Time Course of $^{125}$I-Labeled LDL Accumulation in the Healing, Balloon-Deendothelialized Rabbit Aorta

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We previously showed by qualitative en face autoradiography that after 24 hours of circulation, $^{125}$I-labeled low density lipoprotein (LDL) injected in tracer amounts accumulated focally at the edges of regenerating endothelial islands in the balloon catheter–deendothelialized aorta of the normocholesterolemic rabbit. In the present study with the same animal model, we have used quantitative autoradiography to examine $^{125}$I-LDL accumulation in the healing aorta as a function of LDL circulation time from 2.5 to 40 hours. The results demonstrated that $^{125}$I-LDL accumulation in the healing aorta occurred in two kinetically and biochemically distinct compartments, one of which was in equilibrium with plasma and one of which sequestered LDL. LDL accumulation in the still-deendothelialized aorta (DEA) was diffuse and only moderately intense on autoradiography. It peaked 4 hours after injection; over the following 36 hours the disappearance of $^{125}$I-LDL from DEA paralleled the disappearance of $^{125}$I-LDL from plasma. In contrast, accumulation of $^{125}$I-LDL at the edges of regenerating endothelial islands was focal and intense. LDL accumulation in this compartment also peaked 4 hours after injection but remained elevated even at 40 hours, despite falling plasma levels of LDL. At 24 hours, edge LDL accumulation per unit area was more than five times greater than DEA accumulation. The data indicate that LDL accumulation in specific compartments of the functionally modified arterial wall occurs independently of either acute or chronic hypercholesterolemia. The contrast between labile LDL accumulation in DEA and persistent accumulation at the edges of healing aortic islands indicates that LDL accumulation in the two areas must involve different processes within the arterial wall itself. (Arteriosclerosis and Thrombosis 1992;12:1088–1098)

**KEY WORDS** • low density lipoprotein accumulation • balloon-deendothelialized rabbit aorta • arteriosclerosis

Low density lipoproteins (LDLs), the major carriers of plasma cholesterol, have been implicated as a major factor in atherogenesis. However, the mechanisms of LDL interaction with the arterial wall that lead to the pathological accumulation of LDL cholesterol have not been fully elucidated.

Balloon catheter deendothelialization of the aorta in normocholesterolemic and hyperlipidemic rabbits has been widely used as an in vivo model for the early stages of human atherosclerosis. Endothelial removal is followed by gradual endothelial regeneration (healing) occurring outwardly in a ringlike formation from the ostia of aortic branches, which are spared from the balloon catheter injury. Intimal thickening occurs under the edges of regenerating endothelial islands and, to a lesser extent, in still-deendothelialized areas; the centers of healing islands have little or no intimal thickening. The microscopic changes of intimal thickening have been described in detail and consist of five to 20 layers of smooth muscle cell proliferation with extensive collagen and elastic fiber extracellular matrix production. Healing regions are covered by proliferating endothelial cells, whereas unhealed regions are covered only by smooth muscle cells. In the normocholesterolemic rabbit, which is the model we have studied, no evidence of fatty change or monocyte/macrophage infiltration is observed. This is in contrast to the cholesterol-fed and Watanabe heritable hyperlipidemic (WHHL) rabbit models for atherosclerosis, in which aortic lipid-laden foam cells are prevalent. Nevertheless, although gross lipid accumulation is not evident in the healing aorta of the normocholesterolemic rabbit, there is almost three times more cholesterol ester in areas of regenerating endothelium than in still-deendothelialized areas.

In the normolipidemic rabbit, we have shown previously by using Evans blue dye staining and autoradiography that after 24 hours of circulation, $^{125}$I-labeled LDL accumulation had three levels of intensity in the healing balloon aorta; most striking was the intense, focal accumulation at the healing edges of regenerating endothelial islands. In addition, there was less intense, uniformly diffuse accumulation in the still-deendothelialized regions of the aorta. Minimal accumulation was
present in the centers of endothelium-covered islands. The three-level pattern of accumulation was selective for LDL; radioiodinated human serum albumin and high density lipoprotein did not accumulate in the healing aorta. Our finding that the most intense accumulation of LDL occurred at the edges of healing islands was consistent with the observation of Falcone et al. that the highest concentration of cholesterol ester in the healing aorta occurred in the same location.

