Low Density Lipoprotein Metabolism by Endothelial Cells from Human Umbilical Cord Arteries and Veins

Victor W. M. van Hinsbergh, Louis Havekes, Jef J. Emeis, Emile van Corven, and Marielle Scheffer

Binding and metabolism of low density lipoprotein (LDL) and acetylated LDL were examined in endothelial cells from human umbilical cord arteries and veins. Both high and low affinity LDL interactions were observed. High affinity LDL binding and catabolism were increased five- to sevenfold after preincubation for 18 hours in LPDS containing medium. Subconfluent cells degraded, endocytosed, and bound 1.5 to 2.7 times more LDL by high affinity interaction than confluent cells, when endothelial cell growth supplement (ECGS) was present in the culture system. In the absence of ECGS, these ratios were somewhat less. Low affinity LDL metabolism was less affected by the state of confluency. Binding of LDL and acetylated LDL by venous endothelial cells was more than two- and threefold, respectively, than that by comparable arterial cells. However, the difference in LDL binding was not reflected in an altered LDL catabolism. There apparently is a population of low affinity binding sites not involved in LDL catabolism.

LDL metabolism was identical in cells, which were cultured in medium supplemented with 20% to 100% serum or hirudin- or heparin-treated platelet-poor plasma. Without preincubation in LPDS, high affinity adsorptive endocytosis mediated the main part of LDL uptake only at low LDL concentrations (5 to 20 μg protein/ml). However, at physiological LDL concentrations (550 μg/ml), we estimated that this process mediated only 17% of the LDL uptake. We calculated that fluid endocytosis and low affinity adsorptive endocytosis of LDL accounted for the remaining 12% and 70%, respectively, of the LDL uptake at physiological LDL concentrations.

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Low density lipoprotein (LDL) is a risk factor for atherosclerosis. The plasma level of LDL depends on the balance between its formation and its catabolism. To become catabolized, LDL has to enter or pass through the endothelium that lines the blood vessels. Only in the liver, bone marrow, and spleen does blood plasma have direct access to the tissue parenchymal cells via open fenestrations. Clearing by the liver accounts for 35% to 67% of the LDL removal in pigs, rabbits, and rats. Possibly, LDL also passes through the fenestrations of other visceral capillaries, e.g., those in the gut and the adrenal glands. However, the fenestrations in these tissues, which account for 10% to 15% of the LDL metabolism, are commonly closed by a thin diaphragm. LDL metabolism by the remaining tissues (15% to 35%) must involve handling by endothelial cells. This suggests that these cells are of quantitative importance for whole body LDL metabolism.

Furthermore, endothelial cells play an important role in the penetration and accumulation of LDL cholesterol in the arterial wall. It has been suggested that the integrity of the endothelium prevents lipid accumulation in the vascular wall. On the other hand, Minick et al. observed that, after deendothelialization of the aorta of hypercholesterolemic rabbits, lipid accumulation was only found in reendothelialized areas. Smith & Staples found a nearly two-fold higher LDL concentration in intact aorta intima than in the corresponding blood plasma. Intact endothelium was found to be a prerequisite for this accumulation. The active involvement of the endothelium in lipid accumulation in the arterial wall may, however, depend on the functional state of the endothelial cells.
Important data on endothelial cell lipoprotein metabolism have been obtained with cultured endothelial cells,13-22 although final conclusions must await studies on intact blood vessels and on animals. We began a study on the metabolism of LDL by cultured endothelial cells to investigate whether modulation of endothelial function may lead to an altered vascular or whole body metabolism of lipoproteins. At present, no data are available on lipoprotein metabolism by human arterial endothelial cells. Therefore, we compared LDL metabolism by arterial and venous endothelial cells from human umbilical cord. We also determined the metabolism of acetylated LDL, which is degraded by endothelial cells,19 but does not bind to the LDL receptor.23

High affinity LDL catabolism in bovine endothelial cells, which were cultured in the presence of fibroblast growth factor (FGF),24 decreased markedly when the cells became confluent.15 However, only a slight decrease was found under the same conditions with venous endothelial cells from human umbilical cord vein.17 We studied the effect of confluency on high and low affinity LDL metabolism by human arterial endothelial cells to see whether differences in cell type or in species account for these variant data. In these studies we also used an endothelial cell growth supplement (EGCS),25,26 which is a powerful growth-supporting substance for human endothelial cells.27

It has been shown in other cells that the high affinity LDL receptor is repressed in the presence of serum. Therefore, we also investigated LDL metabolism in endothelial cells that were not exposed to lipoprotein-depleted serum before assay of LDL metabolism. By using full serum and platelet-poor plasma, we estimated the LDL metabolism at physiologic LDL concentration by human endothelial cells. The data on LDL endocytosis were compared with the rate of uptake of sucrose to discriminate between fluid and adsorptive endocytosis processes.28

