of the chemical treatments required by any labeling secures a better preservation of LDL particles which facilitates the contrasting procedures designed to enhance LDL electron opacity. The LDL preservation can be further improved by addition of LDS, by supplementing the fixative with DAB (that increases the cross-linking), and by mordanting with tannic acid, which, in addition to enhancing contrast, also prevents extensive extraction during specimen dehydration (56).

The immunocytochemical reaction of LDL by anti LDL-HRP allows the unequivocal identification of LDL particles, with concomitant enhancement of their electron opacity. Since, in the case of LDL, the immunocytochemical procedure detects individual particles, the technique has less limitations than the cytochemical methods in which the final mass reaction product does not allow any quantification.

Human LDL and Rat LDL

It has been shown that, at least in culture conditions, human LDL binds rather poorly to rat LDL receptors in fibroblasts and smooth muscle cells (12, 15, 32, 66). In other experimental conditions, however, an increased binding of human LDL to liver membranes from rats treated with 17-α-ethinyl estradiol (37), and a hepatic catabolism of human LDL similar to that of rat LDL in intact rats (55) and in the rats treated with estradiol (10, 74), were reported. Similar observations have been made in other species, e.g., a comparable hepatic catabolism of intravenously administered human or canine ^{125}I-labeled LDL, in dog, despite different apparent affinities for receptors (38). In recent work by Pittman et al. (48), on tissue sites of catabolism of rat and human LDL in rats, it has been pointed out that, despite the low affinity of human LDL for rat LDL receptors, human and rat LDL are catabolized at similar rates and at similar sites. A well-founded explanation of such apparent discrepancies has not been yet produced.

As far as we know, there is no reported information on the distribution and cellular localization of LDL receptors of the endothelial cell in situ. In our experiments, we used human LDL because it is a homogeneous fraction containing almost exclusively apolipoprotein B. Human LDL, although at a lower rate, can reveal LDL-binding sites exclusively. As such, the high-affinity uptake detected in the LDL_h experiments should be considered underestimated. The data that emerged from these experiments have been used as relative values which may reflect the nature and not the absolute magnitude of the process observed. The rat LDL used in parallel experiments has the advantage of the homologus high binding to rat LDL receptors but has the disadvantage of being a heterogeneous fraction which contains small but definite amounts of apolipoprotein E (10–15% of LDL protein). This suggests that the binding sites and pathways detected with rat LDL reflect, to some degree, the sites for uptake and routes for transport of HDL_1 as well. Accordingly, no quantitation was made for the results obtained with rat LDL.

Physiologic vs. High Concentration of LDL

In the male adult R white rats used in our experiments, the serum level of LDL cholesterol was found to be ~22 mg/dl. In the literature, a surprisingly broad spectrum of LDL concentrations has been reported; the difference occurs not only between various strains of rats but in animals of the same strain. For example, in fed, male Sprague-Dawley rats, a threefold range in both LDL and HDL levels is apparent. These discrepancies are difficult to explain entirely (12). In the strains of rats used in the last decade by many investigators, the LDL cholesterol had values of 10–15 mg/dl. As described above, the LDL cholesterol concentrations used in some of our experiments were in the range of 10 mg/dl or close to the physiological range (30 mg/dl). The LDL cholesterol concentrations (80–220 mg/dl) used in other experiments were well above the normal physiological range.

Results in General: the LDL Dual Pathway

The methodology used in this study to investigate LDL interaction with vascular endothelium in situ was that generally applied to trace the intracellular pathway(s) taken by water-soluble macromolecules across epithelia and endothelia. On such studies involving pulse-chase experiments carried out at successive time intervals and electron micrographic recording of the cellular events on large sampling is largely based our current knowledge on the cellular aspects of transendothelial passage of macromolecules (47).

Results obtained in these experiments, as well as those

previously reported (69), indicate that LDL enters the vessel wall from both faces: the luminal endothelium and the endothelium of the vasa vasorum. During its passage through the endothelium, LDL behaves as a water-soluble macromolecule and is carried across the endothelial cell by two commonly involved basic processes, namely receptor-mediated endocytosis (coated pits/vesicles, endosomes, lysosomes) and transcellular passage or transcytosis (57, 58) (plasmmalemmal vesicles). Both processes occur simultaneously; the distinction between them is based mostly on: (*a*) successive LDL labeling of the structures participating in receptor-mediated endocytosis (coated pits, endosomes, lysosomes), (*b*) progressive LDL labeling of plasmalemmal vesicles from the luminal front to the abluminal front with the occurrence on the latter (at later time points) of LDL within vesicles open and apparently discharging their content, and (*c*) the appearance, at later time points, of LDL in the subendothelial space, where it increases in amount with time and concentration.

