Low-Density Lipoprotein Modification and Arterial Wall Accumulation in a Rabbit Model of Atherosclerosis†

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ABSTRACT: Chemically or enzymatically modified low-density lipoproteins (LDL), with and without changes in surface charge, were studied *in vivo* in the healing, balloon catheter-deendothelialized rabbit aorta to determine the effect of LDL modification on its accumulation in arterial lesions. In this model, in which healing (reendothelialization) proceeds radially outward from individual aortic branch arteries, it was previously shown by autoradiography that two kinetically distinct compartments accumulated ^{125}I-labeled LDL. In aortic regions which were still deendothelialized, accumulation was diffuse and labile. In contrast, at the edges of the islands of regenerating endothelium, LDL accumulation was intensely focal, as it is in human atherosclerotic lesions, and persisted for at least 40 h after injection in spite of falling levels of radiolabeled LDL in plasma [Chang, M. Y., et al. (1992) *Arterioscler. Thromb. 12*, 1088–1098]. In the present study, modified LDLs with gradations in charge change were prepared to clarify the role of changes in surface charge on focal aortic LDL accumulation. Oxidized LDL (weakly anionized), desialated LDL (weakly cationized), and reductively methylated LDL (no change in net charge) all accumulated focally. Focal accumulation of native LDL also occurred in ballooned rabbits fed probucol to inhibit LDL oxidation. Strongly anionized succinylated and diazobenzenearsonylated LDL and strongly cationized dimethylpropanediamine LDL did not accumulate focally. The results support the concept that focal sequestration of LDL in arterial lesions is mediated by specific, oxidation-independent patterns of charge and polarity on LDL which are disrupted by major changes in LDL surface charge.

The presence of cholesteryl esters in atherosclerotic lesions has long been recognized (Antischkow, 1913; Smith & Ashall, 1983; Yomantas et al., 1984). Low-density lipoprotein (LDL),[1] the major carrier of plasma cholesteryl ester, has been implicated as the agent by which cholesteryl ester enters lesions. However, the mechanisms of LDL interaction with arterial lesions have not been fully elucidated.

Balloon catheter deendothelialization of the rabbit aorta has been used previously as an *in vivo* model for human atherosclerosis (Faxon et al., 1982; Minick et al., 1979; Stemerman et al., 1977). With this model, we have shown that ^{125}I-labeled LDL accumulates with three levels of intensity in the "ballooned" aorta after 24 h of circulation (Roberts et al., 1983). Most striking was the intense focal accumulation at the healing edges of regenerating endothelial islands. Less intense, diffuse accumulation was present in still-deendothelialized regions of the aorta, and no accumulation was observed in the centers of reendothelialized islands.

The effects of LDL modification on binding to the high-affinity cell surface apolipoprotein B/E receptor (Weisgraber et al., 1978; Mahley et al., 1979a), to the scavenger receptor (Haberland et al., 1984), and to heparin (Mahley et al., 1979b) have been described by others. The present study was designed to determine if modification of LDL would affect its *in vivo* interaction with arterial lesions, an interaction that occurs independently of cell surface receptors (Fischman et al., 1987). We prepared modified LDLs by succinylation, diazobenzenearsonylation, copper-ion oxidation, reductive methylation, desialation, and diamine cationization. We also tested the effect of feeding probucol to ballooned rabbits before injecting radiolabeled native LDL to determine whether inhibition of LDL oxidation *in vivo* would affect its accumulation in healing aortic lesions.

The accumulation of ^{125}I-radiolabeled modified lipoproteins in the ballooned rabbit was compared with that of ^{125}I native LDL by three techniques: (i) Plasma disappearance curves were obtained and their equations derived, yielding information about rates of removal and plasma half-lives. (ii) Autoradiographs of aortas were analyzed qualitatively to identify patterns of accumulation. (iii) Aortic accumulation was quantified by image digitization of autoradiographs.

EXPERIMENTAL PROCEDURES

Preparation of Lipoproteins. Human LDL was isolated from the blood of fasting normolipemic volunteers by sequential flotation between densities of 1.025 and 1.050 g/mL, as described previously (Hatch & Lees, 1968). LDL protein, for both native and modified LDL, was determined by the method of Lowry et al. (1951) and corrected according to the method of Margolis and Langdon (1966). Lipoprotein purity

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[1] Abbreviations: LDL, low-density lipoproteins; DMPA, *N,N*-dimethyl-1,3-propanediamine; AA, abdominal aorta; TA, thoracic aorta; DEA, deendothelialized area; REA, reendothelialized area; AC, accumulation; CL, clearance; MPR, mean plasma radioactivity; R, radioactivity.