The hypothesis that discrete domains of the LDL protein moiety, apolipoprotein (apo) B, are responsible for specific functions of the lipoprotein has been explored previously. To see if defined domains of apo B were important for LDL accumulation in the injured arterial wall, we synthesized short peptides, with sequences corresponding to specific regions of apo B, and examined their ability to accumulate in the healing rabbit aorta. (An amino-terminal tyrosine was added to all of the peptides to facilitate 125I-labeling.) The synthetic peptide SP-4 (tyrosyl-apo B residues 1,000-1,016) accumulated focally at the healing edges of regenerating endothelial islands but not in still-deendothelialized regions. This was in contrast to the behavior of two other synthetic peptides, SP-11 (tyrosyl-apo B residues 3,352-3,375) and SP-2 (tyrosyl-apo E residues 129-148). SP-11 represented a heparin-binding domain of apo B, and SP-2 had considerable homology to another heparin-binding domain of apo B (residues 3,359-3,367). SP-2 and SP-11 accumulated in a uniformly diffuse manner throughout the still-deendothelialized regions of the aorta. The clearly differing patterns of accumulation of peptides derived from different domains of apo B implied that differing molecular sites in the arterial wall were involved in interactions with specific domains of LDL.

To specify further the nature of the heterogeneous interactions of LDL with the injured arterial wall, the distribution of tracer doses of 125I-LDL in the healing aorta of the normocholesterolemic rabbit was studied as a function of time of exposure to circulating radiolabeled LDL. Animals were killed, each rabbit was injected intravenously with 6 ml of a 0.5% Evans blue dye solution, and plasma radioactivity was measured. To determine plasma disappearance kinetics, serial blood samples were obtained from the central artery of the ear, and plasma radioactivity that was precipitable by 10% TCA was measured. This information was also used to establish the integrity of the 125I-LDL in the circulation. Fifteen minutes before the animals were killed, each rabbit was injected intravenously with 6 ml of a 0.5% Evans blue dye solution, which stained areas of deendothelialized aorta blue. Immediately after each rabbit was killed, the aorta was removed and rinsed thoroughly with saline to remove any free radioactive iodide and radioactive blood. The adventitia was gently stripped away and the aorta opened along the ventral surface. The specimen was then pinned out, fixed with 10% TCA for several hours, opened along the ventral surface, and the specimen was examined. (An amino-terminal tyrosine was added to all of the peptides to facilitate 125I-labeling.)

Methods

Preparation of Lipoproteins

Human LDL was isolated from the blood of fasting normolipemic volunteers by sequential flotation in the ultracentrifuge between densities of 1.025 and 1.050 g/mL, according to the method of Hatch and Lees. After dialysis against 0.2 M NaCl and 1.0 mM Na2 EDTA (pH 9), the LDL was passed through a 0.22-μm filter and stored under nitrogen. LDL was quantified by its protein content, which was determined by the method of Lowry et al. as corrected according to Margolis and Langdon.

Lipoprotein purity was confirmed by paper electrophoresis and Ouchterlony double immunodiffusion against the following rabbit anti-human antisera: anti-human whole serum, anti-human LDL, anti-human very low density lipoprotein, anti-human high density lipo-

Quantification of Lipid Peroxidation

Oxidation of LDL lipids was estimated by assaying for thiobarbituric acid reactive substances (TBARS). Briefly, 60 μg LDL in 1.0 ml was precipitated with 2.0 ml of a solution containing 15% (wt/vol) trichloroacetic acid (TCA), 0.375% (wt/vol) thiobarbituric acid, and 0.25N HCl. The mixture was heated for 15 minutes in a boiling water bath and then cooled, and the flocculent precipitate was removed by centrifugation. Malondialdehyde standards were obtained by rapid acid hydrolysis of 1,1,3,3-tetramethoxypropane with concentrated hydrochloric acid. Spectroscopic measurements were made at 535 nm, and the degree of lipoprotein oxidation was expressed in nanomoles of malondialdehyde equivalent per milligram of LDL cholesterol. TBARS values for native LDL were typically 0–0.7 nmol/mg, indicating that no lipid peroxidation had occurred.

Radiolabeling of LDL

LDL was radioiodinated with Na[125I] (17.4 Ci/mg in 0.1N NaOH) by a modification of the iodine monochloride technique. Specific activities were 200–500 cpm/ng protein. Greater than 97% of the radiolabel was associated with LDL protein, as determined by 10% TCA precipitability; <5% of the radiolabel was associated with lipid as measured by extraction with chloroform/methanol (2:1, vol/vol).

