Measurement In Vivo of Irreversible Degradation of Low Density Lipoprotein in the Rabbit Aorta

Predominance of Intimal Degradation

Thomas E. Carew, Ray C. Pittman, E. Roger Marchand, and Daniel Steinberg

The development of a highly sensitive method for assessing, tissue by tissue, the rates of irreversible protein degradation in vivo has allowed us to quantify low density lipoprotein degradation in the normal rabbit aorta and to localize it. The method depends upon the fact that tyramine-cellobiose, like sucrose used in previous studies, can be covalently attached to proteins, enter cells with them, and then remains trapped within the cell after the remainder of the protein molecule has been degraded. Rabbit LDL (d = 1.02 to d = 1.06 g/ml) was labeled with 125I-tyramine-cellobiose and injected into rabbits. Aortic 125I content 24 hours later served as a cumulative measure of degraded LDL (after appropriate corrections for any intact, nondegraded LDL present). Calculated aortic degradation of LDL averaged 9.4 x 10^-3 percent of the plasma pool per g aortic wet weight per day (n = 6). Intimal cells, obtained by gentle swabbing, accounted for fully 40% of total aortic degradation even though the Intima represented less than 5% of the aortic mass. Autoradiography confirmed the high concentration of label in the intima. Degradation of unmodified and reductively methylated LDL were compared. The fractional rate of degradation of methylated LDL by the Intima was 50% to 60% of that for native LDL, indicating that 40% to 50% of LDL degradation in the Intima, predominantly endothelial cells, is mediated by LDL receptors. (Arteriosclerosis 4:214-224, May/June 1984)

A number of lines of evidence support the conclusion that most of the cholesterol and other lipids accumulating in atherosclerotic lesions arise from plasma lipoproteins.1-5 Particularly in early lesions, cholesterol is found both intracellularly and extracellularly,6 and the presence of intact low density lipoprotein (LDL) in lesions has been demonstrated.7-11 Measurements have been made of the rates of penetration of plasma lipoproteins into the artery wall, particularly by Zilversmit and coworkers,12-21 but it has not been possible to distinguish clearly between entry into the extracellular space, on the one hand, and uptake and degradation by cells of the artery wall, on the other.

For a number of reasons, it would be valuable to know the true rate of lipoprotein degradation in normal vessels and in developing lesions. Even more valuable would be the ability to determine which cell types in the normal and atherosclerotic artery contribute to lipoprotein degradation. For example, lipid-laden foam cells are a prominent feature of early lesions, presumably because uptake of lipoproteins exceeds the capacity of the cells to release stored lipids. But how rapidly is the cell turning over its stores of lipids in vivo? Direct measurement of rates of irreversible lipoprotein degradation by these cells could provide an index of this at steady state. In this paper we describe a method that we hope will provide this kind of information. We apply it initially to a study of the role of the normal aortic intima in LDL degradation.

The principle of the approach has previously been described and validated in a number of in vitro and in vivo systems.22-26 The method depends upon the covalent labeling of LDL with 125I-tyramine-cellobiose, a method that has been previously described and validated in an in vitro system.27 The method is similar to that used by Zilversmit and coworkers22-26 for determining rates of penetration of plasma lipoproteins into the artery wall. However, our method allows the measurement of rates of irreversible degradation, whereas the method described by Zilversmit and coworkers allows the measurement of uptake only. This distinction is important because it allows the determination of the fraction of LDL taken up by cells that is degraded.

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vivo studies of the metabolism of LDL, serum albumin, and apolipoprotein A-1. A marker molecule, originally 14C-sucrose but now 125I-tyramine-cellobiose, is covalently attached to the plasma protein of interest so that it will enter with it into cells. The principle is illustrated in Figure 1. When a conventionally iodinated protein enters the lysosome, it is degraded to its constituent amino acids, including labeled iodotyrosine, all of which rapidly cross the lysosomal membrane (Figure 1, left). Since iodotyrosine is not reused, it rapidly escapes from the cell and is excreted, predominantly in urine. Thus, in the average tissue, the content of label following injection of conventionally iodinated protein represents primarily undegraded protein and bears no necessary direct relationship to the rate of degradation in that tissue.

Proteins labeled with iodinated tyramine-cellobiose (Figure 1, right) upon entering the lysosome are also degraded to their constituent amino acids and to iodinated tyramine-cellobiose (I-TC) which remains attached to short peptide fragments. These I-TC fragments do not escape readily but remain predominantly trapped in the cell. The tissue content of I-TC degradation products thus represents a cumulative tally of labeled protein degraded by that tissue. If there is any extralysosomal degradation, the trapped ligand would probably also accumulate intracellularly.

