LDL, Scavenger, and β-VLDL Receptors on Aortic Endothelial Cells

Derek P. Baker, Brian J. Van Lenten, Alan M. Fogelman, Peter A. Edwards, Charles Kean, and Judith A. Berliner

Primary and first passage aortic endothelial cells were shown to possess a high affinity receptor for β-migrating very low density lipoproteins (β-VLDL) distinct from the low density lipoprotein (LDL) receptor and scavenger receptor on these cells. In bovine aortic endothelial cells, 125I-rabbit β-VLDL was taken up and degraded by a high affinity process that was competed for by unlabeled rabbit β-VLDL and unlabeled postprandial VLDL from a fat-fed normal subject. However, unlabeled human or rabbit LDL, human LDL modified by malondialdehyde (MDA-LDL), or VLDL from a fasted normal human or a rabbit were not effective competitors for the degradation of 125I-rabbit β-VLDL. In contrast to the receptor-mediated degradation of 125I-human or rabbit LDL and 125I-human-MDA-LDL, cell density did not affect the receptor-mediated degradation of 125I-rabbit β-VLDL. Endothelial cells from a Watanabe heritable hyperlipidemic (WHHL) rabbit virtually did not degrade rabbit LDL, but degraded rabbit β-VLDL at a rate equal to that seen in normal rabbit endothelial cells. It was concluded that the β-VLDL receptor on endothelial cells is genetically distinct from the LDL receptor. Incubation of cells for 3 days with 100 μg/ml protein of unlabeled β-VLDL caused an 88% increase in cellular cholesterol content, even though the β-VLDL receptor activity was down-regulated by 60%. Endothelial cells and monocyte-macrophages are thus far the only cells known to possess the LDL receptor, the scavenger receptor, and the β-VLDL receptor. (Arteriosclerosis 4:248-255, May/June 1984)

Lipoprotein uptake by vascular endothelial cells has been implicated in the initiation and development of atherosclerosis.1-3 In animals fed a high cholesterol diet, there is the appearance of cholesterol-rich lipoproteins that float at a density of less than 1.006 g/ml and have β-electrophoretic mobility, referred to as β-VLDL.3 A specific receptor on macrophages that recognizes β-VLDL has been recently identified.4-6 The uptake of β-VLDL by its receptor has been shown to cause cholesteryl ester accumulation in macrophages to the degree seen in foam cells.6,7 Macrophages also accumulate massive amounts of cholesteryl ester when they are exposed to acetyl-LDL8 or malondialdehyde-altered LDL (MDA-LDL).9 The uptake of these ligands is mediated by a receptor that recognizes negatively charged proteins, the scavenger receptor.10 Endothelial cells have been shown to possess both the LDL and scavenger receptors.11,12 Thus, we thought it important to determine which, if any, of these receptors were present on the primary and first passage cultures of adult aortic endothelium, and to examine the control of receptor function.

Methods

Materials

The following materials were used in these studies: sodium 125I-iodide (16.5mCi/μg) (Amersham, Arlington Heights, Illinois); phosphate-buffered saline (Gibco 310-4200, Gibco Laboratories, Grand Island, New York); Waymouth’s medium (Gibco 320-1220); Dulbecco’s modified Eagle’s medium (DME) (Gibco 430-1600); Costar multiwell tissue culture plates (Costar 3524, Costar Incorporated, Cambridge, Massachusetts, and Costar 3548); normal serum albumin (human) 25% USP from Cutter Biological (Berkeley, California); fetal bovine serum (Hyclone); malondialdehyde bis (dimethyl acetal) (Cat. No. 10.838-3, Aldrich Chemical Company, Milwaukee, Wisconsin); cholesterol enzymatic kit (T11-1182, T11-1183, Technicon Instruments Corporation, Tarrytown, New York); pancreatin (Gibco 6105725); and collagenase (Worthington Biochemicals, Freehold, New Jersey).