Animal Procedures

The abdominal aortas of normocholesterolemic male New Zealand White rabbits (2–3 kg) were balloon-catheter deendothelialized by the Baumgartner technique by using a 4F Fogarty embolectomy catheter. Animals were maintained on a diet of normal rabbit chow (Prolab, Agway Laboratory Feeds, Ithaca, N.Y.). All animal procedures conformed to state and federal laws and to guidelines set by the Deaconess Hospital Animal Care and Use Committee.

Four weeks after the aortas were deendothelialized by balloononing, 125I-LDL (1–3 mg) was injected into the marginal ear vein and allowed to circulate for varying times up to 40 hours. Injected doses ranged from 95 to 450 μCi. To determine plasma disappearance kinetics, serial blood samples were obtained from the central artery of the ear, and plasma radioactivity that was precipitable by 10% TCA was measured. This information was also used to establish the integrity of the 125I-LDL in the circulation. Fifteen minutes before the animals were killed, each rabbit was injected intravenously with 6 ml of a 0.5% Evans blue dye solution, which stained areas of deendothelialized aorta blue. Immediately after each rabbit was killed, the aorta was removed and rinsed thoroughly with saline to remove any free radioactive iodide and radioactive blood. The adventitia was gently stripped away and the aorta opened along the ventral surface. The specimen was then pinned out, fixed with 10% TCA for several hours or overnight, and photographed. (TCA precipitation also allowed non–protein-bound radioactivity to diffuse out of the aortic tissue.) Total 125I radioactivity (in disintegrations per minute) in the abdominal (R\textsubscript{a}\textsubscript{A}) and thoracic (R\textsubscript{a}\textsubscript{T}) aortas was determined by counting the
whole tissue in a Packard Auto-Gamma 5650 counter. All surgical procedures were carried out under aseptic conditions.

**En Face Autoradiography**

The distribution of radioactivity in the balloon-deendothelialized aorta was examined by macroscopic autoradiography. The TCA-fixed opened vessels were washed with saline, and excess moisture was removed. The vessels were covered with one layer of plastic wrap, placed on Kodak X-OMAT AR high-speed x-ray film, and stored in a Spectronics stainless steel cassette at -70°C before development in a Kodak X-OMAT processor. Exposure times ranged from 3 to 13 days and were adjusted for the total 125I radioactivity in the aorta.

**Image Analysis of Photographs**

**Extent of healing.** Photographs of the Evans blue-stained vessels were digitized by using BarneyScan (BarneyScan Corp., Alameda, Calif.) to facilitate measurement. The surface areas of the whole abdominal aorta (AA) and the whole (uninjured) thoracic aorta (TA) were determined as units of area by computer image processing of digitized photographs by using PHOTOSHOP (Adobe, Berkeley, Calif.) and ULTIMAGE (Grafttek, Meudon-la-Foret, France) software. Within the abdominal aorta, the total surface of reendothelialized areas (REAs) was also determined as arbitrary units of area. The surface of still-deendothelialized areas (DEAs) was calculated by subtraction. The extent of healing was expressed as a percentage of the surface area of the whole abdominal aorta ([REAA/AA]x100).

The perimeters (edges) of healing islands were also measured on the photographs. Evans blue staining could not differentiate the width of regenerating edges, nor could the width of the edges be determined from autoradiographs because of radioactive scatter. Because we knew from the work of Minick et al that the regenerating edge in the normcholesterolemic rabbit was narrow, we approximated the area by assigning to the edge a width of 1 unit (pixel) and multiplying its perimeter by that width. For a circle with an edge of 1 unit, the conversion of perimeter to area would give a constant overestimate of pi for the area of a healing ring. Because the mean perimeter of the rings was 293 ± 275 units, this error was < 1%.

**Mean plasma radioactivity.** To calculate the clearance of 125I-LDL by the aorta, the average exposure of the aorta to plasma radioactivity was estimated. The mean plasma radioactivity (MPR) expressed as disintegrations per minute per microliter of plasma was a measure of the average specific activity of the plasma and was determined as

\[
\text{MPR} = \frac{[\text{AUC}(\text{ID})]}{(V)(t)}
\]

where AUC \[ \Sigma \] (percent injected dose remaining) \[(t)\] was the total area under the plasma disappearance curve determined according to the trapezoidal rule, and ID was the injected dose in disintegrations per minute. Thus, the quantity \[ [\text{AUC}(\text{ID})] \] was a measure of the amount of radioactivity in the plasma over the entire experimental period. V was the plasma volume (in microliters), and t was the time period (hours) of an experiment.

