Accumulation of Native and Methylated Low Density Lipoproteins by Healing Rabbit Arterial Wall

Alan J. Fischman, Ann M. Lees, Robert S. Lees, Martha Barlai-Kovach, and H. William Strauss

To determine whether healing arterial wall accumulation of low density lipoproteins (LDL) is mediated by the high affinity LDL receptor, normocholesterolemic rabbits were injected with [125I]-LDL, [99mTc]-MeLDL, or the reductively methylated analog of these compounds (125I-MeLDL, 99mTc-MeLDL), 1 month after balloon catheter denudation of the abdominal aorta. If the mechanism of accumulation requires interaction with the LDL receptor, reductively methylated lipoproteins which do not bind to the receptor should not accumulate in healing arterial wall. Twenty-four hours after injection of labelled lipoproteins, each animal was injected with Evans blue dye, in order to distinguish reendothelialized from denudated aorta. One hour after dye injection, the aorta was fixed, removed, divided into abdominal (ballooned) and thoracic (unballooned) regions and counted. For all lipoprotein preparations, there were three to four times as many counts in the abdominal as in the thoracic aorta. En face autoradiographs were made of the aortas that had been exposed to 125I-labelled lipoproteins. In the autoradiographs, the areas of the lowest activity corresponded to the centers of healing endothelial islands. The most intense radioactivity for both lipoproteins occurred in the region of the leading edge of the endothelial islands where active endothelial regeneration was in progress. The overall distribution of native and MeLDL accumulation was the same. The results suggest that low density lipoproteins are accumulated in areas of active endothelial regeneration by a mechanism that does not involve the high affinity LDL receptor.

(Arteriosclerosis 7:361–366, July/August 1987)

There is abundant evidence that the cholesterol in developing atherosclerotic lesions is derived from circulating low density lipoproteins (LDL); however, the mechanism of accumulation remains unclear.1–13 Most available data suggest that LDL penetrates the monolayer of endothelial cells lining the arterial wall by transcellular vesicular transport.14,15 The mechanism of transendothelial LDL transport could be receptor-mediated endocytosis, nonreceptor-mediated endocytosis, or some combination of both processes.

The balloon-cather denudated rabbit aorta is widely used as an in vivo model for human atherosclerosis. We have previously demonstrated with this system14 that in normocholesterolemic rabbits, healing abdominal aortic wall selectively accumulated 125I-labelled low density lipoproteins (125I-LDL). The amount of labelled lipoprotein accumulated in 24 hours by abdominal aorta increased steadily over several weeks as the time since injury increased and closely paralleled the degree of reendothelialization. The maximum 24-hour accumulation occurred 4 to 8 weeks after injury. In contrast, the amount of 99mTc-albumin and 125I-labelled high density lipoprotein (125I-HDL) in balloon-damaged abdominal aorta, and the amounts of 125I-LDL, 125I-HDL, and 99mTc-albumin in undamaged thoracic aorta showed no such increase. Recently, we established that 99mTc-LDL can be employed for the external imaging of LDL accumulation by tissues, including the injured healing arterial wall and the adrenal cortex.17,18

Modification of native LDL by derivatization of the guanidino groups of arginyl residues with 1,2-cyclohexanedione or the epsilon-amino groups of lysyl residues by reductive methylation (MeLDL) abolishes the binding of the lipoprotein to LDL receptors, and causes the cellular uptake of these derivatized molecules to be mediated by apparently nonspecific mechanisms.16,20 It has been shown recently that the initial uptake of native and reductively methylated LDL is not significantly different in normal rabbit aorta.21 However, intramural accumulation of the two LDLs was not measured, nor was the effect of healing on arterial wall accumulation of the LDLs tested.