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Cell Cultures

Bovine Cells

Bovine endothelial cells were obtained from segments of adult bovine thoracic aortas and were isolated using a 0.5% collagenase solution, as previously described.13-15 Cells were maintained at 37°C in a 5% CO₂ atmosphere in Waymouth's medium containing 20 U mycostatin, 100 U penicillin, and 100 μg streptomycin per ml (Medium A) supplemented with 8% fetal bovine serum (FBS).

Only primary and first passage cultures of endothelial cells at a density of 1-5 × 10⁵ cells/cm² were used in these studies. These cells displayed the morphological and growth characteristics of endothelial cells, forming a monolayer of flattened polygonal cells. At a density of 4-5 × 10⁵ cells/cm², more than 95% of the cells exhibited strict contact-inhibition as verified by thymidine incorporation studies with autoradiography16 and by positive immunofluorescence for the presence of Factor VIII antigen.17 Cultures at low cell density (1-2 × 10⁵ cells/cm²) appeared confluent, but were not contact-inhibited as assessed by thymidine incorporation.

Rabbit Aortic Endothelial Cells

Rabbit aortic endothelial cells were isolated from normal New Zealand white rabbits and Watanabe heritable hyperlipidemic (WHHL) rabbits weighing approximately 2 kg each by methods similar to those described previously,18 except that pancreatin was substituted for viokase. Aortic segments were split longitudinally and placed on top of a small amount of collagenase and pancreatin in DME and incubated for 10 minutes. Cells were dislodged by pipetting a stream of enzyme solution against the walls. Cells were plated into 48-well dishes in DME containing antibiotics as for bovine cells and 15% FBS. After 1 day, the cells were washed, and DME containing 5% FBS + 10% clottable rabbit plasma-derived serum was added to the cells. After an additional day, the clotted medium was removed along with many adherent cells. The remaining cells in the wells were then placed in DME containing conditioned medium from bovine aortic endothelial cells plus 15% FBS. Cells were kept in this medium until they were used (1-2 weeks), at which time the contact-inhibited state was obtained. The cell cultures at this time had the typical appearance of an endothelial monolayer with cobblestone morphology (Figure 1). Only primary cultures were used.

Lipoproteins

Human VLDL (d < 1.006 g/ml) from normal, fasting subjects or from normal subjects fed a liquid fat meal, and rabbit or human LDL (d = 1.019 to 1.063 g/ml) were isolated by ultracentrifugation as previously described. The cholesterol/protein ratio of the rabbit β-VLDL was approximately 10:1. The d < 1.006 g/ml fraction from cholesterol-fed rabbits showed a single β-migrating band on agarose electrophoresis. Four separate β-VLDL preparations were used. All lipoproteins were filtered through a 0.45 μm filter just before addition to the cells.

Assays

Before the addition of radioactive lipoproteins, the cells were washed three times with 1 ml of DME containing 10 mM Hepes (Medium C). Radioactive lipoproteins were added in the same medium supplemented with 24 mM NaHCO₃ and 2 mg/ml glucose (Medium D). The degradation of ¹²⁵I-LDL, ¹²⁵I-MDA-LDL, and ¹²⁵I-rabbit β-VLDL were measured by assaying the amount of ¹²⁵I-labeled trichloroacetic acid-soluble (noniodide) material formed by the cells and excreted into the culture medium as described by Goldstein and Brown.20 The protein content of extracts and lipoproteins was determined by the method of Lowry et al.21 with bovine serum albumin as a standard. The total cholesterol content of the cells was determined after the cells had been incubated with 100 μg/ml protein of each lipoprotein for 72 hours. On the day of the harvest, the cells were washed three times in the dishes with 5 ml of phosphate-buffered saline (PBS) containing 1% bovine serum albumin. The cells were scraped with a rubber policeman in PBS and layered on 3% human serum albumin in 30 ml siliconized tubes. The tubes were
centrifuged at 10,000 g for 30 minutes and the supernatants were removed. The pellets were washed three times with 20 ml PBS and suspended in an appropriate volume of isopropyl alcohol (0.5–1.0 ml/mg cell protein). The cells were then sonicated with a microprobe (140 x 30 mm) for 10 seconds with a "lo" setting of 5 on a Ystrom sonicator (Technic International, Bergenfield, New Jersey). After centrifugation for 15 minutes at 800 g, the clear supernatant was removed and the total cholesterol was measured enzymatically as described by Allain et al. The residue was dissolved in 0.1 N NaOH and an aliquot was removed for protein determination. Statistical analyses were carried out using the Student t distribution; a p value of < 0.05 was considered significant.