The average specific activity in the plasma ranged from 683 to 5,393 dpm/µL. The wide variability primarily reflected differences in injected dose and time of circulation.

**Aortic clearance of 125I-LDL.** Aortic accumulation was expressed as clearance of 125I-LDL from the plasma by the aorta. Clearance (CL) represents the plasma volume equivalent of 125I-LDL that was accumulated in a unit area of arterial wall (microliters per unit area) and was determined by normalization of 125I-LDL uptake per unit area of abdominal or thoracic aorta for mean plasma radioactivity as

\[
\text{CL}_{\text{AA or TA}} = \frac{R_{\text{AA or TA}}/\text{unit area}_{\text{AA or TA}}}{\text{MPR}}
\]

Because the aortic clearance of 125I-LDL was a function of the independent parameters of injected dose, plasma volume, and circulation time as well as differences in plasma removal kinetics, normalization was necessary to compare the clearance in different animals.

**Image Analysis of Autoradiographs**

**125I-LDL accumulation at edges of regenerating endothelium and in still-deendothelialized regions.** Autoradiographs were also digitized as described above for Evans blue-stained aortas for determination of radioactivity associated with regenerating endothelium at the edges of healing islands versus that associated with still-deendothelialized regions. 125I activity was concentrated at the edges of healing islands with essentially no radioactivity detected in the centers of the islands, as discussed in "Results." One assumption was required for image analysis. The intensity, I (grays on a scale of 0–255, where 0 represented white and 255 represented black) of silver grain development in the regions of interest could not be saturated and the intensity within a given region had to be uniform. These criteria were met. The high sensitivity of image analysis enabled precise intensity measurements, which showed that no region of interest used for analysis was saturated. Uniformity within a region of interest was demonstrated by histograms of gray-scale intensity within the region; the standard deviation of intensity was < 15% of the mean intensity. Thus, autoradiographic intensity at edges (I_{edge}) and still-deendothelialized areas (I_{DEA}) was a valid relative measure of 125I-LDL accumulation in the two regions, and intensities could be related to total radioactivity (disintegrations per minute) to allow calculation of radioactivity per unit area in each region as described below.

The intensity of radiolabel accumulation in the edge of each healing island of each aorta was evaluated by densitometric analysis of the digitized autoradiograph. To do this, a representative region of interest was drawn at the edges of each healing island with essentially no background radioactivity. The criteria for selection of regions of interest included uniform intensity within a region and the same area for all regions (measured in pixels). Thus, measurement of intensity was independent of differences in the width of edges. Edges were classified into
Mean accumulation was determined by averaging all five $A_{\text{edge}}$ or $A_{\text{DEA}}$ values obtained for each aorta. This term represents the plasma volume equivalent of $^{125}\text{I}$-LDL that was accumulated in the entire edge or still-deendothelialized region and therefore is not the same as the clearance term, which represents uptake per unit area of region as defined above. Total $^{125}\text{I}$-activity in the individual compartments of the abdominal aorta was not converted into clearance terms because of the limitation involved in measurement of the area of edges, as discussed above.

**Statistical Analysis**

Average values are presented as mean±SD. Significance was examined by the $t$ test for comparison of pairs of groups in a one-way analysis of variance.

**Results**

**Pattern and Quantification of Endothelial Regeneration**

The pattern of endothelial regeneration 4 weeks after balloon catheter injury of the abdominal aorta is shown in the photographs (left) in Figures 1–4. In vivo Evans blue staining of the aortas clearly differentiated unhealed areas from healed areas. Still-deendothelialized regions of the abdominal aorta were stained with Evans blue dye, whereas reendothelialized islands and the thoracic aorta remained white.

The areas of all reendothelialized islands were summed and divided by total abdominal luminal surface area to measure the extent of healing by image digitization. Four weeks after injury, the mean±SD ($n=13$) extent of healing was 37±9%. The healing edge constituted 2% or less of the healed areas (see "Methods").