If the specific activity of the protein being metabolized remains constant (as in studies in vitro) or if the time-averaged specific activity of the in vivo pool from which the protein is being taken up is known, one can calculate from the amount of radioactivity trapped in each tissue the absolute amount of protein degraded. Some of the theoretical and practical considerations in the application of this approach have been dealt with in detail elsewhere.

Using 14C-sucrose as the marker, we have previously reported preliminary data on degradation of LDL in the normal rabbit aorta. However, the radioactivity in the aorta was at the limits of reliable measurement. By using radioiodinated tyramine-cellobiose, we have increased sensitivity as much as 1,000-fold. Measurements of regional and even cellular uptake are now feasible.

**Methods**

**Lipoprotein Preparation**

LDL (d = 1.020–1.060 g/ml) was prepared by differential ultracentrifugation from fresh rabbit plasma containing disodium EDTA (1 mg/ml). Plasma was initially adjusted to d = 1.022 g/ml and centrifuged at 11°C for 18 hours at 50,000 rpm in a 60 Ti rotor (Beckman Instruments). The supernate was removed by slicing the tube, and the density of the clear solution immediately beneath was verified to be d = 1.020 g/ml using a density meter (Mettler/Par, Graz, Austria). The infranate containing the LDL was "washed" by restoring it to its initial volume with a d = 1.020 g/ml stock solution of NaBr and again centrifuging for 18 hours as above. After removing the d < 1.020 supernate, the density of the infranatant fraction was adjusted to 1.065 g/ml by addition of solid NaBr or addition of an appropriate NaBr stock solution. The samples were then centrifuged at 11°C in polycarbonate bottles in a 60 Ti rotor for 24 hours at 50,000 rpm. The density of the clear infranate after this centrifugation was 1.060 g/ml. The supernate containing the LDL was washed by centrifuging again under the same conditions. The final LDL preparation was dialyzed exhaustively against a buffer containing 0.15S M NaCl, 0.01% EDTA and 20 mM sodium phosphate, pH 7.4 (Buffer A). All LDL preparations were radioiodinated and used within 12 days of their initial isolation.

**Lipoprotein Labeling**

As will be explained under Results, most of the present studies required simultaneous measurement of the tissue contents of conventionally iodinated LDL and of tyramine-cellobiose-labeled LDL. Consequently most LDL preparations were doubly labeled, first with 131I by a conventional direct iodination technique and then with 125I-tyramine-cellobiose as described in detail elsewhere. Briefly, the tyramine-cellobiose (TC) ligand (0.1 μmol TC for each 10 mg LDL protein to be reacted) was iodinated with carrier-free Na 125I and 1,3,4,6-tetrachloro-3,6-diphenylglycouril (lodogen; Pierce Chemical Company, Westchester, Pennsylvania). The radioiodinated TC ligand was removed from the lodogen-coated vessel to a fresh vessel, quenched by the addition of NaHSO3 and NaI and then activated by addition of 1 mol equiv. of the cross-linking agent, cyanuric chloride. After 10 to 30 seconds, 3 mol equiv. of acetic acid was added. The resulting activated ligand was used immediately to bind to 4–12 mg of LDL adjusted to pH 9.5 by the addition of 0.3 M
borate buffer. After a binding period of at least 1 hour, preparations were exhaustively dialyzed against Buffer A and sterilized by filtration (0.45 μM filter; Millipore, Bedford, Massachusetts). The extent of derivation was less than one tyramine-cellobiose residue per 100,000 Da of LDL protein. Specific activities ranged from 330 to 761 cpm/ng protein.

Most labeled preparations were analyzed by SDS polyacrylamide gel electrophoresis on 4% gels. More than 95% of both the $^{131}$I and the $^{125}$I TC labels was associated with apoprotein B.

Reductively methylated LDL was prepared as described by Weisgraber et al. 34 by the addition of formaldehyde in the presence of sodium borohydride. When preparations were labeled with the TC ligand was associated with apoprotein B.