Reproducibility of Data

The number of replicate assays for each data point is indicated in the legends. The average variation for replicate well analyses in the different assays used in the endothelial cell incubation studies was less than 10%. All experiments were repeated on at least two or more occasions with similar results.

Results

Degradation of 125I-Labeled Lipoproteins

Figure 2 shows the degradation of 125I-human LDL, 125I-human MDA-LDL and 125I-rabbit β-VLDL by bovine endothelial cells. 125I-MDA-LDL, a lipoprotein recognized by the scavenger receptor in human monocyte-macrophages, was taken up by a saturable process with maximal velocity of approximately 10 µg/ml protein. The rate of degradation of 125I-rabbit β-VLDL was clearly greater than that of LDL. The uptake of 125I-LDL was saturated at approximately 20 µg/ml protein, whereas the apparent saturation of 125I-rabbit β-VLDL was approximately 60 µg/ml protein.

As shown in Figure 3, LDL was not recognized by the scavenger receptor. The degradation of 125I-MDA-LDL was progressively inhibited by increasing concentrations of unlabeled MDA-LDL. However, 125I-MDA-LDL degradation was not inhibited by the addition of unlabeled LDL at concentrations as high as 400 µg/ml protein (Figure 3 A). When unlabeled MDA-LDL and LDL were compared for their abilities to compete for 125I-LDL degradation (Figure 3 B), MDA-LDL at concentrations up to 400 µg/ml protein did not inhibit the degradation of 125I-LDL.

To determine if human LDL and rabbit β-VLDL compete for receptor-mediated degradation via the same receptor on bovine endothelial cells, we performed the experiments shown in Figure 4. The addition of increasing concentrations of unlabeled rabbit β-VLDL progressively inhibited the degradation of 125I-rabbit β-VLDL, whereas the addition of increasing concentrations of unlabeled LDL was less effective (Figure 4 A).

The degradation of 125I-human LDL, on the other hand, was markedly inhibited by increasing concentrations of both unlabeled LDL and rabbit β-VLDL (Figure 4 B). The results of the experiments shown in Figure 4 suggest that the LDL receptor on bovine endothelial cells has a greater affinity for rabbit β-VLDL than for LDL itself. Thus, at low concentrations a significant portion of the total 125I-rabbit β-VLDL degradation may be mediated via the LDL receptor. This is similar to the results reported for the β-VLDL receptor in human monocyte-macrophages. Therefore, unlabeled human LDL was included in incubations when measuring the degradation of 125I-labeled rabbit β-VLDL to determine the β-VLDL receptor activity independent of the LDL receptor in the experiments to follow.

It has been shown that the β-VLDL receptor on macrophages recognizes triglyceride-rich lipoproteins from hypertriglyceridemic subjects and from postprandial plasma of normal subjects. To determine if similar lipoproteins can compete for the receptor-mediated degradation of β-VLDL in endothelial cells, we performed the experiments shown in Figure 5. A 20-fold excess of unlabeled VLDL from a normal fat-fed subject was almost as effective as unlabeled rabbit β-VLDL in inhibiting the degradation.
of \( ^{125}\)I-rabbit-\( \beta \)-VLDL. In contrast, a 20-fold excess of either unlabeled human-VLDL from a fasted normal subject, human MDA-LDL, or human LDL was much less effective in competing with \( ^{125}\)I-rabbit \( \beta \)-VLDL degradation. In other experiments, unlabeled VLDL and LDL from fasted rabbits were as ineffective in competing for \( ^{125}\)I-rabbit \( \beta \)-VLDL degradation as were the respective human lipoproteins (data not shown).

