Influx and Cellular Degradation of Low Density Lipoproteins in Rabbit Aorta Determined in an In Vitro Perfusion System

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The accumulation of 125I-low density lipoprotein (LDL) into normal and atherosclerotic arterial tissue and cellular uptake in arterial cells were studied in an in vitro perfusion system for rabbit aorta. The accumulation of 125I-LDL in normal tissue could be fitted to an inverse exponential function with an initial influx rate of 1.39 ml/mg wet weight/hour and an equilibration volume of about 2% of the tissue volume. The influx rate into atherosclerotic plaques was about 10 times faster and the equilibration volume, 50 times higher. In atherosclerotic tissue there was a steep concentration gradient between the plaque and the underlying media. The accumulation of 125I-LDL in the media under plaque and in normal tissue adjacent to plaques was similar to that seen in normal tissue. For studies of cellular uptake of LDL a trace label, 125I-tyramine-cellulobiase (TC), was used. Normal or atherosclerotic rabbit aorta was perfused in vitro with medium containing 125I-TC-LDL. After perfusion the tissue was digested and the cells were isolated by density gradient centrifugation. Two main cell fractions with characteristics of smooth muscle cells and foam cells, respectively, were obtained. A 70-fold higher increase was seen in the foam cells. In conclusion, these studies suggest a higher influx rate into atherosclerotic plaques, as well as a high LDL concentration in the plaque, compared with normal tissue or underlying media. We suggest that most of the cellular uptake of LDL in the arterial wall is caused by the foam cells.

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Cholesterol deposited in atherosclerotic lesions appears to be derived from plasma lipoproteins, primarily low density lipoprotein (LDL). Thus, in atherosclerosis, apo B is deposited in the arterial tissue. Information concerning the influx of LDL and the local cellular uptake of LDL is therefore of fundamental significance for the understanding of atherogenesis. Influx of cholesterol into the arterial wall has been studied in vivo in various animal models as well as in humans. However, no matter what tracer was used, these studies are difficult to interpret in quantitative terms, since the tracer concentrations in plasma are affected by lipoprotein catabolism in nonvascular tissues and by exchange and transfer between different lipoproteins. We attempted to overcome such problems by developing an in vitro perfusion system for arterial tissue that would allow us to study uptake of LDL under more easily controlled conditions.

After uptake from plasma, LDL may be degraded in the vascular cells. This degradation has been estimated both from the hydrolysis of labelled cholesteryl ester and from the cellular uptake of LDL labelled in its protein moiety. Studies on the uptake of protein-labelled LDL have been facilitated by the development of a “trace-label” methodology that makes use of 125I-tyramine cellulobiase as an intracellularly trapped undegradable marker for the cellular LDL degradation. In our present study, this technique was combined with the in vitro perfusion system for study of cellular uptake in normal and atherosclerotic arterial tissue.

In the atherosclerotic lesion, two major cell fractions are found — macrophage-derived cells and smooth muscle cells. It has been suggested that smooth muscle cells, as well as macrophages, may develop into foam cells. Both cell types express the B,E receptor which allows a regulated uptake of LDL in relation to the intracellular cholesterol levels. Obviously, interaction of LDL with this receptor does not lead to the formation of foam cells because the receptor is down-regulated if intracellular cholesterol levels increase. However, the macrophage also expresses a receptor for modified LDL that is not subject to down-regulation. It is unclear, though, if modification of lipoprotein occurs in vivo. Moreover, the significance of such a process in arterial tissue may be difficult to evaluate in vivo due to the receptors for modified LDL that are present in the reticuloendothelial system of the body. Such processes may be easier to study in vitro in the arterial perfusion system.