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Just as shown previously, not all of the degradation products of TC-LDL are soluble in trichloroacetic acid and thus degradation would be underestimated if only the soluble products were counted. It follows that the radioactivity insoluble in trichloroacetic acid includes TC-LDL degradation products in addition to the intact TC-LDL degradation products. If one can make an independent estimate of the radioactivity attributable to intact TC-LDL, that can be subtracted from the total radioactivity due to the TC label to derive the radioactivity that represents degradation products. This is accomplished by taking advantage of the fact that the degradation products of conventionally iodinated LDL are all soluble in trichloroacetic acid. Thus, the radioactivity insoluble in trichloroacetic acid measures only the intact LDL (that in extracellular spaces and that in the cells but not yet degraded). LDL is degraded very rapidly after uptake so that the protein-bound radiiodine in tissue represents almost exclusively intact LDL in the extracellular spaces. What is done, then, is to inject LDL conventionally iodinated with $^{131}$I and also labeled with $^{125}$I-TC; on tissue sampling, the tissue content of $^{131}$I insoluble in 10% trichloroacetic acid is used as the measure of the nondegraded LDL present. That amount, converted to a corresponding amount of $^{125}$I from the relative specific radioactivities of the two forms of LDL, can be subtracted from the total amount of TC radioactivity in the tissue to derive a value for TC degradation products.

**Determination of Degradation Products in Individual Tissues**

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**Animal Studies**

Indwelling Silastic catheters were placed in the external jugular veins of New Zealand white rabbits (2.8 to 3.1 kg body weight) under general anesthesia (intramuscular ketamine, 35 mg/kg and xylazine, 5 mg/kg). Catheters were used for injection of labeled LDL and for blood sampling. Plasma decay kinetics were determined in terms of TCA-precipitable radioactivity in plasma. Sequestration of radiiodide was prevented by injection of 3 mg NaI just before injection of the labeled LDL. At termination of the experiments, generally 24 hours after injection, the rabbits were anesthetized and the systemic circulation was perfused at 100 mm Hg pressure using Buffer A. Perfusion was generally completed within 3 minutes. The thoracic aorta was rapidly, but gently, removed and loose adventitial tissue was dissected away.

In most experiments the descending thoracic aorta was divided into four segments weighing 0.08 to 0.12 g and placed directly into counting tubes and assayed for radiiodine. Immediately after radioassay, the aortic segments were opened longitudinally, pinned flat, and the intimal surface was gently brushed with a moistened cotton swab to remove endothelium (and possibly other intimal cells). The brushed segments and the cotton swabs containing the intimal cells were placed in counting tubes and radioassayed. Recovery of both $^{125}$I and $^{131}$I averaged 94.3% of total aortic activity previously determined. The $^{131}$I activity soluble in 10% trichloroacetic acid was determined in homogenates of the brushed aortic segments. This was used to determine trichloroacetic acid-precipitable $^{131}$I, which, in turn, was used to correct the aortic content of total $^{125}$I for that present in undegraded LDL. The TCA-soluble $^{131}$I activity per unit volume of aorta in all animals was approximately 20% to 35% of the TCA-soluble $^{131}$I activity per unit volume of plasma at the termination of the experiment, assuming that the density of aorta was 1.0 g/cc. It is probable that the majority of the TCA-soluble $^{131}$I in the aorta resulted not from local aortic catabolism of $^{131}$I-LDL but from equilibration of $^{131}$I-iodide and iodotyrosine between the plasma and the extracellular fluid of the aorta.

**Radioassay**

Radioassay of $^{125}$I and $^{131}$I were performed in a gamma scintillation counter using appropriate correction factors to separate the energy spectra of the two isotopes. All activities were corrected for physical decay of the two isotopes.

**Protein Determinations**

Protein determinations were performed by the method of Lowry et al. 35 using bovine serum albumin as standard. The protein content of the intima was determined on an alkaline digest of the cotton swabs. Swabs were incubated overnight in 0.15 N NaOH at 37°C, the samples were centrifuged, and residual cotton fibers and insoluble matrix proteins were removed by filtration before aliquots were taken for protein assay. The soluble protein content of whole aortic segments was determined from neutralized alkaline digests of their homogenates.

**Data Analysis**

Plasma decay data were analyzed as previously described. 28 All curves were adequately fit to biexponential functions.
The fractional rate of catabolism of LDL by a given tissue was determined as the product of two quantities: 1) the whole body LDL fractional catabolic rate, determined from the plasma decay curve; 2) the fraction of total body LDL catabolism attributable to the tissue of interest determined from the tissue's contents of $^{125}$I-TC label (corrected for $^{125}$I-TC present on undegraded LDL, as discussed above). At sufficiently long times after injection of the labeled protein, this fraction is represented by the tissue's content of degradation products divided by the total degradation products in the whole body.$^{27}$