**Effect of Cell Density on Receptor Activities**

The expression of LDL and scavenger receptor activities on endothelial cell lines has been shown to be dependent on cell density.\(^{24,25}\) The experiments,

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**Figure 3.** The ability of unlabeled human LDL and human MDA-LDL to inhibit the degradation of \( ^{125}\)I-human MDA-LDL (A) and \( ^{125}\)I-human LDL (B) by bovine endothelial cells. Each monolayer of normal endothelial cells was washed three times with Medium C (1 ml/wash) and incubated with 0.5 ml of Medium D containing either 5 \( \mu \)g/ml protein of \( ^{125}\)I-MDA-LDL (163 cpm/ng) (A) or 15 \( \mu \)g/ml protein of \( ^{125}\)I-LDL (289 cpm/ng) (B) and the indicated concentrations of unlabeled LDL (•) or unlabeled MDA-LDL (○). After incubation for 4 hours at 37°C, the medium was removed and the \( ^{125}\)I-labeled acid-soluble content was determined. The 100% of control values with no addition were 2248 and 130 ng degraded \( 4 \text{ hr}^{-1} \text{ mg protein}^{-1} \) for A and B, respectively. Each value is the average from duplicate wells.

**Figure 4.** The ability of unlabeled human LDL and rabbit \( \beta \)-VLDL to inhibit the degradation of \( ^{125}\)I-rabbit \( \beta \)-VLDL (A) and \( ^{125}\)I-human LDL (B) by bovine endothelial cells. Each monolayer of normal endothelial cells was washed three times with Medium C (1 ml/wash) and incubated with 0.5 ml of Medium D containing either 15 \( \mu \)g/ml protein of \( ^{125}\)I-\( \beta \)-VLDL (96 cpm/ng) (A) or 20 \( \mu \)g/ml protein of \( ^{125}\)I-LDL (103 cpm/ng) (B) and the indicated concentrations of unlabeled LDL (•) or unlabeled \( \beta \)-VLDL (○). After incubation for 4 hours at 37°C, the medium was removed and the \( ^{125}\)I-labeled acid soluble content was determined. The 100% of control values with no addition were 455 and 148 ng degraded \( 4 \text{ hr}^{-1} \text{ mg protein}^{-1} \) for A and B, respectively. Values are the means ± SEM from triplicate wells (A) or the average from duplicate wells (B).
fold excess of one of the following unlabeled lipoproteins: 

\[ \text{VLDL by bovine endothelial cells. Each monolayer of normal endothelial cells at low cell density (1-2} \times 10^5 \text{ cells/cm}^2 \text{) was washed three times with Medium C containing either rabbit P-VLDL, fasted human VLDL, fed human LDL, human MDA-LDL, or human MDA-LDL. After incubation for 4 hours at 37°C, the medium was removed and the 125I-labeled acid-soluble content was determined. The 100% of control value with no addition was 157 ng degraded 4 hr\(^{-1}\) mg protein\(^{-1}\). Each point is the mean \pm SEM from triplicate wells.}

**Figure 5.** The effect of unlabeled lipoproteins on 125I-rabbit \(\beta\)-VLDL degradation by bovine endothelial cells. Each monolayer was washed three times with Medium C (1 ml/wash) and incubated in 0.5 ml of Medium D containing 3 \(\mu\)g/ml protein of 125I-\(\beta\)-VLDL (132 cpm/ng) and a 20-fold excess of one of the following unlabeled lipoproteins (left to right): rabbit \(\beta\)-VLDL, fasted human VLDL, fed human LDL, human MDA-LDL, or human MDA-LDL. After incubation for 4 hours at 37°C, the medium was removed and the 125I-labeled acid soluble content was determined. The 100% of control value with no addition was 157 ng degraded 4 hr\(^{-1}\) mg protein\(^{-1}\). Each point is the mean \pm SEM from triplicate wells.