**Methods**

**Materials**

Collagenase was obtained from Millipore Corporation (New Jersey), tissue culture multwells (16 mm diameter) from Costar (Cambridge, Massachusetts), medium 199 from Flow Laboratories (Irvine, Scotland). Hirudin (spec. act. > 1000 ATU/mg) was purchased from Pentapharm (Basel, Switzerland), and heparin from Leo Pharmacology Products (Emmen, The Netherlands). A crude preparation of human fibronectin was a gift from Charles Willems (Red Cross Central Blood Transfusion Laboratory, Amsterdam); purified human fibronectin was obtained from Collaborative Research (Waltham, Massachussetts). Endothelial cell growth supplement (ECGS) was a gift from Thomas Maciag (Boston, Massachussetts). Rabbit antiseraum against human factor VIII-related antigen was obtained from the Red Cross Central Blood Transfusion Laboratory (Amsterdam).

We purchased U-14C-sucrose (673 mCi/mmol), 1-14C-glycine hippuryl-L-histidyl-L-leucine (3.9 mCi/mmol), and 3H-methyl-thymidine (6.7 or 80.1 mCi/mmol) from New England Nuclear (Dreierdenhain, Federal Republic of Germany), and 125I-iodine (13-17 Ci/μg) from Amersham (Amersham, United Kingdom), and Picofluor from Packard (Groningen, The Netherlands).

**Preparation of Serum and Plasma**

Human serum was prepared from freshly collected blood obtained from healthy donors (18 to 25 years old). The sera from 15 to 25 subjects were pooled and stored at −90°C. The serum was depleted from lipoproteins by ultracentrifugation at a density of 1.21 g/ml. The lipoprotein-depleted serum was dialyzed against phosphate-buffered saline and subsequently against M-199 medium with 20 mM Hepes buffer, and sterilized through a 0.22 μm filter.

Platelet-poor plasma was prepared from fresh blood collected under sterile conditions by two centrifugations for 20 minutes at 2000 g and 6000 g, respectively. It was stored at 4°C and used within 1 week.

**Isolation and Culture of Endothelial Cells**

Human umbilical cords were kept after delivery in ice-cold cord buffer (140 mM NaCl, 4 mM KCl, 11 mM D-glucose, 10 mM Hepes, pH 7.3, 100 IU/ml penicillin, 0.10 mg/ml streptomycin). They were collected once a day from the hospital. Endothelial cells were isolated from the arteries and veins as described by Jaffe et al.29 In short, one of the arteries was cannulated and rinsed with cord buffer. The endothelial cells were detached by 20 minute-incubation at 37°C in 0.10% collagenses in M-199 medium, after which the cells were collected by perfusion with M-199 medium. Next, the vein was rinsed and incubated for 15 minutes at 37°C with collagenase. The collected arterial and venous cells were centrifuged (5 minutes 200 g) and suspended in M-199 medium containing 20% human serum and 15 mM Hepes buffer. They were seeded in a rather high density (1–5 x 10⁴ cells/cm²) in six 2-cm² wells, that had been coated with a crude fibronectin solution. Coating was performed at 37°C for 30 minutes. The fibronectin solution was removed by aspiration immediately before the cells were seeded.

The cells were cultured at 37°C in M-199 medium supplemented with 20% pooled human serum (not inactivated), 15 mM Hepes buffer, 100 IU/ml penicillin and 0.1 mg/ml streptomycin under 5% CO₂ in air. The medium (0.2 ml/cm²) was refreshed every 2 or 3 days. At confluency the cells were used, or released with trypsin/EDTA and passaged with a 1:3 split ratio to obtain the subcultures.

Pig aorta endothelial cells were isolated in a similar way from aortas freshly obtained from slaughterhouse pigs. They were seeded in 2-cm² wells that had been coated with pig plasma fibronectin and
isolated according to Vuento and Vaheeri.\textsuperscript{30} The cells were cultured in Dulbecco's ME medium with 20% inactivated fetal calf serum under 5% CO\textsubscript{2} at 37°C.

\textbf{Preparation and Iodination of Lipoproteins}

LDL was prepared from freshly obtained serum from individual healthy donors or from pigs by gradient ultracentrifugation according to the method of Redgrave et al.\textsuperscript{31} The LDL fraction was sliced from the tube and the protein content was measured.\textsuperscript{32} Subsequently, iodination of LDL was performed by the \textsuperscript{125}I-iodine monochloride method described by Bilheimer et al.\textsuperscript{33} After iodination, LDL was dialyzed against 1 liter of PBS (4 hr, 5 refreshments of 1 liter). Thereafter, it was stabilized by the addition of 10% lipoprotein-depleted serum\textsuperscript{34} and dialyzed for 18 hours against M-199 medium with 20 mM Hepes buffer. The \textsuperscript{125}I-LDL preparation was stored at 4°C. Its specific activity ranged from 80 to 150 cpm/ng protein. Although the stabilized LDL preparation is stable for at least 4 weeks, the iodinated lipoprotein preparations were usually used within 2 weeks.