The present study was undertaken to determine whether accumulation of LDL by healing arterial wall is mediated by high affinity LDL receptors on the cells of the vessel wall. We measured the in vivo 24-hour accumulation by abdominal and thoracic aortas of 125I-LDL, 125I-MeLDL, 99mTc-LDL, and 99mTc-MeLDL 1 month after balloon-cather deendothelialization of the abdominal aorta. We also determined the adrenal gland accumulation of the labelled compounds and made autoradiographs of the damaged vessels.
Methods

Lipoprotein Preparation

LDL (density = 1.025 to 1.050 g/ml) was prepared by sequential flotation in the ultracentrifuge. After dialysis against buffer containing 0.2 M NaCl and 1.0 mM disodium EDTA, pH 8.6 (EDTA saline), the LDL was passed through a 0.22-micron filter (Millipore Corporation, Bedford, Massachusetts). Lipoprotein purity was determined by double immunodiffusion in agarose against the following rabbit antisera to human proteins: anti-LDL, anti-very low density lipoprotein (VLDL), anti-high density lipoprotein (HDL), anti-immunoglobulins and anti-whole serum. Protein concentration was determined by the method of Lowry. Reductive methylation of LDL was carried out as described by Weisgraber et al. The extent of derivatization of LDL was greater than 70% of the lysine residues as estimated by the trinitrobenzene sulfonic acid assay.

Lipoprotein Iodination

Iodination of native and reductively methylated LDL was performed by a previously described modification of the McFarlane iodine monochloride technique. After dialysis against EDTA saline, more than 97% of the radioactivity was precipitable by 10% trichloroacetic acid. Final specific activities were between 100 and 400 cpm/ng protein. From 2.5% to 4.5% of the radioactivity was bound to lipid. The radiolabelled lipoproteins used in this study were tested in a previously described in vitro lymphocyte assay for lipoprotein degradation. Native LDL degradation was saturable, consistent with a high affinity receptor-mediated process, while methyl LDL degradation was unsaturable up to 500 µg/ml, consistent with a nonreceptor-mediated process.

Lipoprotein Labelling with Technetium-99m

Technetium-99m labelling of native and reductively methylated LDL was performed by the method previously reported with minor modifications. A standard reaction mixture was prepared by adding an aliquot of 99mTcO₄⁻ (about 30 mCi in 0.5 ml of 0.9% saline) to LDL or MeLDL (1.5 to 2.5 mg protein) previously dialyzed against EDTA saline or 0.2 M bicarbonate buffer, respectively. Sodium dithionite (10 mg, 57.5 µmol) freshly dissolved in 0.1 ml of 0.5 M glycine buffer (pH 9.8) was immediately added and gently mixed with the solution of 99mTcO₄⁻ and lipoproteins. The reaction mixture was allowed to stand at room temperature for 30 minutes and was occasionally mixed. The mixture was then applied to a column of Sephadex G-50 and eluted with bicarbonate buffer (0.1 M NaHCO₃, 0.15 M NaCl), pH 8.0. Labelled lipoproteins were collected from the end of the void volume. Final specific activities were between 3.0 and 7.5 x 10⁴ cpm/ng protein. The preparations were characterized by paper electrophoresis.

Animals and Surgery

Male New Zealand white rabbits (2 to 3 kg each) were obtained from ARI Breeding Labs, West Bridgewater, Massachusetts. They were maintained on a normal diet of Wayne rabbit ration (Allied Mills, Incorporated, Special Feed Division, Chicago, Illinois). Their abdominal aortas were denuded of endothelium by a modification of the Baumgartner technique. After each animal was anesthetized with ketamine and ether, the left femoral artery was isolated; a 4F Fogarty embolectomy catheter (Model 12-040-4F, Edwards Laboratories Incorporated, Santa Ana, California) was introduced through an arterotomy in the femoral artery and was advanced under fluoroscopic visualization to the level of the diaphragm. The catheter was inflated to a pressure of about 3 psi above the balloon inflation pressure with radiographic contrast medium (Conray, Mallinckrodt, St. Louis, Missouri). Three passes were made through the abdominal aorta with the inflated catheter to remove the aortic endothelium before removal of the catheter, ligation of the femoral artery, and closure of the wound. The animals were allowed to heal for a period of 4 to 5 weeks before injection of the labelled lipoproteins.