This LDL dual pathway across the vascular endothelium has to be expected in view of the fact that endothelial cell in situ is a special cell different from all other cells in which the "LDL pathway" has been investigated, mostly in culture (e.g., fibroblast, macrophage, smooth muscle cells). The endothelial cell needs plasma LDL cholesterol for its own membrane synthesis and, at the same time, has to transport LDL or its components to the other cells of the surrounding tissue, including those of the vessel wall itself. The latter process could not be detected in endothelial cells in culture (61) because of the loss of their functional polarity and the absence of concentration gradients across the cell. In vivo, the fibroblasts and smooth muscle cells can actually recognize and metabolize LDL particles, provided these particles have been transported across endothelium by transcytosis from the plasma into the interstitial fluid. The demonstration of the way in which, in vivo, the fibroblast and other cells are handling the LDL which was previously transported through endothelium may largely enhance the physiological significance of the LDL pathway identified in such cells so far in in vitro conditions only.

Endocytosis

As indicated by the kinetics of LDL labeling of the endothelial features involved in endocytosis, at physiologic concentrations of LDL, some particles are taken up by a receptor-mediated process in which, at early time points (2 min), LDL particles are bound to coated pits and then appear internalized in coated vesicles, endosomes and, starting at 5 min, LDL could be detected in multivesicular bodies and lysosomes. The process is similar to the LDL pathway originally described in fibroblasts in culture (3, 23, 24). Since heparin almost completely displaced LDL from coated pits, these features behave in the endothelial cell as well as high-affinity binding sites. It cannot be distinguished whether some LDL particles are also taken up by a receptor-independent process (fluid endocytosis) presumably carried out by a small fraction of plasmalemmal vesicles. Based on observations with other macromolecular tracers, this may be a remote possibility since, during transport of solutes by plasmalemmal vesicles, commonly these shortcut the lysosomal compartment (47, 57, 58). There is no evidence on whether all LDL-labeled coated vesicles or endosomes reach the lysosomes or whether all the LDL taken in by plasmalemmal vesicles is transported across the cell. In the conditions used in our experiments, the endocytosis of LDL does not parallel the increase in LDL concentration (up to 220 mg LDL cholesterol/dl) in the vascular lumen. This suggests that in vivo the high-affinity binding sites on the luminal aspect of the endothelium are probably fully saturated by relatively low concentrations of LDL such as 10–30 mg LDL cholesterol/dl. The low temperature (4°C) remarkably inhibits the LDL internalization by a receptor-mediated process. Rat LDL is taken up by endocytosis more avidly than human LDL: the average number of LDL particles bound to coated pits is almost double (two to five) that observed for LDL_h (one to two); and the percentage of coated pits marked by LDL is almost 80–85% in the case of LDL_r as compared to LDL_h in which this value is approximately 45–50%.

Transcytosis

At physiologic concentrations (10–30 mg LDL cholesterol/dl), LDL particles are also taken up by plasmalemmal vesicles. At early time points (up to 2 min), LDL can be visualized within such vesicles which are associated with the luminal front or which are apparently free in the cytoplasm. At later time points (5 min), more LDL-labeled vesicles can be seen on the tissue front of the endothelium and in the subendothelial space. This process was significantly augmented when high concentrations of LDL (up to 220 mg cholesterol/dl) were perfused. Since heparin only slightly reduced the LDL uptake by plasmalemmal vesicles, these features can be tentatively considered low-affinity sites. (However, the possibility that failure of heparin to displace LDL from plasmalemmal vesicles is due not to low-affinity sites but to mechanical factors—or both—cannot be ruled out.) The characterization of other parameters of these endothelial sites of presumably different affinities for LDL remains to be worked out, including experiments with methylated LDL (6). Transcytosis of LDL appears not to be significantly modified by low temperature. Human LDL and rat LDL are transported at similar rates which appear to be simply proportional to the LDL concentration in the perfusion medium. LDL transcytosis appears to be a nonsaturable uptake process that does not discriminate between homologous and heterologous LDL. Exposure of endothelium to higher concentrations of LDL may generate a higher transcytotic rate. Comparable observations have been recorded in studies on rat and human LDL catabolism by the rat liver (48).

From the experiments reported here, it appears that transcytosis across the endothelial cell is the primary rate—limiting step in LDL transport through the arterial wall.

The relatively small amount of LDL detectable as fully or partially intact particles within the subendothelium (as compared to the number of LDL particles visualized on the cell surface or within plasmalemmal vesicles) may be explained in several ways. One possibility is that LDL degradation starts even during its contact with the endothelial cell membrane or while being transported within plasmalemmal vesicles. Such a process implies the existence, in those locations, of an extrahepatic lipaselike activity comparable to that reported in rat adrenals (33). As a result of this possible extralysosomal hydrolysis, the number of intact LDL particles detectable in the subendothelial space would be much smaller than expected; at least some of the LDL may occur in this location as apoprotein B and cholesterol. It is possible that in vivo such LDL uptake by a low-affinity mechanism and receptor-independent degradation of LDL by the endothelial cell may be a salient process especially in hyperlipoproteinemic conditions.

Since the arterial endothelium is not provided with a particularly rich lysosomal apparatus, a major processing of the excess LDL in vivo within this endothelial compartment with subsequent cholesterol efflux appears unlikely.

The lack of LDL accumulation within the subendothelium, in areas in which the endothelium was accidentally damaged, supports the idea that LDL transcytosis is an active process implemented and monitored by the endothelial cell itself. This inquiry is now being extended to the immunocytochemical detection of autologous LDL in vivo (work in progress).

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