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was confirmed by paper electrophoresis and by Ouchterlony double-immunodiffusion (Hatch & Lees, 1968). LDL was examined for lipid peroxidation by assaying for thiobarbituric acid reactive substances (TBARS) (Buege & Aust, 1978). Results of 0–1.4 nmol of TBARS/mg of protein indicated the absence of measurable lipid peroxidation (Parthasarathy et al., 1989).

Succinylation of LDL. Solid succinic anhydride (Aldrich, Milwaukee, WI) was added in small increments to LDL at room temperature in 0.11 M sodium phosphate buffer (pH 7.8) for a final molar ratio of reagent to LDL lysine residues of 60:1 (Scanu et al., 1968). The pH was maintained between 7.5 and 8.0 with 0.5 N NaOH. Succinyl LDL was dialyzed against 0.11 M sodium phosphate (pH 7.8) with 0.15 M NaCl and was passed through a 0.22-μm low-binding filter.

The extent of derivatization of lysyl residues was quantitatively determined with the trinitrobenzenesulfonic acid (TNBS) assay of Habeeb (1966) as the difference in lysyl residues of modified vs unmodified LDL.

Diazobenzenearsonate Modification of LDL. Fresh diazobenzenearsonic acid was prepared from *p*-arsanilic acid and sodium nitrite (Aldrich), as described (Margolis & Langdon, 1966), and was added to LDL in 0.1 M sodium carbonate (pH 9.9) over a 15-min period at 0 °C. The ratio of reagent to LDL was 6.67 μmol of diazobenzenearsonate/mg of LDL protein. The reaction was complete after 3 h on ice. The solution was stored overnight at 4 °C, and diazobenzenearsonyl LDL was dialyzed against 0.2 M NaCl with 1.0 mM Na_2EDTA (pH 9). The resulting modified LDL was passed through a 0.22-μm filter.

The extent of derivatization of tyrosyl and histidyl residues was determined spectrophotometrically by visible spectrum scanning, after addition of NaOH to the modified LDL for a final concentration of 0.1 N (Tabachnick & Sobotka, 1959). A correction was made for carotenoid absorption by subtraction of the absorption of similarly diluted unmodified LDL (Tabachnick & Sobotka, 1960).

Cu^{2+} Oxidation of LDL. LDL (5–10 mg at 3–8 mg/mL) was dialyzed at 4 °C against phosphate buffer (1.5 mM NaH_2PO_4/3.5 mM Na_2HPO_4, pH 7.5) supplemented with 10 μM cupric acetate (Fluka, Ronkonkoma, NY) at 4 °C (Steinbrecher et al., 1987; Triau et al., 1988). The dialysate was replaced after 2 h and after an additional 6 h, after which time dialysis continued for 24–48 h. Control LDL was prepared simultaneously by dialysis against phosphate buffer supplemented only with 1 mM EDTA; all other conditions were as above. Filtration and estimation of the extent of oxidation were performed by assaying for TBARS.

Desialation of LDL. LDL was incubated with insoluble agarose-bound neuraminidase (*N*-acetylneuraminate glycohydrolase) (Sigma) for 18 h at 37 °C in 0.2 M sodium acetate/0.9% NaCl/0.02% NaN_3/0.01% EDTA (pH 5.0) (Attie et al., 1979). The protein to enzyme ratio was 20 to 1 (milligrams of protein to units of enzyme activity). The agarose-bound enzyme was removed by centrifugation at 4000 rpm for 5 min at 4 °C. By centrifugation on a Centricon 10 filter (Amicon) at 4 °C, cleaved sialic acid was removed and desialated LDL was transferred into 0.02 M sodium phosphate/0.9% NaCl (pH 7.4). An unmodified control was prepared by incubation of LDL without enzyme under the same conditions.

The extent of desialation was determined by the thiobarbituric acid (TBA) assay (Warren, 1959) for free sialic acid after methanol extraction (Blix & Lindberg, 1960) of the cleaved sialic acids and compared with the results for unmodified LDL and an acid-hydrolyzed native LDL control.

