Passage of Low Density Lipoproteins through Monolayers of Human Arterial Endothelial Cells

Effects of Vasoactive Substances In an In Vitro Model

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The endothelium controls the influx of lipoproteins into the arterial wall, a process that may be disturbed in arteriosclerotic blood vessels. We have used an in vitro model to investigate the characteristics of the passage of low density lipoproteins (LDL) through monolayers of human arterial endothelial cells. Umbilical artery, aorta, or carotid artery endothelial cells were cultured on polycarbonate filters and formed a tight monolayer in which the cells were connected by tight junctions. Passage of 125I-LDL through these monolayers proceeded linearly over a 24-hour period. It was threefold lower through monolayers of aorta or carotid artery cells than through monolayers of umbilical artery cells. The LDL passage process did not show saturation with LDL concentrations up to 800 μg/ml LDL-protein (i.e., 1.6 nmol/ml apolipoprotein B) between 2 and 4 hours after addition. However, during the first 30 to 60 minutes after addition of high concentrations of LDL, a reduction of the passage rate of both LDL and peroxidase, resulting in an apparent saturation of the passage process, was observed. The passage rate of the negatively charged acetylated LDL was twofold lower than that of native LDL. Addition of histamine to the endothelial monolayer resulted in a large, but transient, increase in permeability paralleled by a decrease in electrical resistance. The effects of histamine were mediated via an H1 receptor. Thrombin and Ca++ ionophore also induced an increase in permeability of the monolayer, while bradykinin did not. The effects of histamine and thrombin were paralleled by a rapid and marked increase in cytoplasmic Ca++ concentration of the endothelial cells, while bradykinin induced only a small increase. Although the cyclic adenosine 5'-monophosphate-elevating agent, forskolin, markedly decreased the basal rate of LDL passage through the endothelial cell monolayers, it did not change the relative increase in permeability induced by histamine. Thus, histamine induces small, but significant, increases in the permeability of tight endothelial cell monolayers. (Arteriosclerosis 9:550-559, July/August 1989)
bovine aorta and human umbilical vein endothelial cells in vitro is changed after a stimulation by vasoactive substances. Since H1 receptors are present on human coronary arteries and on guinea pig endothelial aortic cells, we wondered whether histamine could also have effects on the permeability characteristics of endothelial cell monolayers of human arteries. In this report, we describe the effects of histamine, thrombin, bradykinin, and calcium ionophore on LDL passage and intracellular calcium concentration of the endothelial cells.

The CAMP-increasing agents are able to alter reversibly the permeability of tight junctions of epithelial cells. Prostaglandins E1 and E2, agents which increase the CAMP concentration in vascular cells, are able to decrease venular permeability in the hamster cheek pouch and dextran transport through porcine arterial endothelial cells. In addition, Antonov et al. suggested that the adenylate cyclase activator, forskolin, intensified the cell-cell contacts between human aorta endothelial cells. We studied the effects of forskolin and isobutyl methyl xanthine (IBMX) on permeability and showed that these agents by themselves decrease the permeability, but that, in the presence of these agents, histamine is still able to raise the permeability.

**Methods**

**Materials**

Medium 199 supplemented with 20 mM HEPES was obtained from Flow Laboratories (Irvine, Scotland); all tissue culture plastics, including Transwells (diameter 0.65 cm, pore size 3 µ) were from Costar (Cambridge, MA). Penicillin/streptomycin was purchased from Boehringer (Mannheim, FRG). A crude preparation of endothelial cell growth factor was prepared from bovine hypothalamus as described by Maciag et al. Human serum was prepared in our laboratory from fresh blood of healthy donors, was pooled (12 to 20 donors), and was stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY), heparin from Leo Pharmaceuticals Products (Weesp, The Netherlands), human serum albumin from the Red Cross Central Blood Transfusion Laboratory (Amsterdam, The Netherlands), Horse radish peroxidase (HRP), histamine, bradykinin, A23187, pyrillamine, cimetidine, and α-thrombin were obtained from Sigma Chemical Company (St. Louis, MO) and IMAX, from Janssen Chimica (Beerse, Belgium).