The present study was undertaken to study: 1) the influx of LDL in normal and atherosclerotic arterial tissue and 2) the uptake and degradation of LDL in different cell populations of the atherosclerotic artery. These processes were studied in an in vitro perfusion system, rather than in vivo, because the experimental conditions were simpler.
Methods

Isolation and Labelling of Lipoproteins

Fresh human EDTA plasma was used for the preparation of lipoproteins. LDL-2 was isolated by sequential ultracentrifugation in the presence of 0.01% EDTA as described earlier. Conventional iodination (125I-LDL) was performed with the ICI-technique as described by McFarlane and modified by Shepherd. For the studies of cellular uptake, iodination was performed with 125I-TC-LDL as described by Pittman et al. Briefly, the TC adduct (0.1 μmol TC for each 10 mg LDL protein to be reacted) was iodinated with Iodogen (Pierce Company, Rockford, Illinois) with carrier-free (Amersham International, Amersham, U.K.). After iodination, the reaction was quenched with NaHSO3 and KI and the labelled adduct was activated by the addition of 1 mol equivalent of cyanuric chloride. The activated ligand was used immediately to bind to 5 mg of LDL at a pH of 9.5 adjusted by the addition of 0.3 M borate buffer (pH 9.5).

For both iodination procedures, the specific activity was 100 to 200 cpm/ng. For 125I-LDL, 97.9% ± 1.1% (mean ± SD) of the radioactivity could be precipitated with 15% TCA, while the equivalent figure for 125I-TC-LDL was 99.0% ± 0.2%.

To evaluate whether modification of LDL might have been induced during preparation or labelling of the LDL, 125I-TC-LDL was incubated for 6 hours with: 1) receptor-induced normal fibroblasts, 2) receptor-repressed fibroblasts, and 3) mouse peritoneal macrophages (which express only the modified LDL receptor). Significant internalization of the labelled LDL was observed only in receptor-induced fibroblasts.

Protein determinations were performed according to the method of Lowry as modified by Markwell et al. Aortas for perfusion were taken from New Zealand White rabbits obtained from a local breeder. They were fed for 2 to 3 months on either normal rabbit chow (Astra-Ewos, Sodertalje, Sweden) or (to induce experimental atherosclerosis) normal rabbit chow with 1% cholesterol. The responses to internalization of the labelled LDL were observed only in receptor-induced fibroblasts.

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In Vitro Perfusion

The in vitro perfusion system used in the present study has been characterized in detail and used to study arterial wall/blood cell interactions and oxygen consumption in arterial tissue. Shortly after dissecting the outer half of the media, the intima-media preparations were divided into four to six segments that were mounted in an incubation chamber. The whole dissection procedure required about 2.5 hours. Contact between the arterial tissue and the medium was restricted to the endothelial surface during perfusion. The tissue preparations maintained a stable metabolic activity for at least 48 hours in this system, and the endothelium was intact for at least 24 hours with more than 95% of the intimal surface covered after 3 days. During the incubation, segments could be removed at different time points for analysis.

In the present study, human LDL was added to Eagle’s minimum essential medium with Earle’s salt, 0.8 mg/ml sodium bicarbonate, and 1% nonessential amino acids (Flow Laboratories, Irvine, U.K.). The medium included 100 μg/ml cefalothin sodium (Lilly, Indianapolis, Indiana), 20 mM HEPES buffer, pH 7.4 (Flow Laboratories), 60 mg/ml bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri) and 10% rabbit lipoprotein-deficient serum (LPDS) prepared by ultracentrifugation at d > 1.21. The final concentration of apo B in the medium after the addition of LDL was 0.37 to 0.92 mg/ml (mean 0.51) with a specific activity of 8 to 32 cpm/ng. The mean cholesterol concentration was 0.90 mg/ml (range 0.73 to 1.82). The medium was allowed to circulate from a reservoir of 50 to 100 ml medium. Perfusion was allowed to proceed for various time intervals up to 48 hours. During the perfusion, 1 ml samples were taken from the medium. No significant changes in apo B immunoreactivity with perfusion time were found as evaluated from the height and appearance of immunoprecipitates in an apo B electroimmunoeassay performed as described previously. The mean apo B concentration in the medium after 48 hours of perfusion was 0.54 mg/ml. The electrophoretic mobility of labelled LDL on agarose gel electrophoresis did not change during perfusion, as evaluated from the radioactivity in excised mm strips counted in a gamma counter (Ria-Gamma; LKB-Produkter AB, Bromma, Sweden). After perfusion, the proportion of trichloroacetic acid (TCA)-precipitable radioactivity for 125I-LDL decreased to 96.8%, while the proportion of TCA-precipitable 125I-TC-LDL was maintained at 98.4%.