Labelled Lipoprotein Injections

Each labelled lipoprotein preparation (150 to 300 µCi of 125I or 1.0 to 1.5 mCi of 99mTc bound to lipoprotein) was injected into the marginal ear vein of the rabbits 4 to 5 weeks after deendothelialization of the aorta. Serial blood samples were obtained from the opposite ear during the ensuing 24 hours and were analyzed for 125I-LDL, 125I-MeLDL, 99mTc-LDL, or 99mTc-MeLDL radioactivity. The labelled protein concentration in the blood sample that was withdrawn 5 minutes after injection was considered as time zero radioactivity in the calculation of average plasma radioactivity. Figure 1 shows representative plasma decay
LIPOPROTEIN ACCUMULATION BY ARTERIAL WALL  Fischman et al. 363

curves for 99mTc-labelled native and methyl LDL. Similar curves (data not shown) were obtained with the corresponding radioiodinated lipoproteins.

Aortic and Adrenal Specimens

Twenty-four hours after injection of the labelled lipoprotein preparations, each animal was injected intravenously with 4 ml of a 0.5% solution of Evans blue dye (Allied Chemical Company, National Aniline Division, New York, New York) which stains areas of deendothelialized aorta blue. After 1 hour, the animal was sacrificed by a lethal injection of pentobarbital. After sacrifice, the aorta was fixed in situ with formalin and was then removed en bloc. The adventitia was removed completely and the remaining aorta was washed in formalin and divided into abdominal (ballooned) and thoracic (unballooned) regions. After removal of the aorta, the adrenal glands were removed and all samples were weighed and counted for 125I or 99mTc in an LKB automatic gamma counter. An aliquot of the injected dose was counted simultaneously.

To compare the relative accumulation of the different labelled lipoproteins in the aorta and adrenal gland, it was necessary to correct for differences in mean plasma concentration of the labelled compounds. The mean concentration of lipoprotein-associated radioactivity was calculated by numerical integration of the plasma decay curves and division by the time since injection of the isotope.

En Face Autoradiography

After counting, the aortic segments from animals that had been injected with 125I-labelled lipoprotein were opened along the ventral surface, were pinned out, were immersed in 10% trichloroacetic acid, and were photographed. The fixed, opened vessels were then covered with a single layer of plastic (Saran) wrap, placed on high speed x-ray film (Kodak Orthofilm OH-1), and stored for 2 to 3 weeks in a Kodak “X-Omatic cassette” (24 x 30 cm) before development in a 90 second X-OMAT.

Statistics

Statistical analyses were performed by two-way analysis of variance followed by unpaired t testing (lipoprotein accumulation by thoracic aorta) or by Duncan’s new multiple range test (other parameters).

Results

Accumulation of 125I-Native and Methyl LDL in Healing and Normal Aorta

After radio-labelled LDL or MeLDL had circulated for 24 hours, aortic lipoprotein accumulation was assessed by gamma counting and autoradiography. Figure 2A shows the accumulation of 125I-labelled native and methyl LDL in healing deendothelialized abdominal aorta and uninjured thoracic aorta, 24 hours after injection of the lipoprotein preparations. The effect of healing on lipoprotein accumulation (mean ± SEM) was significant for both LDL (25.1 ± 3.3 μl/g for the intact thoracic aorta compared with 84.7 ± 6.7 μl/g for the healing abdominal aorta, p < 0.01) and MeLDL (46.3 ± 7.6 μl/g for intact aorta, compared with 142.1 ± 25.3 μl/g for healing aorta, p < 0.01). Lipoprotein methylation appeared to enhance aortic lipoprotein accumulation. Accumulation of 125I-MeLDL was 68% higher than 125I-LDL in abdominal aorta (p < 0.05), and 84% higher in thoracic aorta (p < 0.05).