**Isolation and Culture of Endothelial Cells**

Human umbilical artery endothelial cells were isolated by the method of Jaffe et al. Endothelial cells from human aortas or carotid artery were isolated and characterized as described previously. Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 µg/ml crude endothelial cell growth factor, 5 U/ml heparin, and penicillin/streptomycin; this was kept at 37°C under 5% CO2-95% air.

For passage studies, endothelial cells from umbilical artery (primary), aorta (third passage), or carotid artery (sixth passage) were released with trypsin ethylenediamine-
Passage rates are expressed in pmol/h/cm² or pmol/cm². The clearance rate (nl/h/cm²) was calculated when the passage of different macromolecules was compared.

To control the integrity of the monolayers during experiments in which the passage rate of various lipoproteins was evaluated, peroxidase was added simultaneously with the lipoprotein in the upper compartment. Passaged peroxidase in the sample of the lower compartment was measured spectrophotometrically (408 nm) after incubation with o-dianisidine and hydrogen peroxide.

Vasoactive substances and cAMP-increasing agents were added to both the upper and lower compartments when tested for their ability to influence the passage of macromolecules.

**Transendothelial Electrical Resistance**

The transendothelial electrical resistance (TEER) was measured as previously described. In short, an alternating current (50 μA) was passed across the monolayer (one pulse every minute). The measured electrical potential difference was used to calculate the electrical resistance by Ohm's law.

**Assay of Cytoplasmatic Calcium Concentration**

Confluent monolayers of endothelial cells grown on coverslips were loaded with 2 μmol/l fura-2/AM in serum-containing medium. After 30 to 60 minutes, cells were gently washed with Tyrode's buffer, and the coverslip was placed in a special cuvette in a Kontron SFM 25 fluorimeter. Fluorescence intensity was measured at 37°C over a period of 15 to 30 minutes. Cells were excited alternately at 340 nm and 380 nm, while the emission was measured at 510 nm.

**Results**

**Passage of ¹²⁵I-Low Density Lipoprotein through Monolayers of Human Arterial Endothelial Cells In Vitro**

Human umbilical artery endothelial cells seeded at high density on fibronectin-coated polycarbonate filters formed in 4 days a monolayer of closely apposed cells connected by tight junctions and with an electrical resistance of 17±4 Ohm·cm² (mean±SD, 45 determinations). When ¹²⁵I-LDL (25 μg protein/ml; 49 pmol/ml) in Medium 199 with 20% human serum was added to the upper compartment, a linear passage of ¹²⁵I-LDL was observed over a 24-hour period (Figure 1). No difference was observed in the transendothelial electrical resistance of these monolayers also showed an apparent saturation during the first 30 minutes after addition of the LDL (Figure 2B). When the passage rates of the various LDL concentrations were determined between 2 and 4 hours after the addition, no saturation of the passage process was observed. The passage of peroxidase was, thus, not influenced by the passage of high LDL concentrations (Figures 2A and 2B).

The basal rate of the LDL passage (49 pmol/ml ¹²⁵I-LDL) through monolayers of human aorta endothelial cells was 24.4±8.4 pmol/h/cm² (mean±SD of nine determinations of one culture). This value is about threefold lower than that obtained with monolayers of human umbilical artery endothelial cells. Nevertheless, the LDL passage process through these monolayers also showed an apparent saturation during the first 30 minutes after addition of the LDL (Figure 2C), while no saturation was observed between 2 and 4 hours after the addition (Figure 2C). The passage of peroxidase through these cells was comparable with its passage through human umbilical artery cells (Figures 2B and 2D).

**Comparison of Passage of Various Modified Low Density Lipoprotein Particles**

In Table 1, the passage rates of various modified LDL particles through endothelial cell monolayers are compared. Methylation of the lysine residues of the LDL particle results in a modified LDL particle (Me-LDL) that has the same electrical charge as LDL, but has lost its ability to bind to the B,E receptor. The passage rate of Me-LDL is identical to that of LDL (Table 1), indicating that the B,E receptor is not involved in the passage process. When the surface charge of the LDL particle was more negative by acetylation of the lysine- and arginine-residues, the passage...
rate decreased (Table 1). The passage rate of peroxidase that was measured simultaneously with the different lipoproteins was not influenced by them.