Neuraminidase was tested for proteolytic activity with ^{125}I hemoglobin as substrate. ^{125}I Hemoglobin was incubated with or without insoluble agarose-bound neuraminidase in the same ratio as for LDL and under the same conditions. Protease activity was assayed in terms of ^{125}I-labeled products not precipitable by 10% trichloroacetic acid in the presence of bovine albumin as a carrier protein.

Reductive Methylation of LDL. LDL (5–10 mg at 3–8 mg/mL) was diluted with 0.2 M sodium borate (pH 8.0) to 1.5 times the original volume. Reductive methylation was performed at 0 °C by the addition of 1 mg of sodium borohydride followed by six additions over 30 min of 1 μL of 37% aqueous formaldehyde (Weisgraber et al., 1978). The entire sequence of additions was repeated for a total of 2 additions of sodium borohydride (at 0 and 30 min) and 12 additions of formaldehyde (at zero time and at 6-min intervals for 60 min). Six minutes after the last addition of formaldehyde, the modified LDL was dialyzed against 0.2 M NaCl/1.0 mM EDTA (pH 9). After dialysis, the methyl LDL was passed through a 0.22-μm filter.

The extent of derivatization of lysyl residues was quantitatively determined with the TNBS assay, as described above for succinyl LDL.

DMPA Modification of LDL. N,N-Dimethyl-1,3-propanediamine (2 M; DMPA) (Eastman Kodak) (adjusted to pH 7.0 with HCl) was added to LDL in a weight ratio of reagent to LDL protein of 13 to 1 (Basu et al., 1976). This represented approximately a 10-fold molar excess of DMPA relative to the number of glutamic and aspartic acid residues in LDL. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (Pierce) was added slowly to the mixture in a molar ratio of 1:15 relative to DMPA. The pH was maintained at 7.0 with 0.2 M HCl until the reaction had gone to completion, as indicated by no measurable change in pH (<2 h). The solution was stored overnight at 4 °C, and DMPA LDL was dialyzed against 0.15 M NaCl/0.3 mM EDTA (pH 7.4). The resulting solution was passed through a 0.22-μm filter. Protein content of this cationized LDL was determined according to the method of Lowry et al. (1951), with an additional correction factor of 1.5 to account for the DMPA effect of increasing the color yield (Basu et al., 1977).

Characterization of Modified Lipoproteins. In addition to the specific quantitative analyses for each modification, control and modified lipoproteins were analyzed by paper or agarose electrophoresis to determine the change in electrophoretic mobility after modification (Hatch & Lees, 1968). Double-immunodiffusion against the same antisera as for native LDL was performed to establish that antigenicity of the lipoprotein was maintained after modification.

Radiolabeling of Lipoproteins. Native LDL and modified LDL were radioiodinated with $Na^{125}I$ (17.4 Ci/mg in 0.1 N NaOH) by a modification (Bratzler et al., 1977) of the iodine monochloride technique (McFarlane, 1958). Specific activities were between 200 and 500 cpm/ng of protein. Greater than 96% of the radiolabel was associated with protein, as determined by 10% trichloroacetic acid precipitability. Less than 5% of the radiolabel was associated with native LDL lipid, and less than 7% was associated with modified LDL lipid, as measured by extraction with chloroform/methanol 2:1 (Folch et al., 1957).

Animal Procedures. The abdominal aortas of normal chow-fed (Prolab, Agway Laboratory Feeds, Ithaca, NY) male New Zealand white rabbits (2–3 kg) were balloon catheter-deendothelialized under aseptic conditions according to the Baumgartner technique (Baumgartner, 1963), using a 4-French

Fogarty embolectomy catheter (Roberts et al., 1983). Animals were allowed to heal for 4 weeks before injection of ^{125}I-labeled native or modified lipoprotein (Chang et al., 1992). All use of animals conformed to state and federal laws and to guidelines established by the Deaconess Hospital Animal Care and Use Committee.

Additional rabbits (2–3 kg) were assigned to group A or B to examine the effects of *in vivo* oxidation inhibition on the aortic accumulation of native LDL. Group A rabbits (n = 2) were maintained on normal rabbit chow for 5 weeks after balloon catheter injury. Group B rabbits (n = 2) were fed normal rabbit chow supplemented with 1% (w/w) probucol (gift of Marion Merrell Dow, Cincinnati, OH) beginning 2 days prior to balloon catheterization and continued on this diet for 5 weeks after injury. Pure probucol was added to rabbit chow in diethyl ether, and the chow was allowed to dry for several days before use. Animals in both groups received to 125 g of food per day.