**Autoradiography**

The aortas of two animals were prepared for light microscopic autoradiography. Separate preparations of $^{125}$I-TC-labeled LDL were injected intravenously ($5.3 \times 10^8$ and $1.6 \times 10^8$ cpm), and 24 hours later the animals were exsanguinated. The systemic circulation was perfused with buffered saline as outlined above. The aortas were fixed in modified Karnovsky's fixative containing 2% paraformaldehyde, 1.5% glutaraldehyde, 2.5 mM CaCl$_2$, 0.1 M sodium cacodylate buffer (pH 7.2). In one rabbit the aorta was fixed in situ by perfusion of fixative at 90 mm Hg pressure for 15 minutes. The aorta was dissected, opened longitudinally and pinned on a flat support before continuing fixation overnight at 4°C in the modified Karnovsky's solution. In the other rabbit the aorta was rapidly dissected, opened, and pinned flat before fixation overnight at 4°C. Both methods provided satisfactory fixation, but the latter method allowed easier dissection of the periadventitial tissue and resulted in flatter tissue specimens without any tendency to curl. The pinned aorta specimens were stored in 0.1 M Na cacodylate buffer (pH 7.2) at 4°C. Multiple tissue samples were embedded in Paraplast after dehydration in graded alcohols and clearing with xylene. Through-wall sections of 5 to 6 μm thickness were cut and mounted on albuminized slides. The mounted sections were then deparaffinized, hydrated to water, and then overstained with eosin and resulted in flat tissue specimens without any tendency to curl. The pinned aorta specimens were stored in modified Karnovsky's solution containing 2% paraformaldehyde, 1.5% glutaraldehyde, 2.5 mM CaCl$_2$, and 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C. Multiple tissue samples were embedded in Paraplast after dehydration in graded alcohols and clearing with xylene. Through-wall sections of 5 to 6 μm thickness were cut and mounted on albuminized slides. The mounted sections were then deparaffinized, hydrated to water, and then overstained with eosin and dipped in Kodak NTB-2 nuclear track emulsion. The emulsion-coated sections and appropriate controls were stored in sealed microslide boxes containing CaSO$_4$ and held at 20°C until development.

After 2 to 17 days of exposure, the coated slides were developed in Kodak D-76 developer for 2 minutes at 17°C and fixed in Kodak fixing solution. The sections were counterstained with hematoxylin after development of the photographic emulsion. Photomicrographs of numerous fields were obtained under bright field and dark field illumination and printed at a final magnification of 800X for grain counting.

The adequacy of the fixation of $^{125}$I-TC-labeled degradation products and intact LDL was assessed in preliminary experiments. Samples of aorta and other tissues were obtained from rabbits injected 24 hours previously with LDL labeled both with $^{125}$I-TC and with $^{131}$I by conventional iodination. In several independent tests, blocks of tissue were counted initially and after fixation, dehydration in alcohol (or tetrahydrofuran), clearing in xylene and paraffin infiltration. Fixative solutions, dehydrating agents, and paraffin used in the tests were also radioassayed. When tissues were fixed in modified Karnovsky's solution, there was less than a 5% loss of tissue $^{125}$I-TC activity throughout. As expected, only TCA-precipitable $^{131}$I activity (determined in homogenates of replicate samples) was retained during the fixation and subsequent processing. Other fixatives tested, including formalin, glutaraldehyde alone, Bouin's fixative, and a number of commercially available fixatives, failed to retain more than 60% of the $^{125}$I-TC activity during dehydration of the tissue.

**Results**

**Kinetics of $^{125}$I-TC-Labeled LDL Plasma Decay**

In most rabbits studied, the plasma decay of LDL labeled with both the trapped ligand ($^{125}$I-TC) and labeled conventionally with $^{131}$I was followed for 24 hours after injection. The representative pair of decay curves shown in Figure 2 demonstrate virtual identity of decay of the two labels; the fractional catabolic rates (FCR) did not differ significantly. The mean value of FCR in the present studies using $^{125}$I-TC-labeled LDL, 1.51 ± 0.31 d$^{-1}$ (0.063 ± 0.013 h$^{-1}$) (Table 1), is in good agreement with values from previous studies using $^{14}$C-sucrose-labeled LDL$^{26}$ and the results of others using conventionally iodinated LDL.$^{30}$ At the time of sacrifice, the amount of $^{125}$I-TC-labeled LDL remaining in the plasma compartment averaged 20.3 ± 3.4% of the injected dose (n = 6). The remainder of the activity was accounted for in tissues and excreta as both intact LDL and $^{125}$I-TC-labeled degradation products.