**Effect of Vasoactive Substances on Low Density Lipoprotein Passage**

Addition of histamine ($10^{-5}$ M) to the culture medium resulted in a rapid increase in the passage of $^{125}$I-LDL (Figure 3) and peroxidase (not shown). Upon examination with the electron microscope, the cell contacts remained intact in the control monolayers (Figures 4A and 4B), while partial loss of cell contacts was regularly observed after stimulation with histamine (Figures 4C and 4D). The effect of histamine on the passage rate of LDL was

**Table 1. Comparison of Passage of Native and Modified Low Density Lipoproteins through Human Endothelial Cell Monolayers**

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Passage of lipoprotein (fmol/hr/cm²)</th>
<th>Passage of peroxidase (pmol/hr/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>70±10</td>
<td>ND</td>
</tr>
<tr>
<td>Me-LDL</td>
<td>67±12</td>
<td>ND</td>
</tr>
<tr>
<td>LDL</td>
<td>89±16</td>
<td>645±165</td>
</tr>
<tr>
<td>Ac-LDL</td>
<td>48±5</td>
<td>638±105</td>
</tr>
</tbody>
</table>

Values (fmol/hr/cm²) are the means±range for two different experiments, each performed in triplicate.

The passage rates of various lipoproteins (49 pmol/ml) and peroxidase (125 pmol/ml) through monolayers of human umbilical artery endothelial cells were simultaneously determined. Samples from the lower compartment were taken after a 1-hour incubation. LDL=low density lipoprotein, Me-LDL=methylated LDL, Ac-LDL=acetylated LDL, ND=not determined.
transient (Figure 3). It was largest during the first 2 hours after the addition of histamine and decreased rapidly thereafter. At 4 to 6 hours after the addition of histamine, the passage rates of LDL and peroxidase were comparable to those in control monolayers. Thereafter, even a decrease of the passage rate was observed.

When the effect of histamine on monolayers of human aorta or carotid artery endothelial cells was evaluated,
similar results were obtained (Table 2). In four independent experiments with monolayers from two different aortas and one carotid artery, a 2.1 ± 0.5-fold increase in LDL passage was observed after a 1-hour incubation with 10^{-6} M histamine (mean ± SD).

The increase in LDL passage by histamine appeared to be mediated by H1 receptors (Table 3). It could be prevented by the simultaneous addition of the H1 antagonist, pyrilamine, whereas the addition of the H2 antagonist, cimetidine, did not prevent this. In contrast to histamine, the addition of bradykinin (up to 10^{-4} M) to the medium did not increase the passage of LDL. On the other hand, addition of α-thrombin (1 U/ml) or the calcium ionophore A23187 (10 μM) to the medium resulted in a threefold increased passage rate of LDL and a decrease in the electrical resistance of the monolayers.

Endothelial cells produce prostacyclin upon stimulation by histamine. Prostacyclin appears not to be involved in the histamine-induced increase of the LDL passage, because indomethacin (25 μM) and aspirin (25 μM) did not prevent the effect of histamine (not shown).

Effect of Vasoactive Substances on Cytoplasmic Calcium Concentration of Endothelial Cells

An increase in the cytoplasmic calcium concentration underlies the release of several other endothelial cell products, such as endothelium-derived relaxing factor and von Willebrand factor, and may play a role in inducing endothelial cell contraction. By the fura-2 assay, we found a basal level of 0.11 ± 0.05 μM calcium ions in the endothelial cells. After addition of histamine, an immediate increase in cytoplasmic calcium concentration was observed, as detected fluorimetrically by an increase in fluorescence at excitation 340 nm and emission at 510 nm (Figure 5A). Because the increase in fluorescence was accompanied by a decrease in fluorescence at excitation 380 nm, it is highly unlikely that the increase in fluorescence is the result of morphological changes of the cells. Pyrilamine, but not cimetidine, prevented the histamine-induced increase in cytoplasmatic calcium concentration (Figure 5B). Addition of bradykinin had only a very small effect on the cytoplasmic calcium levels of our cells (Figure 6). On the other hand, addition of α-thrombin resulted in a similar increase in cytoplasmic calcium ions and LDL passage, as did histamine (Figure 6, Table 2). As expected, a marked rise in cellular calcium level was observed after the addition of the calcium ionophore ionomycin.