Four to 5 weeks after aortic ballooning, ^{125}I-labeled protein was injected into the marginal ear vein and allowed to circulate. Injected doses ranged from 95 to 560 μCi. To determine plasma disappearance kinetics, serial blood samples were obtained from the central artery of the ear, and plasma radioactivity precipitated by 10% TCA was measured. The time of sacrifice was determined by the rates of disappearance of ^{125}I activity from plasma; the goal was minimal redistribution of radioactivity from the aortic tissue back into the plasma, so sacrifice occurred when <10% of the injected dose remained in the plasma.

Fifteen minutes prior to sacrifice, animals were injected intravenously with 6 mL of a 0.5% Evans blue dye solution to stain areas of still-deendothelialized aorta blue (Stemerman et al., 1977). Immediately after sacrifice, the aorta was removed and processed as described elsewhere (Chang et al., 1992). Total ^{125}I radioactivity (disintegrations per minute) in the abdominal (R_{AA}) and in the thoracic aorta (R_{TA}) was measured in a Packard Auto-Gamma 5650 counter.

En Face Autoradiography. The distribution of radioactivity in the balloon deendothelialized aorta was examined by macroscopic autoradiography, as previously described (Chang et al., 1992). Briefly, the fixed vessels were placed on high-speed X-ray film in a stainless steel cassette and stored at −70 °C until development. Exposure times ranged from 3 days to 3 weeks and were adjusted for the total ^{125}I radioactivity in the aorta.

Image Analysis of Photography. (*a*) *Extent of Healing.* Photographs of the Evans blue stained vessels were digitized as described (Chang et al., 1992). Briefly, the total surface of **Re**Endothelialized Areas (REA) and the total surface of still-**De**Endothelialized Areas (DEA) of the abdominal aorta were determined in units of area by computer image processing of digitized photographs. The surface areas of the whole abdominal aorta (AA) and the whole uninjured thoracic aorta (TA) also were determined. The extent of reendothelialization (healing) was expressed as a percentage of the total surface area of the abdominal aorta [(REA/AA) × 100]. The edges of healing islands also were measured; as the areas of the regenerating edges could not be precisely determined, this parameter was approximated from measurements of the perimeters of the healing islands (Chang et al., 1992).

(*b*) *Mean Plasma Radioactivity.* Mean plasma radioactivity (MPR), expressed as disintegrations per minute per microliter of plasma, was the average specific activity of the plasma and was a measure of the average exposure of the aorta to plasma radioactivity (Chang et al., 1992). This was determined as

$$\mathrm{MPR} = (\mathrm{AUC})(\mathrm{ID})/Vt$$

AUC [Σ(% of injected dose remaining)(t)] was the total area under the plasma disappearance curve; ID was the injected dose in dpm; and the quantity [(AUC)(ID)] was a measure of the total plasma radioactivity over the entire experimental period. V was the plasma volume (μL), and t was the time period (h) of an experiment.

The average specific activity of the plasma ranged from 26 to 6457 dpm/μL, reflecting differences in plasma removal kinetics and injected dose.

Image Analysis of Autoradiographs. (*a*) *^{125}I Lipoprotein Accumulation at Edges of Regenerating Endothelium and in Still-Deendothelialized Regions.* Autoradiographs were digitized for determination of radioactivity associated with regenerating endothelium at the edges of healing islands vs that associated with still-deendothelialized regions as described previously (Chang et al., 1992). The sole assumption required for the computer image analysis was that the intensity (I) of silver grain development in any region of interest on the autoradiographic film was directly related to the ^{125}I concentration (disintegrations per minute per unit area) in the corresponding aortic region of interest (i.e., edge or DEA). This assumption required that silver grain development in regions of interest was not saturated and that intensity within a given region was uniform; both criteria were met, as discussed previously (Chang et al., 1992). Thus, autoradiographic intensity at edges (I_{edge}) and DEA (I_{DEA}) regions could be related to total radioactivity (R_{AA}) to calculate the total radioactivity in the individual compartments of edge (R_{edge}) and DEA (R_{DEA}) (both in disintegrations per minute).