After perfusion, the aortic samples were taken out of the incubation chamber, were cut open, and were rinsed in saline. In the first short-term incubations the samples were rinsed for 20 seconds. To minimize the influence of free iodine, the samples were later rinsed twice in saline for 10 minutes. In control experiments, we observed that only trace amounts of radioactivity were released during subsequent rinses. After rinsing, tissue wet weight and total tissue radioactivity were determined. In one experiment we also determined the surfaces of the tissue samples by photographing the tissue, cutting out the pictures, and weighing them. For atherosclerotic tissue, plaques were removed by dissection and analyzed separately. Samples were also taken from the tunica media under the plaques and from areas without visible lesions. The tissue accumulation of 125I-LDL in the aortic samples was defined as protein-bound radioactivity and was determined after precipitation of homogenates in 15% TCA for 30 minutes and centrifugation at 400 g for 15 minutes. On the average, 86.5% of the radioactivity in the tissue homogenates was TCA-precipitable. Radioactivity was determined in a gamma counter.

Cell Separation

For studies of the cellular uptake of LDL, tissue was perfused with 125I-TC-LDL-containing medium. After perfusion, the aortic samples were cut into 0.5 mm3 cubes,
which were washed twice in 20 ml Hanks' medium (Flow Laboratories). The tissue pieces were then incubated in a proteolytic solution as described by Haley et al.\textsuperscript{13} and modified in our laboratory.\textsuperscript{23} The proteolytic solution contained 450 U/ml collagenase CLSPA (Cooper Biomedical, Massachusetts); 47 \( \mu \)g/ml elastase E-0127 (Sigma Chemical Company); and 1 mg/ml trypsin inhibitor (Sigma Chemical Company) in Hanks' solution without Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. After digestion for 20 minutes, a supernatant with cells was separated from the tissue debris. The digestion was repeated until the tissue samples were completely dissolved; one to three consecutive incubations were generally needed. In the supernatant of the cell pellet, 10.8% of the radioactivity was TCA-soluble (range 5.7 to 16.7). The cell pellets were combined and fractionated on Percoll as described elsewhere.\textsuperscript{23} As in previous experiments, two major cell fractions were obtained from atherosclerotic samples.\textsuperscript{13,23} One cell fraction had a density of less than 1.06 g/ml and structural characteristics of foam cells. The other cell fraction had a higher density and structural characteristics of smooth muscle cells. The viability of the cells in both fractions was 80% or more, as evaluated with a dye exclusion test. The recovery of foam cells from tissue was estimated to 5% to 15%, and the yield from one aorta, to be \( 4 \times 10^6 \) to \( 20 \times 10^6 \) cells.\textsuperscript{23}

The cell fractions were collected with a syringe and radioactivity was determined in a gamma counter (RIA-Gamma, LKB, Bromma, Sweden). The cells were then collected on a 5.0 \( \mu \)m filter (SMWP, Millipore, Molsheim, France), stained with Mayer's hæmalun and oil red O, and counted under the light microscope.

**Results**

**Accumulation of \( ^{125}\text{I}\)-LDL**

The accumulation of \( ^{125}\text{I}\)-LDL as determined by TCA-precipitable radioactivity in the normal aortic tissue of normocholesterolemic rabbits is summarized in Figure 1. The accumulation was studied for up to 35 hours of incubation, and the activity was normalized to that in the medium, thus expressing the tissue clearance of LDL. Data is given for 25 segments from five different aortas. In this figure the different aortas are represented by different symbols. The accumulation of iodinated LDL could be fitted to an inverse exponential function (\( y = 18.5 (1 - e^{-0.0780x}) \), \( r = 0.89 \), standard error of the slope = 0.009), suggesting an influx rate of 1.39 nl/mg wet weight/hour (corresponding to about 0.7 ng apo B/mg wet weight/hour). Equilibration was reached after 20 hours at 18.5 nl, suggesting a distribution volume for LDL of about 2% of the tissue volume.