**Patterns of Accumulation of $^{125}\text{I}$-LDL**

$^{125}\text{I}$-LDL was allowed to circulate for 2.5, 4, 10, 19, 24, or 40 hours before examination of the aortas of rabbits ballooned 4 weeks earlier.

An autoradiograph of an aorta removed 2.5 hours after injection of $^{125}\text{I}$-LDL is shown in Figure 1 (right) together with the corresponding photograph (left) of the Evans blue-stained aorta. The still-deendothelialized (bare) areas of the abdominal aorta showed a moderate level of uniformly diffuse radioactivity that was clearly greater than the minimal level of radioactivity in the uninjured thoracic aorta. No evidence of focal accumulation at the edges of regenerating endothelialized islands was observed at this early time.

After 4 hours of circulation (Figure 2), focal accumulation at the regenerating edges of endothelial islands began to appear, in addition to the moderate levels of uniformly diffuse radioactivity still present in bare regions.

Autoradiographs of the aortas of rabbits in which $^{125}\text{I}$-LDL was allowed to circulate for 10 hours (Figure 3) revealed even greater intensification of the regenerating edges of endothelialized islands than was observed after 4 hours. Moderate diffuse accumulation was still present in bare regions.

At 19 and 24 hours (Figure 4), accumulation of $^{125}\text{I}$-LDL was intense at the regenerating edges of endothelialized islands, with a low level of diffuse radioactivity in still-deendothelialized regions.
FIGURE 1. Correlation between accumulation of $^{125}$I-low density lipoprotein (LDL) after 2.5 hours of circulation and regenerating endothelium in the balloon catheter-deendothelialized rabbit aorta. $^{125}$I-LDL (95.5 μCi) was injected 4 weeks after deendothelialization and allowed to circulate for 2.5 hours. Evans blue dye injected 15 minutes before the aorta was removed stained the still-deendothelialized areas of the abdominal aorta blue while reendothelialized islands and uninjured thoracic regions remained white (left, photograph). An autoradiograph (right) of the same aorta shows a moderate level of uniformly distributed radioactivity in the still-deendothelialized (stained) areas of the abdominal aorta after 13 days of exposure at $-70^\circ$C. Actual size.

After 40 hours (not shown), the pattern of accumulation was similar to that at 19 and 24 hours.

At all times, radioactivity associated with islands of regenerating endothelium was concentrated at the healing edges of the islands, with minimal radioactivity in the centers.

FIGURE 2. Aortic accumulation of $^{125}$I-low density lipoprotein (LDL) after 4 hours of circulation in the balloon-injured rabbit. $^{125}$I-LDL (111.4 μCi) was injected 4 weeks after deendothelialization and allowed to circulate for 4 hours. A photograph of the Evans blue-stained aorta is shown on the left. The corresponding autoradiograph (right) shows a moderate level of uniformly distributed radioactivity in the still-deendothelialized (stained) areas of the abdominal aorta with initial intensification of the regenerating edges of endothelial islands also shown after 13 days of exposure at $-70^\circ$C. Actual size.
FIGURE 3. Aortic accumulation of $^{125}$I-low density lipoprotein (LDL) after 10 hours of circulation in the balloon-injured rabbit. $^{125}$I-LDL (462.7 μCi) was injected 4 weeks after deendothelialization and allowed to circulate for 10 hours. A photograph of the Evans blue-stained aorta is shown on the left. The corresponding autoradiograph (right) shows intense focal accumulation of radioactivity at the regenerating edges of endothelial islands. Minimal accumulation was observed in deendothelialized regions after 4 days of exposure at −70°C. Actual size.

In general, there was greater accumulation of radioactivity at the healing edges of lesions in the lower abdominal than in the upper abdominal aorta; there was little or no focal accumulation of radiolabeled lipoproteins observed at the boundary of regenerating endothelium between the ballooned abdominal and the unballooned thoracic aorta. The reason for this is unknown but may be related in some way to the documented greater development of atherosclerosis in

FIGURE 4. Aortic accumulation of $^{125}$I-low density lipoprotein (LDL) after 24 hours of circulation in the balloon-injured rabbit. $^{125}$I-LDL (390.5 μCi) was injected 4 weeks after deendothelialization and allowed to circulate for 24 hours. A photograph of the Evans blue-stained aorta is shown on the left. The corresponding autoradiograph (right) shows intense focal accumulation of radioactivity at the regenerating edges of endothelial islands. Minimal accumulation was observed in deendothelialized regions after 10 days of exposure at −70°C. Actual size.
the lower two thirds of the abdominal aorta in humans compared with the upper third of the abdominal aorta.31