To compare ^{125}I activity in edges and in DEA between animals, R_{edge} and R_{DEA} were normalized for MPR. Accumulation (AC) in these compartments (AC_{edge} or AC_{DEA}, microliters in edge or DEA) was calculated as

$$AC_{\text{edge or DEA}} = R_{edge}/\mathrm{MPR}$$

This term represents the plasma volume equivalent of ^{125}I lipoprotein that accumulated in the entire edge or DEA region and is not the same as the clearance term, which represents uptake per unit area of region as defined above.

(*b*) *Statistical Analysis.* Mean values are presented ± standard deviation (SD). Significance was examined by the two-sample t-test for independent samples with equal or unequal variances, as applicable (Rosner, 1990).

RESULTS

Lipoprotein Modifications. Derivatization of LDL lysyl residues with succinic anhydride resulted in replacement of positively charged epsilon-amino groups with negatively charged carboxylic acid groups. Ninety to 95% of the surface-accessible lysyl residues were affected, for a greater than 4-fold increase in relative electrophoretic mobility of this anionized lipoprotein.

Modification with diazobenzenearsonate converted the neutrally charged tyrosyl and histidyl residues of LDL to negatively charged diazobenzenearsonyltyrosyl and diazobenzenearsonylhistidyl residues. The extent of modification was 12–19% for accessible tyrosyl residues and 27–33% for accessible histidyl residues. The increase in electrophoretic mobility was approximately 3-fold, as compared with native LDL.

Modification by incubation with copper cations caused mild free-radical oxidation of LDL lipid (4–12 nmol of TBARS/

mg of protein was detected) with only a small increase in negative charge; the electrophoretic mobility of oxidized LDL was 1.3 relative to that of native LDL.

Reductive methylation of 90–95% accessible lysyl residues in LDL resulted in the formation of dimethyllysyl residues. The net charge of lysine was retained by this modification, and the electrophoretic mobility of methyl LDL was the same as that of the native protein. Methylated LDL is not recognized by any cell surface receptor (Weisgraber et al., 1978).

Treatment with neuraminidase resulted in cleavage of all negatively charged sialic acid residues on the carbohydrate side chains of LDL. In theory, this enzymatic modification should render the LDL more cationic; however, no change in mobility of the modified LDL was detected by paper electrophoresis. Most likely this was a result of the limited sensitivity of the analytical technique since the contribution of sialic acid to the net charge of LDL is minor [LDL contains only 5–9% carbohydrate (Swaminathan & Aledjem, 1976), of which only 10% is sialic acid (Attie et al., 1979)]. Thus, the effect of total cleavage of sialic acid residues on the net charge of LDL was minor and desialated LDL was only weakly cationized. Because of concern that an 18-h incubation at 37 °C might cause undesired lipid peroxidation, all desialated LDL preparations were analyzed for TBARS; results indicated that none of the preparations were oxidized.

Cationization with DMPA converted the negatively charged carboxylic acid functions of aspartic acid and glutamic acid to positively charged amino groups for a net charge change of 2 per residue modified. DMPA-modified LDL was strongly cationized and migrated toward the cathode upon paper electrophoresis, with an R_f of −0.6.

No aggregation of the modified lipoproteins was detected by eye. In five of the six modifications, cross-reactivity with antisera to native LDL was maintained, as evidenced by Ouchterlony immunodiffusion, indicating antigenic determinants of LDL were conserved. Cross-reactivity of DMPA-modified LDL preparations could not be established. While this may have been caused by alteration of the antigenicity as a result of the modification, it was more likely the result of incompatibility of the analytical technique with the sample (electrostatic interactions between the positively charged DMPA LDL and the very slightly negatively charged agarose medium) or nonobvious aggregation of the sample.

Plasma Disappearance of ^{125}I Lipoproteins. Derivatization (except by reductive methylation) accelerated the plasma removal of ^{125}I-labeled modified LDL (Figure 1), although the removal of oxidized and desialated LDLs was only slightly greater than that of native LDL (Figure 1a). Fractional catabolic rates (FCR) were calculated from the disappearance data. As reported previously (Chang et al., 1992), the FCR for native LDL was 0.12 pool/h. FCRs of desialated LDL and oxidized LDL were 1.3 and 1.5 times faster, respectively, than that of native LDL. Plasma disappearance of succinyl LDL, diazobenzenearsonyl LDL, and DMPA LDL (Figure 1b) was markedly accelerated over that of native LDL; for these rapidly removed preparations, less than 10% of the injected dose remained in the plasma after 10 min of circulation. Reductive methylation resulted in a decreased rate of removal of LDL from plasma (Figure 1a). The FCR of ^{125}I methyl LDL was 0.05 pool/h, in agreement with previously reported values (Mahley et al., 1980).