Degradation of Rabbit LDL and Rabbit \(\beta\)-VLDL by Rabbit Endothelial Cells

The different effects of cell density on LDL and \(\beta\)-VLDL degradation are physiological evidence of the separate identities of these two receptors. To show that these receptors were also genetically distinct from each other, we compared the uptake and degradation of 125I-rabbit LDL and 125I-rabbit \(\beta\)-VLDL by endothelial cells from normal and WHHL rabbits (Figure 7). In cells from a normal rabbit, the degradation of 125I-LDL was inhibited by a 43-fold excess of nonradioactive LDL, demonstrating specific high affinity degradation. In contrast, the degradation of 125I-LDL by cells from a WHHL rabbit was negligible. The specific degradation of 125I-\(\beta\)-VLDL was similar in both the normal and WHHL cells. In normal rabbit and in normal bovine cells, unlabeled LDL was a poor competitor for 125I-rabbit \(\beta\)-VLDL uptake and degradation; however, unlabeled rabbit \(\beta\)-VLDL suppressed degradation of 125I-rabbit \(\beta\)-VLDL by 90% to 95% in both cell types. The experiment in Figure 7 is representative of experiments using cells from four normal and two WHHL rabbits. We concluded that the \(\beta\)-VLDL receptor is genetically distinct from the LDL receptor on endothelial cells.

**Figure 6.** The effect of cell density on the degradation of 125I-human LDL, 125I-human MDA-LDL, and 125I-rabbit \(\beta\)-VLDL by bovine endothelial cells. Each monolayer of normal endothelial cells at low cell density (1-2 \(\times\) 10^5 cells/cm^2, open symbols) and at high cell density (4-5 \(\times\) 10^5 cells/cm^2, closed symbols) was washed three times with Medium C (1 ml/wash) and incubated with 0.5 ml of Medium D containing either 125I-LDL, 103 cpm/ng (A), 125I-MDA-LDL, 174 cpm/ng (B), or 125I-\(\beta\)-VLDL, 90 cpm/ng (C) in the presence of a 25-fold excess of unlabeled human LDL (C) at the concentrations indicated. After incubation for 4 hours at 37°C, the medium was removed and the 125I-labeled acid-soluble content was determined. Each point is the mean from triplicate wells with a 12% coefficient of variation.

**Down-Regulation of Receptor Activities**

After reaching confluency, contact-inhibited monolayers of endothelial cells were preincubated for 3 days in the presence of unlabeled human LDL, human MDA-LDL, or rabbit \(\beta\)-VLDL at a concentration of 100 pg/ml of protein to determine if the degradation of these lipoproteins showed regulation. Figure 8 shows that after preincubation, the receptor-mediated degradation of 125I-human LDL, 125I-human MDA-LDL and 125I-rabbit \(\beta\)-VLDL were all decreased. The more than two-fold decrease in LDL and \(\beta\)-VLDL receptor activities is similar to previous
findings on cultured fibroblasts and on monocyte-macrophages. It was found that in macrophages the activity of the scavenger receptor was not regulated by preincubation with lipoproteins. In the present study, the decrease in scavenger receptor activity could be the result of an increase in the number of "sprouting" cells in the population which was observed after incubation with MDA-LDL (data not shown). Sprouting cells are phenotypically altered endothelial cells that are known to differ in the biosynthesis of some secreted proteins which also display reduced contact inhibition.

**Effects of LDL, MDA-LDL, and β-VLDL on Cellular Cholesterol Content**

Receptor-mediated degradation via the scavenger receptor on endothelial cell lines and via the β-VLDL receptor on monocyte-macrophages has led to cellular cholesterol accumulation in vitro. To determine the effects of exposure to lipoproteins on cellular cholesterol levels in early passage cultures, we incubated confluent 3-day-old primary cultures of bovine endothelial cells for 72 hours with 100 μg/ml of protein from unlabeled human LDL, human MDA-LDL, or rabbit β-VLDL. As shown in Table 1, the cells incubated with MDA-LDL or with rabbit β-VLDL had a significantly higher cholesterol content than control cells incubated with LDL appeared slightly higher, the difference was not statistically significant.