Plasma Disappearance of 125I-LDL

Plasma disappearance of 125I-LDL is shown in Figure 5 as the percentage of initial dose remaining (\( [C_p/C_0] \times 100 \)) versus time after injection, where \( C_0 \) was the plasma radioactivity at any time, and \( C_p \) was the initial dose determined by extrapolation to zero time of early plasma radioactivity samples. Data from all rabbits (\( n = 13 \)) were combined to determine the biexponential equation of disappearance;24,32 the curve shown represents the best-fit curve derived by least-squares approximation.

The fractional catabolic rate (FCR) of 125I-LDL as determined from the biexponential equation was 0.12 pool/hr, in close agreement with previously reported values.24-33

The integrity of the radiolabeled LDL in the circulation was established by monitoring the plasma samples for TCA-precipitable radioactivity. Plasma radioactivity was 97.3±2.8% (mean±SD) protein-bound over the experimental period of up to 40 hours.

125I-LDL Aortic Clearance

Clearance of 125I-LDL by the whole thoracic and the whole abdominal aorta was not related to circulation time as shown in Figure 5. The mean clearance by the healing abdominal aorta was 6.1×10^{-4}±1.9×10^{-4} \mu l/unit area. This was approximately 10 times greater than the mean clearance by the uninjured thoracic aorta (0.6×10^{-4}±0.2×10^{-4} \mu l/unit area). The significant difference (\( p < 0.05 \)) in CLA versus CLT values reflected the altered physiological state of the abdominal aorta after balloon-catheter injury rather than simply a normal difference in metabolism between the abdominal and thoracic aortas, since Roberts et al.12 have shown that in nonballooned control rabbits, the accumulation of 125I-LDL in the abdominal aorta was the same as that in the thoracic aorta.

125I-LDL Accumulation at Edges of Regenerating Endothelium and in Still-Deendothelialized Regions

Measurement of 125I-LDL accumulation indicated the existence of two distinct compartments (still-deendothelialized regions and edges of reendothelialized regions) within the healing abdominal aorta that accumulated LDL. Time-related changes for 125I-LDL accumulation in these compartments showed that the still-deendothelialized area was in equilibrium with plasma while the edges of the reendothelialized regions were not. Because the ratio of intensity of radioactivity (Ratio) at edges (\( I_{edge} \)) to that in the still-deendothelialized areas (\( I_{area} \)) within an aorta was directly related to the absolute amount of radioactivity (disintegrations per minute) in each of these compartments, the 125I activity per unit area in edges (\( R_{edge} \)) and in the still-deendothelialized areas (\( R_{area} \)) was calculated from the measured radioactivity (disintegrations per minute) in the whole abdominal aorta. \( R_{edge} \) and \( R_{area} \) values then were normalized for mean plasma radioactivity to determine the accumulation per unit area of 125I-LDL in each of these compartments (\( AC_{edge} \) and \( AC_{area} \)). These results are shown in Figure 6. Maximal accumulation of 125I-LDL in still-deendothelialized regions occurred early, for a peak value of 14.77±2.95 \mu l at 4 hours, and declined steadily over the subsequent 36 hours to 5.04±0.13 \mu l at 40 hours. Still-deendothelialized area accumulation at 40 hours was approximately 66% lower than the peak value at 4 hours; plasma radioactivity decreased approximately 96% over the same time period. These decreases were in contrast to the time course of 125I-LDL accumulation in the edge compartment. Although no 125I-activity at edges was detected by 2.5 hours, \( AC_{edge} \) was 22.85±3.62 \mu l by 4 hours and remained constant from 4 hours up to 40 hours despite...
We have shown that 125I-LDL accumulation in the healing, balloon catheter–deendothelialized rabbit aorta occurred in two separate compartments, one of which was in equilibrium with plasma and one of which sequestered LDL. The larger compartment was the still-deendothelialized region, which covered an average of 63% of the abdominal aorta 4 weeks after ballooning. Still-deendothelialized area accumulation was diffuse and peaked 4 hours after injection of radiolabel. From 4 hours onward, still-deendothelialized region accumulation decreased in parallel with the disappearance of 125I-LDL from plasma. The smaller compartment was at the edges of reendothelialized islands where endothelium was actively regenerating. The edges occupied <2% of the average 37% of the abdominal aorta that had healed by 4 weeks after ballooning; thus, the edges occupied <1% of the total abdominal aortic area. Edge accumulation of radiolabel was intensely focal; it also peaked 4 hours after injection but remained steady up to 40 hours as plasma LDL levels decreased. At 24 hours, edge LDL accumulation per unit area was over five times greater than still-deendothelialized area accumulation. The reason for the absence of measurable edge accumulation at 2.5 hours is unclear. One likely explanation is that LDL binding sites associated with edges are less accessible to plasma LDL than still-deendothelialized binding sites and thus take longer to become occupied. If edge binding were less than still-deendothelialized binding at 2.5 hours, the edges could not be distinguished from the centers of the islands; if it were equal to still-deendothelialized area binding, edges could not be distinguished from still-deendothelialized areas. The persistent sequestration of LDL at high concentration in a localized compartment of the injured arterial wall provides a mechanistic explanation of the demonstrated ability to image experimental arterial lesions\(^\text{11-12}\) as well as human atheromas.\(^\text{34-35}\)