The accumulation of \( ^{125}\text{I}\)-LDL in atherosclerotic plaques is shown in Figure 2. The accumulation was close to linear for up to 16 hours, with an estimated initial influx rate of 14.7 nl/mg wet weight/hour (\( y = -6.92 + 14.7x \), \( r = 0.99 \), i.e., about ten times higher than for normal tissue. Samples incubated for longer times showed a wide scattering and could not be fitted to the curve. However, the accumulation seemed to equilibrate at about 1000 nl/mg wet weight after 24 hours (the observed accumulated activity after 24, 32, 38, and 48 hours were 1140, 1133, 851, and 1163 nl/mg wet weight, respectively).
related to wet weight, the uptake per surface unit (mm$^2$) was about ten times higher in plaques than in normal tissue or in the underlying tunica media.

**Cellular Uptake of LDL in Arterial Wall**

We measured cellular uptake by the accumulation of $^{125}$I-TC within cells isolated from the aortas after perfusion. Table 2 shows the results of four experiments. The denser of the two major cell fractions consisted of oval or elongated oil red O-negative cells (Figure 3) that had the structural characteristics of smooth muscle cells. These cells showed a low uptake of radioactivity, corresponding to a clearance of 10.69 nl medium/10$^6$ cells/24 hours. The lighter cell fraction (Figure 4) consisted of a majority (> 75%) of rounded oil red O-positive foam cells. The uptake of radioactivity in these cells was high with a mean estimated clearance of 713 nl/10$^6$ cells/24 hours. Thus, the total uptake and degradation of $^{125}$I-TC-LDL in these cells was approximately 70 times higher than that in smooth muscle cells.

**Discussion**

We have studied the influx of labelled LDL into normal and atherosclerotic arterial tissue in an in vitro perfusion system. The reason we selected this system was to avoid some of the complex effects of the in vivo system on the circulating labelled lipoproteins.

Assuming a simple, two-compartment model for the accumulation of LDL in tissue, the data were fitted to an inverse exponential function. This may be an overly simple model, but the function adequately describes the observed accumulation and allows the influx rate, as well as the equilibration volume, to be estimated. The influx rate was found to be 1.39 nl/mg wet weight/hour, and the equilibration volume was about 2% of the tissue volume. It is difficult to compare the influx rates we observed with those from in vivo experiments because of the differences in experimental conditions and ways of standardizing the data. However, the influx rates from in vivo studies are certainly in the same range as ours.

In atherosclerotic plaques, the influx rate was about ten-fold higher, and the equilibration volume, about 50-fold

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Cells from enzymatically digested aortic intima-media from cholesterol-fed and chow-fed rabbits were separated by density gradient centrifugation. Cells with d > 1.06 were collected on Millipore filters and stained with Mayers hemalum and oil red O. In this fraction oval or elongated cells can be seen. No cells stained with oil red O. Bars = 50 μm.

**Table 1. Accumulation of $^{125}$I-Low Density Lipoprotein in Tissue**

<table>
<thead>
<tr>
<th>Hours of perfusion</th>
<th>Plaques</th>
<th>Media below plaques</th>
<th>Normal tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>23</td>
<td>2.7</td>
<td>2.11</td>
</tr>
<tr>
<td>17</td>
<td>29</td>
<td>2.3</td>
<td>2.55</td>
</tr>
<tr>
<td>24</td>
<td>72</td>
<td>3.8</td>
<td>5.80</td>
</tr>
<tr>
<td>32</td>
<td>133</td>
<td>3.06</td>
<td>5.53</td>
</tr>
</tbody>
</table>

Data are given in relation to surface of tissue sample (cpm/mm$^2$) or (cpm/ml medium)$^{-1}$ for four segments of aorta from one cholesterol-fed rabbit.