![Figure 2. Plasma decay curve of $^{125}$I-TC-LDL and conventionally iodinated $^{131}$I-LDL injected simultaneously into a rabbit. The biexponential curve was obtained by non-linear regression analysis of the composite data.](http://atvb.ahajournals.org/Downloaded from)
The whole body catabolism of $^{125}$I-TC LDL was calculated from the plasma decay curve using the kinetic model shown in Figure 3. One can determine the fraction of injected dose irreversibly degraded at the time the experiment terminates as follows:

$$\text{Fraction of injected dose irreversibly degraded} = \frac{b_2}{b_2 - b_1} \left(1 - e^{-b_1 t}\right) - \frac{b_1}{b_2 - b_1} \left(1 - e^{-b_2 t}\right)$$

where $b_1$ and $b_2$ are the slower and faster exponential decay rates determined from the plasma decay curve and $t$ is time. The applicability of this equation to our studies was tested in two experiments in which all tissues were sampled as previously described to obtain a direct measure of the catabolic products in the whole body. The fractions of injected dose recovered as degradation products at 24 hours were 0.615 and 0.706, respectively. The values predicted by the kinetic model were 0.681 and 0.669, respectively, both within about 10% of the experimentally determined values.

From the calculated fraction of initial dose irreversibly degraded at 24 hours and from the total $^{125}$I cleared from plasma, it was estimated that, on average, 69% of tissue $^{125}$I should represent degradation products of LDL and only 31% intact LDL. As will be seen later, these values are quite close to the experimental values in the whole aorta.

Degradation of LDL by Aorta and Aortic Intima In Vivo

Catabolism of low density lipoprotein attributable to the full thickness of the thoracic aorta and to the aortic intima were determined using doubly labeled LDL as described under Methods. Degradation rates for the intima were based on the activity recovered by gently removing the intimal cells with a cotton swab. The results are shown in Table 1. Degradation

### Table 1. Fractional Catabolic Rates of Low Density Lipoprotein

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Whole body</th>
<th>Thoracic aorta</th>
<th>Thoracic aortic intima</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction of plasma pool/day</td>
<td>% of plasma pool/g aortic wet weight/day $(\times 10^3)$</td>
<td>% of plasma pool/g aortic wet weight/day $(\times 10^3)$</td>
</tr>
<tr>
<td>1</td>
<td>2.6</td>
<td>14.5 ± 1.8</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>1.79</td>
<td>10.9 ± 2.1</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>1.27</td>
<td>10.2 ± 1.0</td>
<td>4.1 ± 1.6</td>
</tr>
<tr>
<td>4</td>
<td>1.20</td>
<td>9.4 ± 2.4</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>5</td>
<td>1.27</td>
<td>5.4 ± 1.9</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>1.44</td>
<td>5.9 ± 0.9</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>Mean</td>
<td>1.51 ± 0.31</td>
<td>9.4 ± 3.4</td>
<td>4.1 ± 2.1</td>
</tr>
</tbody>
</table>

Counting accuracy in 2 to 3 minutes was better than ±2%. Values are means ± SD for four segments of thoracic aorta. Only one sample of intima was obtained from Rabbits 1 and 2; values for Rabbits 3 to 6 are means ± SD for four samples of intima.

*Percentage of total aortic uptake attributable to intima.

†Mean protein content of intima: 1.98 mg/g wet weight; mean alkaline-soluble protein content of media/adventitia: 107 mg/g aortic wet weight.
rates are expressed as the percentage of the plasma LDL pool catabolized per unit wet weight per day \((\times 10^3)\). As shown in Figure 4, virtually all intimal cells were removed by this method. In numerous histological sections of swabbed aortas, there were only very rare indications that any medial cells (i.e., cells below the internal elastic lamina) had been removed. Occasionally, an isolated intimal smooth muscle cell could be seen adherent to the internal elastic lamina after swabbing the intimal surface.

Tissue degradation rates are expressed here as the percentage of the plasma LDL pool catabolized per unit weight per day. Expressed this way, the data are independent of absolute LDL concentration. Even though aortic degradation of LDL is low, it was possible to inject sufficient 125I-labeled LDL to permit accurate radioassay using very small tissue samples (50 mg to 100 mg wet weight). The fractional catabolic rates for aorta and its subfractions are shown in Table 1. Four samples of descending thoracic aorta were obtained from each animal. Neither analysis of variance with repeated measures nor nonparametric regression analysis revealed any significant trend of catabolic activity along the length of the descending aorta between the ductus scar and the diaphragm. Accordingly, the data for the four samples from each animal were averaged. The mean value for the fractional catabolic rate of LDL by aorta was \(9.4 \times 10^{-3} \pm 3.4 \times 10^{-3}\) percent of the plasma LDL pool/g aorta/day, which agrees reasonably well with our previous estimates of LDL degradation by aorta obtained using 14C-sucrose-LDL. The mean aortic FCR in that study was \(16 \times 10^{-3} \pm 24 \times 10^{-3}\) percent of the LDL plasma pool/g aorta/day. Considering the relatively low specific activity achievable with the 14C-sucrose-label and the consequent very low count rates obtained in tissues such as aorta, it was not surprising to find a considerable improvement in accuracy using the 125I-TCLigand.