Acetylated LDL was prepared from freshly isolated LDL by acetic anhydride treatment according to the method of Basu et al.\textsuperscript{35} Iodination of acetylated LDL was performed as described for LDL. Its specific activity ranged from 80–100 cpm/ng protein.

\textbf{Binding, Internalization and Degradation of LDL and Acetylated LDL}

Metabolism of lipoproteins by human endothelial cells was determined after an 18-hour preincubation of the cells in 20% lipoprotein-depleted human serum, unless otherwise mentioned. When serum was used during the preincubation period, the cells were washed twice with 1% bovine serum albumin (BSA) in M-199 medium before the experiment. The growth medium was replaced by 0.3 ml M-199 medium (Hanks salts, 15 mM Hepes, pH 7.4) containing 15% lipoprotein-depleted human serum and varying amounts of human \textsuperscript{125}I-LDL or \textsuperscript{125}I-acetylated LDL. The presence of lipoprotein-depleted serum prevents possible cytotoxic effects of the added lipoproteins.\textsuperscript{36} After a 2.5-hour incubation at 37°C, the medium was removed for determination of lipoprotein degradation (vide infra). Each monolayer was washed five times with ice cold 0.2% BSA in PBS, subsequently incubated twice for 10 minutes in the same buffer at 0°C and finally washed twice with buffer without albumin. Thereafter, the cells were released by incubation with 0.05% trypsin in buffer A (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, and 0.02% EDTA) for 10 minutes at room temperature. Further proteolysis was prevented by the addition of 0.5 ml ice-cold 0.2% BSA in PBS. The cells were immediately centrifuged for 3 minutes at 200 g at 4°C, and an aliquot of the supernatant was removed and counted in a γ-counter. Trypsin releasable radiolabel reflects binding of the labeled lipoprotein to the exterior of the cells. The cell pellet was resuspended in PBS and centrifuged for 5 minutes at 10,000 g. The radioactivity of the pellet was counted in a gamma counter and represents the intracellular presence of the lipoprotein (trypsin-resistant radiolabel). After counting, the pellet was dissolved in 0.2 M NaOH for protein determination.

Lipoprotein degradation was determined in the incubation medium as 10% trichloroacetic acid-soluble radiolabeled products. The trichloroacetic acid-soluble radioactivity was corrected for labeled-free iodide as described by Goldstein and Brown.\textsuperscript{37} Endocytosis of LDL was calculated from the sum of intracellular and degraded LDL.

Binding studies at low temperature were performed on ice, tentatively indicated as at 0°C. Incubation and washings at 0°C occurred in duplicate as described above. The cells were finally washed with PBS instead of trypsin in Buffer A, and immediately dissolved in 0.3 ml 0.2 M NaOH. The radioactivity of an aliquot of this solution was counted and represents binding. Thereafter protein was assayed.

The metabolism of pig lipoproteins by pig endothelial cells was determined similarly after preincubation in 20% lipoprotein-depleted fetal calf serum. All data were corrected for cell-free blanks.

\textbf{Other Assays}

Fluid pinocytosis was measured as the uptake of \textsuperscript{14}C-sucrose (5 \textmu Ci/ml) according to the method of Davies et al.\textsuperscript{38} Eighteen hours after renewal of the serum-containing medium, a tracer amount of \textsuperscript{14}C-sucrose was added and the cells were incubated for 3 hours at 37°C. The cells were subsequently washed five times with 2% BSA in PBS, incubated for 10 minutes at 37°C in 20% human serum in M-199 plus 15 mM Hepes buffer, washed twice with PBS and dissolved in 0.3 ml 0.3 M NaOH. After the addition of 0.06 ml 1.5 N HCl, the radioactivity was counted in 10 ml Picofluor in a liquid scintillation counter.

The incorporation of \textsuperscript{3}H-thymidine (100 \textmu Ci/ml) was measured after a 2-hour incubation at 37°C. The cells were subsequently washed twice with PBS, four times with 5% trichloroacetic acid, and twice with 96% ethanol; they were air-dried and dissolved in 0.3 ml 0.3 M NaOH for liquid scintillation counting. Cellular angiotensin-converting enzyme activity was measured in Triton X-100 extracts (final concentration 0.1%) in 50 mM Heps, 100 mM NaCl, 750 mM NaSO\textsubscript{4}, pH 7.85 with 100 \textmu M \textsuperscript{4}C-glycine-hippuryl-L-histidyl-L-leucine as substrate in a final volume of 50 \textmu l. After a 3-hour incubation at 25°C the mixture was extracted with 300 \textmu l ethylacetate, of which 200 \textmu l was counted in 10 ml Picofluor in a liquid scintillation counter.