The persistent accumulation of 125I-LDL in regions of endothelial regrowth when plasma levels of LDL were falling strongly suggested that the intense focal accumulation of LDL was a result of binding to a component of extracellular matrix. Intracellular LDL accumulation via a high-affinity, receptor-mediated mechanism accounts for two thirds of total LDL metabolism in the rabbit\(^\text{*}\) and results in rapid lysosomal metabolism followed by rapid diffusion of radiolabel and other degradation products from cells back into the circulation\(^\text{37}\); neither focal nor diffuse accumulation would be observed if radiolabel were accumulated intracellularly via a receptor-mediated mechanism. The remaining one third of total-body LDL catabolism occurs by scavenger...
receptor and/or receptor-independent pathways. Even if LDL did not enter the lysosomal compartment from one of these pathways, LDL has been shown to be degraded extralysosomally, although more slowly than by lysosomal mechanisms, and degradation products are released back into the circulation. Thus, it is unlikely that either the focal or the diffuse accumulation patterns were due to intracellular trapping of LDL or its degradation products. The diffuse accumulation of 125I-LDL in still-deendothelialized areas most likely was also mediated by binding to extracellular matrix; however, the composition of extracellular matrix in still-deendothelialized regions has been reported to differ from that associated with regenerating endothelium. Our results suggest that regional differences from normal arterial extracellular matrix composition are important in the pathological accumulation of LDL.

Although the identities of the LDL binding moieties in regenerating and still-deendothelialized regions are unknown, we postulate that hydrophobic interactions with elastin and/or other matrix components are responsible for the persistent focal LDL binding associated with proliferating endothelium and that ionic interactions with glycosaminoglycans are responsible for the less intense accumulation in still-deendothelialized regions. Consideration of the relative binding affinities of LDL for elastin, glycosaminoglycans, and the cellular high-affinity LDL receptor supports this hypothesis. The interaction between LDL and elastin has been reported to be of moderately high affinity with an apparent dissociation constant \( K_d \) of \( 3.6 \times 10^{-8} \) M. A weaker interaction has been demonstrated between LDL and heparin, with a \( K_d \) between \( 1 \times 10^{-6} \) and \( 1 \times 10^{-7} \) M; the affinity of LDL for several other glycosaminoglycans, including dermatan sulfate, heparan sulfate, and chondroitin sulfate, has been reported to be even weaker than the affinity of LDL for heparin. Although the affinity of LDL for its high-affinity cell-surface receptor \( (K_d = 2.8 \times 10^{-9} \) M\) is one order of magnitude greater than the affinity of LDL for elastin, binding to the LDL receptor is not likely to be responsible for the persistent focal accumulation of LDL as explained above. The lack of LDL receptor involvement in arterial wall LDL accumulation was also demonstrated by the earlier finding that 125I-methyl LDL, which is not recognized by any cell-surface receptor, accumulated focally in regions of regenerating endothelium in a pattern identical to that of native LDL.