As indicated in Table 1, the intima, which consists mostly of endothelial cells in these normal animals, accounted for fully 40% of the LDL degradation attributable to the whole thoracic aorta. As can be seen in Figure 5 by comparing intimal contents of degradation.
ed and intact $^{125}$I-TC-LDL, fully 87% of the total $^{125}$I activity in the intima was present as degradation products of $^{125}$I-TC-LDL (determined as discussed under Methods). Thus very little of the intimal radioactivity could be attributed to nonspecific adherence of intact labeled LDL to the luminal surface. In the media and adventitia, on the other hand, only 66.1% of the total $^{125}$I represented degradation products. For the artery as a whole, 72% of the $^{125}$I activity was present as degradation products, in reasonable agreement with the whole body average of 69% which would be predicted from the kinetic analysis described above.

The fractional catabolic rate for the intima when expressed per mg protein was $2.0 \times 10^{-3} \pm 1.17 \times 10^{-3}$ percent of the plasma LDL pool/mg protein/day. This was more than 40 times higher than the fractional catabolic rate per mg protein for the media plus adventitia. The value for media plus adventitia was $0.049 \times 10^{-3} \pm 0.013 \times 10^{-3}$ percent of the plasma LDL pool/mg protein/day. In several animals in which the fractional catabolic rate for liver was simultaneously determined, the hepatic rate was only about 4.5 times that in the aortic intima ($12.4 \times 10^{-3} \pm 2.3 \times 10^{-3}$ percent of the plasma LDL pool/mg protein/day), in good agreement with previous results. Next to the adrenal gland, the liver is the most active organ in LDL degradation on a unit weight basis. The finding that the aortic intima degrades LDL at a rate about 22% of that of liver places it among the tissues most active in LDL degradation.

**Comparison of Intimal Flux of LDL and Intimal LDL Catabolism**

The flux of LDL across the endothelial barrier of arteries is presumed to occur primarily via vesicular transport. Irreversible uptake and degradation of LDL may or may not be related to this process. We sought to compare the amount of LDL irreversibly degraded by endothelial cells to the flux of LDL across the endothelium. Transendothelial flux of LDL was approximated in three rabbits using conventionally iodinated LDL as tracer. The animals were killed 1 hour after injection of $^{125}$I-LDL, the systemic circulation was thoroughly perfused with buffer, and the adventitia was carefully removed before assaying the aortic segments for $^{125}$I precipitated by trichloroacetic acid.

It was assumed that the protein-bound $^{125}$I in the aortic wall after 1 hour represented LDL transport via

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**Figure 6.** Distribution of autoradiographic grains across the aortic wall 24 hours after injection of $^{125}$I-TC-LDL. A. Dark field micrograph of autoradiogram focused on the silver grains. $\times 360$. B. Bright field view of the same field as A. Scale bar = 50 $\mu$m. Paraffin section; hematoxylin and eosin stain.
the endothelium, and that the time of sampling was sufficiently short that the arterial activity was an adequate measure of unidirectional flux into the wall. These assumptions are supported by previous studies of others and by studies in this laboratory (in collaboration with Dr. Olav Wiklund) showing that uptake at 1 hour is approximately twice that at 30 minutes. The flux was calculated in terms of the volume of plasma "cleared" by the aorta in 1 hour (TCA-precipitable 125I-LDL in the aorta at 1 hour per cm² surface area of aorta divided by the time-averaged concentration of plasma 125I-LDL). Aortic surface area was determined by direct measurement of the opened aorta. The flux of LDL across the endothelium, was 70.2 ± 29.0 nI plasma equivalent/cm² aortic surface area/hour. Expressed in the same units, the intimal LDL degradation rate (from Table 1) averaged 8.1 nI/cm²/hour, while the degradation rate in media-adventitia was equivalent to 12.0 nI/cm²/hour. Thus, only about 10% of the LDL transported into the wall per unit time was degraded irreversibly by cells in the intima, presumably mostly by the endothelial cells themselves. The LDL degradation rate in the media-adventitia could account for about 15% of the LDL entering the arterial wall over any given time. Because there is no evidence to suggest that LDL accumulates at any significant rate in the normal rabbit aorta, these data imply that about 70% to 75% of the LDL flux entering the aorta must be bidirectional for the steady state to be maintained.