Factor VIII-related antigen was visualized in cold acetone-fixed cells by indirect immunofluorescence microscopy. Cells were counted with a hemocytometer. Protein was determined by the method of Lowry et al.\textsuperscript{32} with bovine serum albumin used as a standard.
Results

Cell Characteristics and Fluid Pinocytosis

Cultured cells were identified as endothelial cells on the basis of their morphology at confluency (Figure 1), the presence of factor VIII-related antigen, and the presence of angiotensin-converting enzyme. The cells obtained from a confluent culture covered the whole dish in 3 to 4 days, when they were passed with a split ratio of 1:3. However, they continued to divide slowly for a few more days. About 6 days after passage, the number of cells remained constant. Then the cells had the typical cobblestone pattern (Figure 1). Only these latter cells are designated in this study as confluent. At confluency, the primary cultures of arterial and venous endothelial cells reached densities of $1.79 \pm 0.33 \times 10^5$ and $1.43 \pm 0.43 \times 10^5$ cells per cm$^2$, respectively (mean ± so of eight cultures each). Secondary confluent cultures of arterial and venous cells reached densities of $1.97 \pm 0.27 \times 10^6$ and $1.85 \pm 0.33 \times 10^6$ cells per cm$^2$ (7 and 16 cultures respectively). The incorporation of $^3$H-thymidine per cell was considerably less at confluence than during the growth period (data not shown). The rates of fluid pinocytosis of $^{14}$C-sucrose were equal in arterial and venous cells (Table 1). The pinocytosis rate per dish remained constant once the dish was covered by cells. This led to a decrease in the rate of fluid pinocytosis per cell at confluency (Figure 2).

Table 1. Fluid Endocytosis of $^{14}$C-Sucrose by Confluent Endothelial Cells from Human Umbilical Cord at 37°C

<table>
<thead>
<tr>
<th>Endothelial cells</th>
<th>Fluid endocytosis</th>
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<tbody>
<tr>
<td></td>
<td>nl/hr per 10$^6$ cells</td>
</tr>
<tr>
<td>Arterial EC</td>
<td></td>
</tr>
<tr>
<td>primary (7)</td>
<td>69 ± 33</td>
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<tr>
<td>secondary (3)</td>
<td>43 ± 13</td>
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<tr>
<td>Venous EC</td>
<td></td>
</tr>
<tr>
<td>primary (8)</td>
<td>75 ± 37</td>
</tr>
<tr>
<td>secondary (8)</td>
<td>56 ± 15</td>
</tr>
</tbody>
</table>

Fluid endocytosis of $^{14}$C-sucrose ($5 \mu$Ci/ml) by endothelial cells (EC) was measured in the presence of 20% human serum in M-199 medium at 37°C as described in Methods. The data represent means ± so of the number of experiments with separate cell cultures indicated within parentheses. ND = not determined.
Figure 2. Effect of growth phase on fluid endocytosis of 14C-sucrose by human endothelial cells. Fluid endocytosis of 14C-sucrose (5 μCi/ml) by arterial (●) and venous (○) endothelial cells from the same umbilical cords was assayed at different time intervals after passage. The first assay was 2 hours after passage. A. Sucrose endocytosis on cell base. B. Sucrose endocytosis in relation to the surface area of the well. C. The number of cells in parallel wells. Data represent means of triplicate incubations from a representative endothelial cell culture.

Figure 3. The effect of preincubation conditions on LDL binding (A, C) and on endocytosis (B, D) by confluent arterial endothelial cells from human umbilical cord. The culture medium was changed into medium supplemented with 20% LPDS (A, B) or renewed with medium containing 20% serum (C, D). The amount of unlabeled LDL in the serum (505 μg/ml) was assayed by radial immunodiffusion of apo B. At different time intervals, 125I-LDL was added, so that final LDL concentrations of 10 (△), 50 (○) or 150 (●) μg LDL protein/ml were reached. LDL metabolism was subsequently assayed over a 2.5-hour period at 37°C.
Effect of Preincubation Conditions on Overall LDL Metabolism by Confluent Endothelial Cells

LDL metabolism increased rapidly during the first 14 to 18 hours after changing from medium containing 20% serum to medium supplemented with 20% LPDS (Figure 3 A, B). Like other cell types, the increase was predominantly due to high affinity LDL interaction. At 10 µg/ml [125I]-LDL binding and endocytosis of LDL by human arterial endothelial cells increased seven and 16-fold, respectively, after a 24-hour preincubation in medium with 20% LPDS. Longer periods of preincubation in LPDS (up to 42 hours) did not result in a significant further increase of LDL catabolism. No change in LDL metabolism was observed after a change to fresh medium containing 20% human serum (Figure 3 C, D). Binding and endocytosis of LDL were two- and fourfold higher, respectively, when they were measured at the same final LDL concentration (150 µg/ml) but after a 24-hour incubation period in medium with 20% LPDS.