**Table 2. Uptake of $^{125}$I-Tyramine Cellobiose Low Density Lipoprotein**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Degree of atherosclerosis (%)</th>
<th>Fraction density (g/l)</th>
<th>Oil red O positive cells (%)</th>
<th>Apo B concentration in medium (mg/ml)</th>
<th>Clearance of medium (nl/10$^6$ cells/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>&gt;1.06</td>
<td>0</td>
<td>0.45</td>
<td>12.6</td>
</tr>
<tr>
<td>II</td>
<td>80</td>
<td>&lt;1.06</td>
<td>75</td>
<td>0.40</td>
<td>973</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>&gt;1.06</td>
<td>0</td>
<td>0.92</td>
<td>8.80</td>
</tr>
<tr>
<td>IV</td>
<td>20</td>
<td>&lt;1.06</td>
<td>80</td>
<td>0.49</td>
<td>449</td>
</tr>
</tbody>
</table>
higher than in normal tissue. These observations are compatible with results from in vivo studies. In our earlier studies, we observed similarly impressive differences in the uptake of lipoprotein-bound labelled cholesterol between normal tissue and experimental atherosclerotic lesions. These differences were concentrated in nonendothelialized areas, and we suggested that they might be related to a barrier function of the intact arterial endothelium. Other researchers used other systems and contested these data, suggesting an increased uptake of lipoproteins through regenerating endothelium. In unmanipulated arteries, areas with an increased frequency of injured endothelium, as well as endothelial regeneration, are characterized by an increased uptake of labelled lipoproteins. Whether the higher rate of flux into atherosclerotic plaques observed in the present study is due to dysfunctional or defective endothelium is still unclear but is being investigated. A drastic difference in accumulation between normal and atherosclerotic tissue may be explained if one considers that the basic difference between these tissues is the relative proportion between the intima and the media. As the present study, as well as others, show, there is a steep concentration gradient between the intima and the media, with a several-fold higher concentration in the intima corresponding to the higher equilibration volume of the intimal plaques. Actually Smith and Ashai have suggested that apo B concentration in the interstitial fluid of the intima is two- to threefold higher than in plasma.

The uptake of LDL was 70-fold higher in foam cells than in smooth muscle cells, the two major cell types in these lesions. Our experimental design did not allow conclusions in quantitative terms about the relation between inflow and cellular uptake in the two cell fractions. The recovery of cells with the present isolation procedure was low, estimated at from 5% to 15% of the cells in tissue. Furthermore, we cannot exclude a possible difference in recovery between the two cell fractions. A comparison of the incorporated activities in the two cell fractions would require estimations of the specific activities of LDL in the extracellular compartment; this must await the development of methods to determine apo B in rabbit aorta. It should also be noted that the foam cells are exposed to a higher LDL concentration than the medial smooth muscle cells. However, in view of the impressive difference in incorporated activity between the two cell fractions, it seems safe to propose that LDL in the atherosclerotic arterial wall is mainly taken up by intimal foam cells, with a resultant intracellular cholesterol accumulation. The foam cells, which are characteristic of the atherosclerotic lesion, appear to be derived primarily from monocytes even though some data suggest that some of the foam cells may be of smooth muscle origin. The mechanisms for the intracellular lipid accumulation in foam cells is still poorly understood. In vitro, monocyte-derived macrophages cannot be transformed into foam cells by exposure to native LDL. However, various modifications of LDL allow an uncontrolled uptake of LDL into macrophages, transforming them into foam cells. Examples of such modification of LDL are: acetylation, malondialdehyde treatment, oxidation, or exposure to endothelial cells in culture. However, it has not been shown that these modifications have a role in vivo. In contrast to the receptors for native LDL, the receptors for modified LDL are not subject to down-regulation in response to an increased cellular content of cholesterol. Therefore, uptake of LDL through receptors for modified LDL may explain the present observations. We did not find any evidence for modification of LDL in the perfusion medium. Local modification of LDL is possible; such modification might take place after exposure to endothelial cells, after complex formation with arterial proteoglycans, or after exposure to superoxides formed by the macrophages themselves.

Figure 4. Cells from enzymatically digested aortic intima-media from cholesterol-fed rabbits were separated by density gradient centrifugation. Cells with d < 1.06 were collected on Millipore filters and stained with Mayers hemalun and oil red O. In this fraction a majority of "foamy," oil red O-positive cells can be seen. Bars = 50 μm.
In conclusion, our observations in the present study once again emphasize the significance of local factors for lipoprotein deposition during atherogenesis. The higher rate of influx suggests a failing barrier function in the atherosclerotic tissue, which could be explained by defective or dysfunctional endothelium. The intimal volume in itself appears to determine the amount of LDL that is accumulated in the arterial wall. In this process such factors as the proteoglycan constituents of the intima might be crucial. The present study also suggests that LDL is directed toward the macrophage-derived foam cells, which show a higher uptake than do smooth muscle cells. Since these cells express few receptors for native LDL, this observation might imply uptake by the modified LDL receptor after local modification of LDL in the arterial tissue. The presence of, and the mechanisms for, such modification will be investigated further.

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