**Autoradiographic Studies**

The finding that intimal cells degrade LDL at a substantial rate relative to the rest of the aortic wall was confirmed using autoradiographic techniques. In these studies rabbits were injected with singly labeled 125I-TC-LDL (2.7 to 5.2 x 10⁹ cpn; specific activity, 400 to 861 cpm/ng LDL protein). The animals were killed 24 hours later, and the aortas were removed and fixed as described under Methods. As shown in Figure 6 A, the distribution of silver grains in the autoradiogram when viewed by dark field illumination clearly indicates a predominant localization over the extremely thin intima of the normal rabbit aorta. For orientation the bright field view of the same section is shown in Figure 6 B. The distribution of activity through the aortic wall was determined on representative sections by direct grain counting of photomicrographs.

The activity profile after correction for background is illustrated in Figure 7. The profile demonstrates a marked concentration of grains at the intima relative to the media and adventitia. The distribution over the latter areas was essentially flat and there was no evidence of any increase in LDL degradation associated with the vasa vasorum located in the adventitia. Approximately 40% of the area under the activity profile coincided with the intima. These results are in excellent agreement with the biochemical results presented in Table 1.

The 125I activity demonstrated in the autoradiograms includes both degradation products and intact LDL. However, the tissue samples were obtained at a time following injection when the radioactivity in the aorta, and especially in the intima, had been shown by direct analysis to represent predominantly degradation products. In the studies described above using doubly labeled LDL, an average of 87% of the intimal activity and 66% of the activity in the media-adventitia represented degradation products. (In the count profile shown in Figure 7, no correction was made for undegraded LDL.)

**Estimation of Receptor-Mediated Degradation of LDL in Aortic Intima**

Reductive methylation of LDL blocks its recognition by the LDL receptor on cell membranes. In principle, then, the contribution of the apo B/E receptor to LDL degradation can be determined by subtracting the degradation of reductively methylated LDL (representing only LDL receptor-independent processes) from that of native LDL (representing the sum of receptor-dependent and receptor-independent processes). Figure 8 shows results from the simultaneous injection of 131I-TC-LDL and 125I-TC-MeLDL into three rabbits. Data were calculated using the total intimal contents of 131I and 125I. In this experiment one animal was killed 24 hours after injection and two were killed 48 hours after injection. The 48-hour studies were carried out to increase the fraction of methylated LDL degraded and thus to decrease further the potential source of error dis-
Figure 7. Average distribution of grains across the aorta. Aortic wall thickness was normalized. The internal elastic lamina is at 0 on the thickness scale.

Discuss in the footnote. A representative pair of plasma decay curves is shown in Figure 8 A; in all cases the decay of methylated LDL was considerably slower than that of the unmodified LDL. The calculated plasma fractional catabolic rates obtained from the plasma decay curves are shown in Figure 8 B. The apparent LDL receptor-mediated degradation in the whole rabbit was 60 ± 11% of total LDL catabolism, which agrees reasonably well with previous estimates from this laboratory and others.

Results for individual tissues may be considered in an analogous manner. The activities of methylated 125I-TC-LDL and unmodified 131I-TC-LDL in a given tissue, when expressed as fractional rates of catabolism as described under Methods, provide a measure of receptor-independent and total LDL degradation rates, respectively, in that tissue (Figure 8 C). The bars represent the mean results for four segments of descending thoracic aorta examined in each animal. The fraction of receptor-mediated LDL degradation was relatively constant among segments; in the animal killed 24 hours after injection, 24 ± 8.3% of LDL degradation was calculated to be receptor-mediated. In the animals killed at 48 hours, presumably providing an improved estimate of MeLDL degradation, the fraction of total uptake attributable to receptor mediation was somewhat greater, as predicted. In these two cases 47.6 ± 5.0% and 48.3 ± 4.1% of intimal LDL degradation was calculated to be receptor-mediated. Thus, while there is a significant receptor contribution to intimal LDL degradation, it is somewhat less than the average percentage of receptor-mediated degradation for the whole animal.