Considerable metabolism of LDL was not only found with confluent endothelial cell cultures from neonatal human umbilical cord blood vessels, but also with those from adult pig aorta after preincubation in LPDS containing medium. Binding, intracellular presence, and degradation of 50 µg/ml [125I]-LDL by the latter cells amounted to 550 ± 180, 260 ± 135 and 750 ± 310 µg [125I]-LDL/2.5h/mg protein (mean ± SD of six confluent primary cultures).

High and Low Affinity LDL Metabolism by Human Endothelial Cells

LDL metabolism was measured at various LDL concentrations in human endothelial cells, which were preincubated in medium supplemented with 20% human LPDS (Figure 4). LDL binding, degradation, and endocytosis increased rapidly with increasing LDL concentrations up to 20 µg/ml LDL protein. Thereafter, these parameters still increased although less rapidly. The contribution of high affinity interaction at low LDL concentrations can be tentatively estimated by subtracting the amount of [125I]-LDL, that interacts in the presence of an excess of unlabeled LDL (250–300 µg/ml LDL protein), from the [125I]-LDL interaction without unlabeled LDL. By such calculation, it can be estimated from Figures 4B and 4D that high affinity LDL interaction contributes

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Figure 4. The metabolism of [125I]-labeled LDL by confluent endothelial cells from human umbilical cord arteries was determined at 37°C after preincubation of the cells for 18 hours in lipoprotein-depleted human serum. Binding (A), degradation (C, D) and endocytosis (C, D) were determined as described in Methods. Endocytosis represents the sum of intracellular present and degraded LDL. The insets (A) and (B) represent a representative experiment, in which high affinity LDL binding (A, ●) and high affinity-mediated endocytosis (D, ●) were calculated by subtracting the amount of [125I]-LDL that interacts in the presence of 300 µg/ml unlabeled LDL (x) from that which interacts without excess unlabeled LDL (●). The data (●, □) represent two independent experiments with secondary cultures from different umbilical cords and LDL preparations from different healthy human donors.
considerably to LDL metabolism at LDL concentrations up to 50 µg/ml LDL protein. The high affinity binding sites are saturated for at least 90% at 20 µg/ml LDL protein.

High and low affinity LDL metabolism was also observed in confluent human arterial endothelial cells, which were kept in medium supplemented with 20% human serum instead of lipoprotein-depleted serum (Figure 5). Particularly at low LDL concentrations LDL metabolism proceeded at a much lower rate.

Metabolism of Acetylated LDL by Confluent Endothelial Cells

Endothelial cells from human umbilical cord arteries and veins metabolized acetylated LDL after preincubation for 18 hours in medium with 20% LPDS (Figure 6). Binding and catabolism of acetylated LDL did not compete with that of LDL at concentrations of 10 to 30 µg/ml of both lipoproteins (not shown). Binding, endocytosis, and degradation rates of acetylated LDL increased with increasing substrate concentrations (Figure 6). No saturation of these parameters was observed with concentrations of acetylated LDL up to 100 µg/ml (Figure 6). No significant changes were observed in the metabolism of acetylated LDL (50 µg/ml) between primary and secondary cultures of human arterial and venous endothelial cells (not shown).

Arterial and Venous Endothelial Cells and Metabolism of LDL and Acetylated LDL

Arterial and venous endothelial cells were compared concerning metabolism of LDL and acetylated LDL. To avoid individual variations of the umbilical cords and of the LDL preparations, we cultured arterial and venous endothelial cells from the same umbilical cords, and measured lipoprotein metabolism by confluent monolayers of these cells simultaneously. A marked difference was observed between arterial and venous cells in the binding of LDL and acetylated LDL (Table 2). Since receptor binding of LDL at 37°C might be affected by some receptor downregulation even during the 2.5-hour incubation period, LDL binding to both types of endothelial cells was studied in more detail at 0°C (Figure 7 A). Binding of 125I-LDL to venous cells was higher than to arterial cells at all LDL concentrations measured. Analysis of these binding data according to the method of Scatchard40 (Figure 7 B) reveals a curvilinear plot, which indicates the presence of low and high affinity binding sites. A good curve fitting was obtained with the program of Wahren.41 assuming two types of saturable binding sites. The maximal amounts of high affinity binding were 79 × 10^{-15} and 69 × 10^{-15} µmol/mg cell protein for arterial and venous cells, respectively. The high affinity K_d were 3.7 × 10^3 and 5.8 × 10^3 ml/µmol, respectively. This suggests that the difference in the binding between arterial and venous cells is due to a more pronounced low affinity LDL binding to venous cells.
The increased LDL binding to venous endothelial cells as compared to arterial cells does not lead to proportionally higher rates of internalization and degradation (Table 2). Apparently, there is a population of low affinity binding sites, which are not involved in adsorptive endocytosis of LDL. The threefold higher binding of acetylated LDL to venous endothelial cells as compared to arterial cells likewise is not reflected in a similar increase of the rates of internalization and degradation of this modified lipoprotein.