Effect of cAMP-Increasing Agents

Addition of forskolin or IBMX, agents that increase the cellular cAMP level, increased the electrical resistance of the endothelial cell monolayers (not shown) and decreased the passage rate of LDL (Figure 7). The effect of forskolin on the passage of LDL through the monolayers was instantaneous and remained constant over a 24-hour period. When endothelial cells were incubated with forskolin or IBMX, they still responded to histamine. Interestingly, in both nontreated cells and cells that had been treated with forskolin or IBMX, histamine induced a threefold increase in passage rate. Furthermore, in the presence of forskolin, again no saturation of LDL passage through human umbilical artery endothelial cell monolayers was observed at concentrations up to 800 μg/ml LDL protein (1.6 nmol/ml) (Figure 8).

Discussion

The availability of an in vitro model to study the passage of macromolecules through monolayers of human arterial endothelial cells on porous membranes enabled us to investigate the characteristics of the passage of lipoproteins through endothelial monolayers. These endothelial cell monolayers displayed: 1) tight junctions, 2) an electrical resistance of 17 ± 4 Ohm·cm², 3) molecular sieving characteristics, and 4) a selectivity on electrical charge of the molecule. These characteristics resemble in vivo features of animal endothelial cells. However, it should be noted that in vivo data suggest a marked variation between various micro- and macrovascular endothelia, including the relative involvement of vesicles and inter/intracellular pores in the passage of macromolecules. Limited information is available about the arterial endothelium. Therefore, it is not yet possible to ascertain whether our endothelial cell monolayers reflect all aspects of the barrier function of the arterial endothelium or that they differ in certain aspects. It has been suggested that in vitro monolayers of endothelial cells contain local gaps. We did not find such gaps between the cells of our monolayers by electron microscopic exam-
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Table 3. Effect of Histamine and Histamine Antagonists, Bradykinin, Thrombin, and A23187 on LDL Passage and Electrical Resistance across Human Umbilical Artery Endothelial Monolayers

<table>
<thead>
<tr>
<th>Addition</th>
<th>LDL passage (ng/ml)</th>
<th>Electrical resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Histamine (10^{-5} M)</td>
<td>233±66 (10)</td>
<td>68±8 (8)</td>
</tr>
<tr>
<td>Histamine (10^{-5} M) + pyrilamine (10^{-4} M)</td>
<td>132±14 (3)</td>
<td>95±7 (5)</td>
</tr>
<tr>
<td>Histamine (10^{-5} M) + cimetidine (10^{-4} M)</td>
<td>180±21 (3)</td>
<td>75±2 (5)</td>
</tr>
<tr>
<td>Bradykinin (10^{-4} M)</td>
<td>92±2 (3)</td>
<td>92±1 (4)</td>
</tr>
<tr>
<td>Thrombin (1U/ml)</td>
<td>290±76 (4)</td>
<td>65±13 (4)</td>
</tr>
<tr>
<td>A23187 (10^{-5} M)</td>
<td>261±26 (3)</td>
<td>54±25 (4)</td>
</tr>
</tbody>
</table>

Values (% of control) represent the means±SD of independent experiments (number in parentheses) performed with endothelial cells from different donors. Passage of ^{125}I-LDL (49 pmol/ml) and electrical resistance across human umbilical artery endothelial monolayers was determined in the absence or presence of histamine (with the H1 antagonist, pyrilamine, or the H2 antagonist, cimetidine), bradykinin, thrombin, and A23187. Passage of ^{125}I-LDL was determined in triplicate after a 1-hour incubation and is expressed as percentage of the value observed in control monolayers (determined in triplicate). Electrical resistance was determined before and 10 minutes after addition of the indicated substance(s) to the incubation medium. LDL-low density lipoprotein.

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Figure 5. A rapid increase in cytoplasmatic calcium concentration of human umbilical arterial endothelial cells was observed after stimulation of fura-2-loaded endothelial cells with 10^{-5} M histamine. A. A full record of the changes in fluorescence intensity at excitation wavelengths of 340 and 380 nm (emission wavelength of 510 nm) was made by combination of the data of four overlapping experiments with cells of the same batch. B. Changes in fluorescence intensity were measured at 340 nm excitation and 510 nm emission. The effect of histamine on cytosolic calcium concentration could be prevented by the H1 antagonist, pyrilamine, but not by the H2 antagonist, cimetidine.