Figure 8. Comparison of the whole body and aortic intimal catabolism of 131I-TC-LDL and 125I-TC-methylated LDL (MeLDL). A. Plasma decay kinetics of the two LDL preparations. B. Fractional catabolic rates calculated from plasma decay curves. Difference in FCRs represents a measure of LDL receptor-mediated degradation in the whole animal. Open bars = control LDL. Hatched bars = methylated LDL. C. Calculated intimal degradation rates for unmodified and methylated LDL either 24 hours (Rabbit 1) or 48 hours (Rabbits 2 and 3) after injection of labeled LDL preparations. Bars represent means and standard deviations of results from four segments from each aorta.
Discussion

Accumulation of cholesterol in the artery wall can occur: 1) by deposition of lipoproteins in the extracellular space without prior cellular metabolism; 2) by cellular uptake followed by release of cholesterol to the extracellular space (by secretion or as a result of cell death); or 3) by uptake and degradation of lipoproteins with consequent accumulation intracellularly. Studies of the fatty acid composition of cholesterol esters in the artery wall show a predominance of cholesterol oleate in some lesions, particularly in early lesions where cholesterol ester accumulation is predominantly intracellular. Because plasma cholesterol esters contain more linoleate than oleate, these oleate-enriched esters are probably generated during cellular metabolism. It has been clearly shown that when cells take up lipoprotein cholesterol and store it as esters, they use oleic acid preferentially for such esterification. Thus, the uptake and degradation of lipoproteins by cells appears to be critically important in the genesis of cholesterol ester-rich lesions.

Many investigators have attempted to quantify the entrance of lipoproteins or their components into the artery wall. Most studies have dealt exclusively with the cholesterol moiety. Measurements of rates of lipoprotein penetration have generally involved use of conventionally iodinated lipoproteins. As discussed in the introduction, these studies do not measure LDL degradation. Initial rates of entry can be measured and there is a reasonable amount of information about such rates. However, only some fraction of the protein entering the tissue goes on to be taken up by cells and degraded. Furthermore, that fraction may differ greatly from tissue to tissue.

The present studies are perhaps the first to provide a direct measure of lipoprotein degradation by the artery wall under physiologic conditions in vivo. The most striking finding was the dominant role of the intima in irreversible degradation of LDL in the normal rabbits. The calculated degradation rate per tissue protein in the intimal layer was fully 40 times that for the media and adventitia. This dominant role of the intimal layer was documented both by autoradiography and by direct assay of the stripped intima. This finding can reflect an intrinsically greater activity of intimal cells in LDL degradation or it could simply reflect the fact that the cells on the endothelial surface are exposed to full plasma concentrations of LDL while the medial cells are exposed to a lower concentration (or some combination of these two possibilities).

If we assume that the LDL degraded by the artery is taken up as intact LDL particles, we calculate a delivery of LDL cholesterol to the artery of about 2.9 μg per gram of wet weight tissue per day (or 0.1 μg cholesterol per square centimeter of aortic surface per day). If there were continuing net delivery of cholesterol to the artery at such a rate without concomitant removal of cholesterol by some mechanism, the levels of cholesterol in the artery would build up to enormous values in a relatively short time. Clearly there must be, as in the case of other tissues, some mechanism for reverse cholesterol transport.

It is possible that the high rate of LDL catabolism by endothelium is directly related to the atherogenic process. Under some conditions LDL is toxic to endothelial cells in culture. This injury can be prevented by HDL, implying that the injury may be due to accumulation of LDL cholesterol. Another way in which cholesterol loading of endothelial cells may be atherogenic is by delivery of excess sterol, not back into the plasma but into the subendothelial space. It is generally accepted that endothelial cells of the capillaries provide a barrier against the movement of larger molecules from vascular to extravascular space. The endothelial lining of major arteries is assumed to play a similar role. However, the concentration of LDL in the subendothelial space has been found to be very high. For example, in the human aorta, Smith and coworkers actually found a higher concentration there than in the plasma. They suggested that the endothelium provides a barrier to the outward movement of LDL.

Endothelial cells in culture have been shown to express the high-affinity LDL receptor. However, it has been suggested that highly confluent endothelial cells do not express significant numbers of such receptors. From the present studies, we conclude that about 40% of the LDL degradation occurring in the intimal layer in vivo occurs by way of the high-affinity, specific LDL receptor. This is the first demonstration that these cells use the receptor in vivo, at least for that portion of uptake that is associated with lipoprotein degradation. It does not necessarily follow that the transendothelial transport of LDL also occurs by way of the high-affinity receptor. Further studies will be needed to address that question. However, the present study of the normal aorta in vivo provides evidence that normal confluent endothelium does express LDL receptor activity.

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