**Effect of ECGS and Confluency on LDL Metabolism by Human Endothelial Cells**

The effect of the state of confluency on LDL metabolism was evaluated by the use of an endothelial cell growth supplement (ECGS) and from the comparison of confluent and subconfluent cells.

Cells obtained from primary cultures, cultured in the presence of ECGS, were divided into two groups, which were subsequently grown to confluency in 20% human serum containing medium in the presence and absence of 100 μg/ml ECGS. The cells that were cultured in the presence of ECGS metabolized somewhat less LDL than those cultured without ECGS (Table 3). At an LDL concentration of 10 μg protein/ml LDL, binding and the thereby mediated LDL uptake and degradation were decreased to 76%, 89%, and 76%, respectively, when ECGS was also added in the preincubation and assay media. LDL metabolism at 110 μg/ml LDL protein was similarly influenced by the presence of ECGS (Table 3).

Subconfluent and confluent secondary cultures of human umbilical cord artery endothelial cells were obtained by seeding endothelial cells from the same cultures at different densities (0.8-5 × 10⁴ cells/cm²) on fibronectin-coated wells. Cells were grown for 6 days in the presence of 100 μg/ml ECGS. They were preincubated for 18 hours in medium supplemented with guest on April 19, 2017 http://atvb.ahajournals.org/ Downloaded from
Table 3. Effect of Endothelial Cell Growth Supplement (ECGS) on LDL Metabolism by Human Endothelial Cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Culture medium supplemented with:</th>
<th>10 μg/ml 125I-LDL</th>
<th>110 μg/ml 125I-LDL</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>20% serum</td>
<td>20% serum + ECGS</td>
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<tr>
<td>Binding</td>
<td></td>
<td>193 ± 16</td>
<td>439 ± 23</td>
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<td></td>
<td></td>
<td>146 ± 9</td>
<td>310 ± 2</td>
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<tr>
<td></td>
<td></td>
<td>1036 ± 85</td>
<td>2356 ± 364</td>
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<tr>
<td></td>
<td></td>
<td>927 ± 87</td>
<td>2100 ± 225</td>
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<tr>
<td></td>
<td></td>
<td>611 ± 15</td>
<td>978 ± 24</td>
</tr>
<tr>
<td>Intracellular presence</td>
<td></td>
<td>611 ± 137</td>
<td>1135 ± 35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>462 ± 20</td>
<td>978 ± 24</td>
</tr>
<tr>
<td>Degradation</td>
<td></td>
<td>1.39 ± 0.16</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.99 ± 0.71</td>
<td>1.45 ± 0.13</td>
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<tr>
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<td></td>
<td>1.47 ± 0.02</td>
<td>1.20 ± 0.02</td>
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<tr>
<td></td>
<td></td>
<td>2.56 ± 0.71</td>
<td>1.81 ± 0.02</td>
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<td></td>
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<td>0.90 ± 0.16</td>
<td>0.96 ± 0.24</td>
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<td></td>
<td></td>
<td>1.34 ± 0.16</td>
<td>1.10 ± 0.13</td>
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Metabolism of 10 and 110 μg/ml 125I-LDL was determined in secondary cultures of arterial endothelial cells from human umbilical cord after 18 hours of preincubation in medium with 20% LPDS. Data are expressed in ng 125I-LDL 2½ h⁻¹ mg protein⁻¹ and represent the mean ± SD of three different arterial endothelial cell cultures.

Table 4. Ratio between LDL Metabolism of Subconfluent and Confluent Human Endothelial Cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Medium supplemented with:</th>
<th>15 μg/ml 125I-LDL</th>
<th>140 μg/ml 125I-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20% serum</td>
<td>1.39 ± 0.16</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>20% serum + ECGS</td>
<td>1.99 ± 0.71</td>
<td>1.45 ± 0.13</td>
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<tr>
<td></td>
<td>1.47 ± 0.02</td>
<td>1.20 ± 0.02</td>
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<tr>
<td></td>
<td>2.56 ± 0.71</td>
<td>1.81 ± 0.02</td>
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<td></td>
<td>0.90 ± 0.16</td>
<td>0.96 ± 0.24</td>
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<td></td>
<td>1.34 ± 0.16</td>
<td>1.10 ± 0.13</td>
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</table>

Subconfluent and confluent secondary cultures of human umbilical cord artery endothelial cells were cultured and assayed in the presence and absence of 100 μg/ml ECGS. Metabolism of 15 and 140 μg/ml 125I-LDL protein was measured for 2½ hours at 37°C. The ratios of high affinity interaction between subconfluent and confluent cells at 15 μg/ml 125I-LDL are given within parentheses. Data represent the mean of three separate endothelial cell cultures.

with 20% LPDS before assay of LDL metabolism. The presence of ECGS was maintained in the preincubation and assay media. During assay of LDL metabolism, the cell densities were 0.52–0.74 × 10⁵ cells/cm² for subconfluent cultures and 1.68–2.01 × 10⁵ cells/cm² for confluent cultures. High affinity LDL interaction at 15 μg/ml 125I-LDL was tentatively estimated by subtracting 11% of the amount of 125I-LDL metabolized at 140 μg/ml from that at 15 μg/ml 125I-LDL. It was higher in subconfluent than in confluent cells (Table 4), particularly when ECGS was present in the culture medium.