The intense focal accumulation of 125I-LDL at edges was not simply a result of greater intimal thickening at edges with a resultant increase in volume of distribution. In normcholesterolemic balloon-injured rabbits, the intima associated with the edges of regenerating endothelium was found to be 2.3 times thicker than the intima in adjacent still-deendothelialized areas; intimal thickening did not vary with time and was constant from 8 weeks to 20 weeks after balloon catheterization. Although we did not measure intimal thickening because we wanted to preserve the whole aorta for en face autoradiography, we expect that the lack of correlation between intimal thickening and time after balloononing was also true in our rabbits, which were examined 4 weeks after injury, and that the intima associated with the edges of regenerating endothelium was also approximtely 2.3 times thicker than the intima associated with still-deendothelialized areas. In contrast, autoradiographic intensity at edges was as much as five times greater than in still-deendothelialized regions, a difference of more than twice that of intimal thickening. Persistent focal LDL accumulation has also been observed in spontaneous lesions of the WHHL rabbit (authors’ unpublished data). Evidently the regenerating endothelium of healing islands and the endothelium overlying spontaneous atherosclerotic lesions share the ability to mediate focal accumulation of LDL.

Use of autoradiographic intensity measurements to quantify radioactivity in the different aortic compartments had several advantages over possible alternative methods. The absolute amount of radioactivity that accumulated in either compartment could have been determined by dissecting out, weighing, and counting the still-deendothelialized regions and the edges of healing islands. However, we decided that this method would be inaccurate because of the very small size of the rabbit aorta and the narrowness of the healing edges of islands. Theoretically, the most accurate method would have been to include radioisotope standards with each aorta being autoradiographed. However, 125I standards were not available in the appropriate dose range at the time of this study, and attempts to develop our own were unsuccessful. Although it was not possible to combine en face autoradiography with histology, we knew from the work of Minick et al that the edges of healing islands were narrow, so that assigning edges a width of 1 unit was a reasonable approximation of edge width to use for calculating edge area.

The present study is in general agreement with earlier evidence for a more stable pool of 125I-LDL in regions of regenerating endothelium than in the still-deendothelialized aorta. However, Falcone et al reported a maximal ratio of 125I activity in edges of healing islands to that in still-deendothelialized region of only 1.3:1 at 48 hours. The results of Alavi and Moore indicate a ratio of approximately 0.8:1 at 48 hours. Based on either of these results, one would not expect to observe the intense focal accumulation of 125I-LDL at edges of regenerating endothelial islands that is evident by en face autoradiography. Falcone et al dissected out edges of healing islands and regions of unhealed aorta and expressed their results as radioactivity per gram rather than radioactivity per unit of surface area. This would result in an underestimation of the accumulation of 125I-LDL at the edges and could be one reason for the difference in results. They also used a lipoprotein fraction prepared from the plasma of hypercholesterolemic rabbits, which included low, intermediate, and very low density lipoproteins. The particle size of this fraction was larger than that of the human LDL fraction that we used. Because particle size is inversely related to the rate at which plasma proteins cross the endothelium, entry of the larger lipoprotein fraction into regions of regenerating endothelium may have been impeded; this also could contribute to lower edge accumulation. Alavi and Moore did not observe focal accumulation because in the absence of en face autoradiography, they assumed that 125I-activity was uniformly distributed throughout the entire healing island rather than concentrated at the edges.
Whereas some experimental studies attempt to define the earliest events in atherogenesis by observing the effects of elevated levels of circulating lipoproteins on LDL accumulation in the initially normal arterial wall,1-10,48 the work presented here describes the effects of functional modification of the arterial wall on arterial LDL accumulation in the absence of either acute or chronic hypercholesterolemia. The work attempts to elucidate initial events that may be common among a variety of risk factors, namely changes in the arterial wall itself.

The results indicate that the accumulation of LDL by the healing arterial wall occurs via two kinetically and biochemically different mechanisms. These data are consistent with the recent findings that different domains of apo B mediate distinctly different aspects of LDL metabolism14-17 and point to the need for a more detailed understanding of which components of both LDL and the arterial wall are responsible for the pathological accumulation of LDL in atherosclerosis. Persistent LDL sequestration is focal and apparently extracellular; it appears to be directed by functionally modified endothelial cells that overlie the healing edge and is independent of hypercholesterolemia. Defining the alterations that lead to LDL accumulation in the injured and healing arterial wall should clarify our understanding of atherogenesis and ultimately lead to successful measures for prevention.

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