Evidence of focal LDL sequestration in regions of regenerating endothelium came from en face autoradiographs of the entire rabbit aorta. In the thoracic region the autoradiographic images from rabbits injected with either 125I-LDL or 125I-MeLDL were faint, diffuse, and relatively uniform in intensity. In reendothelializing abdominal aorta, three levels of radioactivity were apparent and each activity level, when compared to the Evans blue staining, corresponded to a specific anatomic region in the abdominal aorta (Figure 3). The centers of regenerating endothelial islands, which did not stain with Evans blue dye, had very low levels of radioactivity. The deendothelialized regions, which did stain with Evans blue, corresponded with areas of moderate radioactivity. The actively regenerating edges of endothelial islands showed intense focal accumulation of radioactivity. Although there was some variation in the intensity of activity among different animals or different healing edges in the same vessels, there was no differ-
Figure 3. En face autoradiographs of the abdominal aorta of rabbits injected with $^\text{125I}}$-LDL (A) and $^\text{125I}}$-MeLDL (B). The labelled lipoproteins were injected 1 month after deendothelialization of the abdominal aorta and were allowed to circulate for 24 hours. One hour before removal of the aorta, Evans blue dye was injected. Comparison of the autoradiographs with photographs of the Evans blue staining (not shown) showed that the areas of lowest activity in the autoradiographs corresponded to the centers of endothelial islands, which did not stain. The middle level of radioactivity corresponded with areas that lacked endothelial coverage and, thus, did stain. The most pronounced areas of radioactivity occurred around the leading edges of the endothelial islands where active regeneration was in progress.

ence in the pattern of distribution of $^\text{125I}}$-LDL or $^\text{125I}}$-MeLDL in any of the aortic areas described.

**Accumulation of $^{99m}\text{Tc}}$-Native and Methyl LDL In Healing and Normal Aorta**

Figure 2B shows the accumulation of $^{99m}\text{Tc}}$-native and methyl LDL in healing deendothelialized abdominal aorta and uninjured thoracic aorta, 24 hours after injection of the lipoprotein preparations. As with the $^\text{125I}}$-labelled compounds, the effect of healing on lipoprotein accumulation was significant for both LDL ($23.5 \pm 3.3 \mu\text{g/g}$ for the intact thoracic aorta compared with $74.4 \pm 21.3 \mu\text{g/g}$ for the healing abdominal aorta, $p<0.05$) and MeLDL ($59.0 \pm 8.7 \mu\text{g/g}$ for intact compared with $229.4 \pm 31.0 \mu\text{g/g}$ for healing aorta, $p<0.01$). Methylation appeared to enhance accumulation of $^{99m}\text{Tc}}$-labelled lipoproteins even more than it did that of $^\text{125I}}$-labelled lipoproteins. Accumulation of $^{99m}\text{Tc}}$-MeLDL was 208% higher than $^{99m}\text{Tc}}$-LDL in the abdominal aorta ($p<0.01$), and 151% higher in the thoracic aorta ($p<0.02$). The difference in accumulation between thoracic and abdominal aorta was significantly greater for methyl than for native $^{99m}\text{Tc}}$-labelled LDL as indicated by a significant interaction between lipoprotein methylation and healing ($p<0.01$).