LDL metabolism at 140 μg/ml LDL protein was much less influenced by the state of confluency. In the presence of ECGS, binding and uptake of LDL were higher in subconfluent cells than in confluent cells; in the absence of ECGS, no difference was observed (Table 4).

LDL Metabolism by Confluent Endothelial Cells Cultured in Platelet-Poor Plasma

LDL metabolism in cells cultured in various percentages of human serum was compared with that in cells cultured in hirudin- or heparin-treated platelet-poor plasma. No differences in LDL metabolism were observed when the cells were cultured in 20% serum or 20% hirudin- or heparin-treated platelet-poor plasma (Table 5). This was also the case when higher concentrations of serum or platelet-poor plasma were used (results not shown).

In a second series of experiments, the cells were divided into three or six groups after passage, and cultured in 20%, 50%, or 100% serum and equal percentages of hirudin-treated platelet-poor plasma. LDL metabolism was determined by adding 50 or 100 μg/ml 125I-LDL directly to these culture media. The final LDL concentration was determined by a radial immunodiffusion assay for apo B.39 LDL binding and catabolism increased with increasing concentrations of serum or hirudin-treated platelet-poor plasma (Figure 8). The increase in LDL metabolism which accompanies the increased LDL concentration, seems to be independent from the presence of other serum of plasma components (see Figure 9). Again, no differences between serum and hirudin-treated platelet-poor plasma could be observed.
Table 5. Metabolism of $^{125}$I-LDL (50 $\mu$g/ml) by Human Venous Endothelial Cells Cultured In Medium Supplemented with Serum or Platelet-Poor Plasma

<table>
<thead>
<tr>
<th>Medium supplemented with</th>
<th>Binding</th>
<th>Intracellular presence</th>
<th>Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% serum</td>
<td>278±64</td>
<td>349±182</td>
<td>301±105</td>
</tr>
<tr>
<td>20% hirudin platelet poor plasma</td>
<td>304±56</td>
<td>426±257</td>
<td>282±41</td>
</tr>
<tr>
<td>20% heparin platelet poor plasma</td>
<td>312±71</td>
<td>656±518</td>
<td>280±62</td>
</tr>
</tbody>
</table>

Human endothelial cells of confluent primary cultures from umbilical cord vein were divided after passage in three comparable groups. They grow to confluency in 6 days in M-199 medium supplemented with 20% human serum, 20% hirudin-treated human platelet-poor plasma, and 20% heparin-treated human platelet-poor plasma. After two washings, metabolism of 50 $\mu$g/ml $^{125}$I-LDL was assayed for 2.5 hours at 37°C in the presence of 15% LPDS. Data represent the mean ± SD of three experiments.

Discussion

The present study examined the contribution of low and high affinity LDL interactions and fluid endocytosis to LDL metabolism of cultured human endothelial cells. It compared confluent arterial and venous endothelial cells and reexamined the effect of confluence on LDL metabolism and fluid endocytosis in these cells. In addition, LDL metabolism was studied at physiological LDL concentrations and without preincubation in lipoprotein-depleted serum in cells, cultured in serum or in platelet-poor plasma.

The data on LDL metabolism are in good agreement with those previously reported for human umbilical vein endothelial cells. Previous studies on human endothelial cells also showed a large variability in the metabolism of LDL. Since intraassay variability was always less than 10% in our experiments, this variability must be contributed to biological variations in the different cell cultures, in the LDL preparations, or in both. Variability was not caused by LDL cytotoxicity, since adequate amounts of LPDS or serum were present in the assay.

LDL metabolism consists of three types of interaction: 1) high affinity or "specific" interaction; 2) low affinity adsorptive interaction; and 3) fluid endocytosis. High affinity binding to confluent human endothelial cells increased sevenfold after preincubation of the cells in medium supplemented with lipoprotein-depleted human serum and was saturated for at
at least 90% at 20 μg/ml LDL protein. Saturation of high affinity LDL binding at 10 to 20 μg/ml LDL was also calculated from data on LDL binding to human venous endothelial cells at 4°C.42