Qualitative and Quantitative Assessment of Endothelial Regeneration. The pattern of endothelial regeneration 4 weeks following balloon catheter injury of the abdominal aorta is shown in the photographs in Figure 2. *In vivo* Evans blue staining of the aortas differentiated still-deendothelialized areas (which stained blue) from reendothelialized islands and the thoracic aorta (which remained white). Four weeks after injury, the mean ±SD (n = 43) extent of healing was 42 ± 13%, and the healing edge constituted 2% or less of the healed areas.

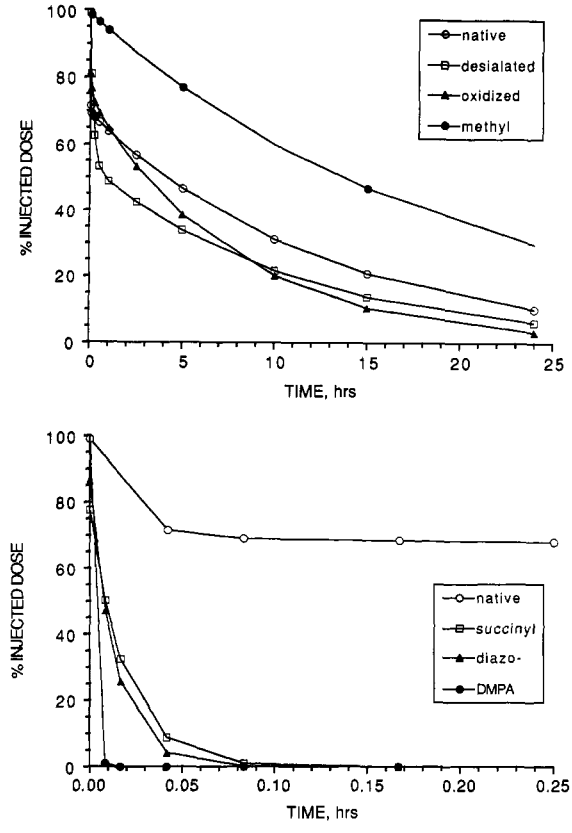

FIGURE 1: (a, top) Plasma disappearance curves of ^{125}I-labeled native, oxidized, desialated, and methyl LDL. Plasma disappearance of ^{125}I native or modified LDL is shown as the percent of initial dose remaining $[(C_p/C_0) \times 100]$ vs time after injection, where C_p was the plasma radioactivity at any time and C_0 was the initial dose determined by extrapolation to zero time of early plasma radioactivity samples. Data from all rabbits injected with ^{125}I native LDL (n = 13), ^{125}I oxidized LDL (n = 6), or ^{125}I desialated LDL (n = 5) were combined to determine the biexponential equation of disappearance (Matthews, 1957) for the given lipoprotein; the curves shown represent the best-fit curves derived by least-squares approximation. Since the disappearance of ^{125}I methyl LDL (n = 3) over the time period of these experiments was monophasic, those data were fit to the monoexponential form of the Matthews equation. (b, bottom) Plasma disappearance curves of ^{125}I-labeled native, succinyl, diazobenzenearsonyl, and DMPA LDL. The average disappearance curves were determined from the combined data of all rabbits injected with ^{125}I native-LDL (n = 13), ^{125}I succinyl LDL (n = 6), ^{125}I diazobenzenearsonyl LDL (n = 5), or ^{125}I DMPA LDL (n = 5). Data for these modified lipoproteins were fit to the monoexponential form of the Matthews equation.