**Table 1. Total Cholesterol Content of Bovine Endothelial Cells Incubated with Lipoproteins**

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Cellular content of cholesterol (µg sterol/mg protein)</th>
<th>Cholesterol content increase over control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>26 ± 1.7 (7)</td>
<td>—</td>
</tr>
<tr>
<td>LDL</td>
<td>32 ± 4.4 (4)</td>
<td>23</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>46 ± 4.6 (5)*</td>
<td>77</td>
</tr>
<tr>
<td>β-VLDL</td>
<td>49 ± 6 (4)*</td>
<td>88</td>
</tr>
</tbody>
</table>

Each monolayer of normal endothelial cells received 5 ml of Medium A containing 100 μg/ml protein of the lipoprotein indicated. After incubation for 72 hours at 37°C, the cells were washed in the dishes three times with phosphate-buffered saline containing 1% BSA (5 ml/wash); harvested, and the total cholesterol content determined. Each value represents the mean ± SEM. The number of monolayers analyzed is given in parentheses in the second column.

*p < 0.05, compared to cells receiving no addition of lipoprotein.
when compared to the control. None of the cells exposed to the lipoproteins under the conditions used here took on the appearance of foam cells when examined by oil red O staining and electron microscopy (data not shown).

Discussion

We have shown in the present study that adult bovine aortic endothelial cells possess at least three distinct receptors: a receptor for LDL, a scavenger receptor, and a receptor for β-VLDL. Our studies were done using primary or first passage cell cultures where minimal phenotypic change would be expected. The scavenger receptor in these cells is distinct from LDL and β-VLDL receptors as shown by competition studies (Figures 3 and 5). That β-VLDL is taken up by a receptor distinct from the LDL receptor has been shown physiologically by the different effects of cell density on the two receptors (Figure 6). Previous studies, showed that in macrophages the degradation of both β-VLDL and LDL is dependent on calcium. Additionally, both receptors can either be up-regulated by preincubating the cells in lipoprotein-deficient serum or down-regulated by preincubating the cells with sterols. Even the decrease in receptor activity over time in culture was similar for LDL and β-VLDL. However, Van Lenten et al., using mutant cells (FH homozygote) that lacked the LDL receptor, conclusively demonstrated that genetically distinct receptors must exist. In a similar manner, studies on endothelial cells from normal and WHHL rabbits (Figure 7) demonstrate that the two receptors are genetically distinct.

The β-VLDL receptor has been previously described only in macrophages and was shown to recognize both triglyceride-rich and cholesterol-rich lipoproteins. Similarly, we have shown that the plasma VLDL fraction of normal fat-fed individuals does not (Figure 5). The morphogenesis and fate of potential and WHHL rabbits (Figure 7) demonstrate that the two receptors are genetically distinct.

Table 1 illustrates that β-VLDL was as effective as MDA-LDL in increasing the cholesterol content of the endothelium. Because of the high cholesterol content of the particle, cholesterol accumulation occurred in spite of the down-regulation of the β-VLDL receptor (Figure 8).

Increases in membrane cholesterol content or changes in fatty acid composition can alter membrane function. It has been shown that an increase in membrane cholesterol leads to a general decrease in membrane phase transitions. Since pinocytosis is a fundamental process in the maintenance of cell homeostasis, even small alterations in the rate of pinocytosis may result in significant changes in intracellular biochemical transport. Increased membrane cholesterol and saturated fatty acid levels have been shown to alter the activities of membrane enzymes such as Na⁺K⁺ ATPase. We have recently found that increased cholesterol content produces a change in the insulin receptor function in endothelial cells.

Endothelial cells and monocyte-macrophages are thus far the only cells known to possess the LDL receptor, the scavenger receptor, and the β-VLDL receptor. Changes in endothelial cells and monocyte-macrophages are among the earliest alterations in the development of the atherosclerotic lesion; however, the precise role of these receptors in this process has yet to be determined in vivo.

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