Vlodavsky et al.15 observed that receptor-dependent LDL catabolism by cultured bovine aorta endothelial cells dropped dramatically when the cells changed from the growing subconfluent to the quiescent confluent state. These cells had been cultured in the presence of a bovine pituitary growth factor.24 Coetzee et al.17 found that the effect of confluency on LDL degradation by bovine aorta endothelial cells was much less at high LDL concentrations and depended mainly on the presence of the growth factor. Furthermore, the difference between dividing and confluent cells was less pronounced with human umbilical vein endothelial cells. Our data confirm and extend these latter observations. Since Coetzee et al.17 found only a limited effect of FGF on LDL metabolism by human endothelial cells, we used a recently described bovine hypothalamus growth supplement ECGS25,26 by which human umbilical vein endothelial cells can be cultured for many passages.27 Human arterial cells also respond to ECGS (van Hinsbergh, unpublished). The growth-promoting and cell-conserving factor in ECGS is very similar to acidic FGF, a potent endothelial cell growth-stimulating factor in brain FGF.43 In the presence of ECGS, high affinity binding of LDL was 2.7 times higher in dividing than in confluent human arterial endothelial cells, which agrees with previously published data on bovine aorta19 and human umbilical vein17 endothelial cells. In contrast to the 10-fold decrease described for bovine endothelial cells,15 high affinity catabolism of LDL was only 1.5–2.4 times less in confluent human cells in our studies. At higher LDL concentrations, LDL metabolism was somewhat less affected by the state of confluency. In the absence of ECGS these effects were even smaller. Thus, a decrease in high affinity LDL metabolism at reaching the state of confluency is found in both bovine and human endothelial cells, but there are important quantitative differences.

Under physiological conditions, pathways not involving the ‘specific’ LDL receptor are also important. The in vivo data indicate that both B,E receptor44 dependent and receptor-independent mechanisms are active in man45 and in animals.46 Studies with rabbit endothelial cells indicate that both low and high affinity sites are present on endothelial cells after exposure to lipoprotein-depleted serum.14 Our data extend this observation to human endothelial cells. Internalization and degradation of LDL by human endothelial cells also occurs without preincubation in LPDS in media supplemented with various amounts of serum or plasma. From the data of Table 1 and Figures 5 and 8, we estimated the relative contribution of the three processes underlying LDL endocytosis at different LDL concentrations (Figure 9). We assumed that LDL uptake via high affinity binding sites is constant at concentrations higher than 50 μg/ml LDL protein. At low LDL concentrations, high affinity-mediated endocytosis has a relatively important contribution. At physiological concentration, however, it represents only 17% of the total LDL endocytosis, while fluid endocytosis and low affinity adsorptive endocytosis represent about 12% and 70%, respectively, of the LDL endocytosis (Figure 9). This indicates that under physiological conditions the low affinity interaction of LDL with endothelial cells is a quantitatively important mechanism. The observation that dividing bovine endothelial cells have a twofold higher rate of fluid endocytosis than confluent cells may be important for LDL metabolism.28 A similar change in fluid endocytosis was...
observed with human arterial and venous endothelial cells, but no decrease in fluid endocytosis per surface area was observed, once the dishes were covered with cells.

The passage of lipoproteins through the vascular endothelium proceeds via endocytotic vesicles. Thus, endocytosis of LDL particles leads either to degradation or to diacytosis. Our assay probably does not measure the amount of LDL that is diacytosed, since the diacytosed LDL particles are released again from behind the cells during trypsinization or during the incubation period by backward diacytosis. Data in vivo and with aorta preparations indicate that 125I-labeled LDL particles pass through the endothelial layer. These particles are precipitable with trichloroacetic acid. It is uncertain whether they have undergone some modification. Besides LDL particles, degradation products of LDL are also found in the vascular wall. Bratzzler et al. observed that 10 minutes after intravenous injection of 125I-LDL in normal conscious rabbits, 30% to 35% of the radiolabel on the intimal side of the media represented degradation products. This percentage may have been underestimated since the flux of degradation products is probably higher than that of the LDL particles, which are much larger, stick to glycans, and are hindered by the lamina elastica interna.

The amount of LDL that is catabolized by human endothelial cells is rather high. It has yet to be established whether endothelial cells from adult vessels and from capillaries show a similar behavior toward lipoproteins. If so, the endothelium of the body, which accounts for several hundred grams, would be an important tissue for the metabolism of LDL in man. However, further studies on animals and intact vessel preparations (cf reference 5) are required before final conclusions can be drawn.

There are regional differences in morphological and metabolic properties of the endothelium in the body. At present, only a few studies have compared metabolic properties of different types of cultured endothelial cells, mainly arterial and venous endothelial cells from human umbilical cord. These studies describe quantitative differences in the production of prostaglandins and prostacyclin, in the amount of angiotensin I-converting enzyme and angiotensinase, and in the number of receptors for insulin and multiplication-stimulating activity. The present study shows clear differences in the amount of LDL binding between both cell types did not result in differences in LDL catabolism. Probably there is another binding factor of yet unknown importance in the extracellular matrix or membrane components of the two cell types. This implies that in studies on LDL metabolism by vascular tissues in vivo or by perfusion technique one should discriminate between LDL that adheres to vascular wall endothelium and LDL that penetrates into or through endothelial cells.

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