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ever, was accompanied by a similar decrease in the passage of peroxidase, suggesting that high concentrations of LDL induce a temporary decrease in the permeability of the monolayer. The mechanism behind this effect is still under investigation. When measured over longer time intervals, the passage rates of peroxidase were independent of the LDL concentration, and the apparent saturation of LDL passage disappeared completely. Therefore, we conclude that the passage of LDL across cultured human endothelial cells is a nonsaturable process at physiological LDL concentrations.
Histamine induced a rapid increase in the passage rate of LDL and peroxidase, concomitant with a decrease in electrical resistance of the endothelial monolayer. The effect of histamine was transient, and several hours after addition of the histamine, the passage rate of LDL was even lower than that across nonstimulated cells. Haddock et al. have shown that desensitization of the histamine response in umbilical vein endothelial cells occurs and that it is due to competitive inhibition of the interaction of histamine with its receptor by an inactive histamine metabolite. This mechanism may explain the transience of the increase in LDL passage rate, but does not explain the subsequent decrease in LDL passage after prolonged exposure to histamine. The effect of histamine on the permeability of the endothelial cell monolayer proceeds via interaction with an H1 receptor. H1 receptors were also detected on human carotid artery,24 guinea pig aorta,25 and human umbilical vein54 endothelial cells. Whether such receptors may play a role in the local regulation of macromolecular permeability of the arterial endothelium remains uncertain.

From in vivo experiments, Majno et al.20 postulated that histamine acted on a contractile mechanism of rat and rabbit venule endothelial cells. In favor of this, Rotrosen et al. demonstrated an increase in cytoplasmatic calcium concentration after stimulation of suspended human umbilical vein endothelial cells, which coincided with a change in F-actin content. An increase in cytoplasmatic calcium concentration was also observed after stimulation of human endothelial cells with thrombin.4144 Here we have demonstrated that in endothelial cell monolayers also the cytoplasmatic calcium level was increased upon stimulation by vasoactive substances (histamine via H1 receptors thrombin and calcium ionophore).

Under our experimental conditions, the addition of bradykinin resulted in only a small increase in cytoplasmatic calcium concentration in the cells, while it had no effect on the permeability of LDL and the electrical resistance across the monolayers. The lack of effect of bradykinin on human endothelial cells has also been found by other authors,23 but this contrasts with observations on animal cells.205556 Possibly human endothelial cells become desensitized for bradykinin in culture.
Because only the large increase in cytoplasmatic calcium elicited by histamine, thrombin, or calcium ionophore was associated with a change in LDL passage through endothelial cells monolayers, while the small increase elicited by bradykinin was not, our data fit in a model in which a marked increase in cytoplasmatic calcium concentration results in contraction of endothelial cells and in an increase of the permeability of the endothelial cell monolayer.

Despite a general resemblance of the passage of lipoproteins in the in vitro model and in vivo, the actual passage rate of LDL was high as compared to in vivo observations. In experiments with monolayers of adult human aorta or carotid artery endothelial cells, we observed passage rates of LDL, which are threefold lower than those of umbilical artery endothelial cells. These values are in the same order of magnitude as those reported for rabbit and bovine aortic endothelial cell monolayers in vitro. Still, the clearance rate of LDL in these models is at the higher limit of LDL influx into atherosclerotic human aortic intima, while this influx is 10-fold higher than that found with unaffected human arteries in vivo. After addition of the CAMP adenylyl cyclase activating agent, forskolin, the passage rate of LDL in vitro becomes 5- to 10-fold lower and approximates the physiological clearance rate of LDL in vivo. Under these conditions, the lack of saturation of LDL passage and the relative increase in permeability by histamine are essentially the same. These data suggest that histamine not only has large effects on the permeability of "leaky" endothelia, but also may induce relatively minute, but significant, increases in the permeability of tight arterial endothelial cell monolayers. The model presented here can be used to evaluate the effects of platelet products, fibrin degradation products, and vasoactive peptides on the permeability characteristics of human endothelial cells. Such studies will be helpful in clarifying the difference in permeability characteristics of normal and atherosclerotic arteries.

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