**Accumulation of $^{99m}\text{Tc}}$-Labelled Native and Methyl LDL by Adrenal Gland**

Figure 4 shows the accumulation of $^\text{125I}}$- and $^{99m}\text{Tc}}$-labelled native and methyl LDL in rabbit adrenal gland, 24

![Graph](attachment:image.png)

**Figure 4.** Accumulation in rabbit adrenal gland of labelled lipoproteins ($\mu\text{g/g}$, mean $\pm$ SEM). Accumulation was measured 24 hours after intravenous injection of: $^\text{125I}}$-labelled native and reductively methylated LDL (hatched bars, $n = 6$), and $^{99m}\text{Tc}}$-labelled native and reductively methylated LDL (open bars, $n = 4$). Adrenal accumulation of labelled lipoproteins was calculated by dividing the measured adrenal radioactivity per gram by average plasma radioactivity as described in Methods.
hours after injection of the lipoprotein preparations. The negative effect of reductive methylation on lipoprotein accumulation was significant for both 125I-labelled lipoproteins (209.9 ± 13.9 μg/g for native LDL compared with 85.7 ± 14.2 μg/g for MeLDL, p < 0.01) and 99mTc-labelled lipoproteins (2165.5 ± 624.9 μg/g for native LDL compared with 567.2 ± 16.5 μg/g for MeLDL, p < 0.01). The observation of greater accumulation of native than MeLDL is particularly significant in light of the fact that the level of residual blood-pool radioactivity was higher for MeLDL than for native LDL with both radiolabels. Also, there was a significant overall effect of the isotope used (p < 0.01), with greater accumulation of 99mTc-labelled lipoproteins than 125I-labelled lipoproteins; for native LDL, 99mTc-labelling led to a tenfold increase in accumulation over 125I-labelling, while for MeLDL, the increase was sevenfold.

Discussion

Experimental animal models have been used by numerous investigators to study the etiology of human atherosclerosis. With the healing, balloon-catherized endoinitialized rabbit aorta model, Minick et al.3,8 demonstrated that the areas of most marked fatty proliferative response were confined to the leading edge of the regenerating endothelium, not to the endoinitialized areas of the aorta as had been expected. With the same model, Falcone et al.32 found three times more free and esterified cholesterol in the endoinitialized vessel wall than in the pseudo-intima. In human vessels, Smith et al.33 found a similar pattern with up to 12 times more LDL in human lesions covered with endothelium than in those that were not endoinitialized. In a previous study,18 we showed that there was focal accumulation of LDL at the leading edge of the regenerating endothelium in healing balloon-catherized rabbit arterial wall.

These studies indicate that the interaction of plasma LDL with regenerating endothelium is an important factor for lipid and lipoprotein accumulation in healing arterial wall. In a recent study, Wiklund, et al.39 demonstrated that interaction with LDL receptors is not necessary for lipoprotein entry into normal rabbit aortic wall. In the present study, we showed by en face autoradiography that LDL and MeLDL were both locally sequestered by the healing rabbit abdominal aorta at the edges of regenerating endothelial islands. Measurement of the total radioactivity in the abdominal aorta confirmed and extended the qualitative information obtained by autoradiography. When 125I was the radiolabel injected, there was a significant difference in lipoprotein accumulation between healing abdominal and intact thoracic aorta, with healing aorta accumulating between three and five times more lipoprotein than intact aorta. Also, there was a greater accumulation of 125I MeLDL as compared with 125I-native LDL by both the abdominal and the thoracic aorta.

When 99mTc was the radiolabel injected, there were again significant differences in lipoprotein accumulation between healing aorta and intact aorta, with healing aorta accumulating between three and four times more lipoprotein than intact aorta. Again both intact and healing aorta accumulated more MeLDL than native LDL. That this occurred (even when the results were corrected for the long-


34. Kovanen PT, Basu SK, Goldstein JL, Brown MS. Low density lipoprotein receptors in bovine adrenal cortex. II. Low density lipoprotein binding to membranes prepared from fresh tissue. Endocrinology 1979;104:610-616


Index Terms: rabbit • balloon deendothelialization • healing aorta • LDL • methyl LDL • iodination • technetium-labelling • adrenal gland
Accumulation of native and methylated low density lipoproteins by healing rabbit arterial wall.
A J Fischman, A M Lees, R S Lees, M Barlai-Kovach and H W Strauss

doi: 10.1161/01.ATV.7.4.361
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1987 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/7/4/361