Qualitative Assessment of ^{125}I Lipoprotein Accumulation. A focal pattern of macroscopic autoradiographic radiolabel distribution characteristic of ^{125}I native LDL in the healing arterial wall (Chang et al., 1992; Fischman et al., 1987; Roberts et al., 1983) was seen with some of the modified lipoproteins but not others. No focal accumulation of radioactivity at healing edges was observed for ^{125}I diazobenzenearsonate LDL (Figure 2B), ^{125}I succinyl LDL (not shown), or ^{125}I DMPA LDL (not shown). Radioautographs for the latter two modified LDLs were similar to that in Figure 2B. Focal accumulation of radiolabel was observed for ^{125}I oxidized LDL

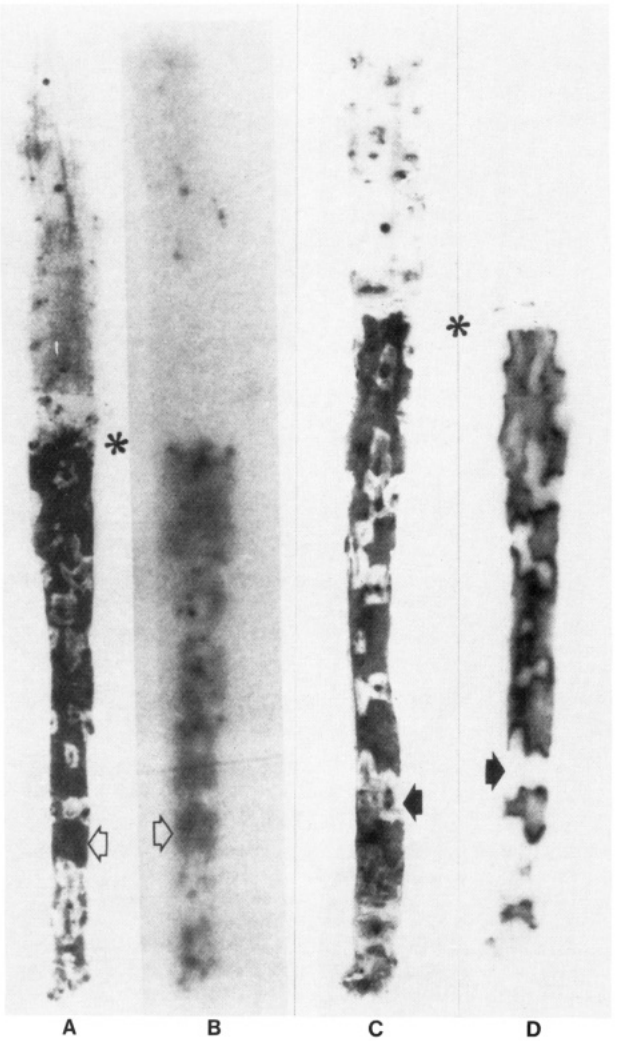

FIGURE 2: Aortic accumulation of ^{125}I diazobenzenearsonate LDL (A, B) in the balloon catheter deendothelialized rabbit aorta and of ^{125}I native LDL in the ballooned aorta of a probucol-fed rabbit (C, D). For each pair of photographs (A, C) and radioautographs (B, D), an asterisk marks the junction of the unballooned thoracic aorta (above the asterisk) and the ballooned abdominal aorta (below the asterisk). Open arrows in (A, B) point to matching deendothelialized areas (DEA are present in both sets of aortas). Solid arrows in (C, D) point to matching healing islands (healing islands are also present in both sets of aortas). Aortic branch arteries are best seen in photograph C and are represented by the small black circular areas in the centers of the white healing islands; branch arteries are present but less easily visible in (A). They are not detectable in radioautographs. (A, B) 165 μCi of ^{125}I diazobenzenearsonate LDL was injected 4 weeks after deendothelialization and allowed to circulate for 1.1 h. Evans blue dye, injected 15 min prior to sacrifice, stained still deendothelialized areas of the abdominal aorta blue, while reendothelialized islands and noninjured thoracic regions remained unstained (A). The corresponding autoradiograph (B) showed only minimal diffuse accumulation of radioactivity in still-deendothelialized regions after 22 days of exposure at –70 °C. (Figure is reproduced here at 75% of its original size.) (C, D) 400 μCi of ^{125}I native LDL was injected into a probucol-fed ballooned rabbit 5 weeks after deendothelialization and allowed to circulate for 10 h. The Evans blue stained aorta is shown in (C). The corresponding autoradiograph (D) showed intense focal accumulation at the edges of healing islands with moderate accumulation in still-deendothelialized regions after 4 days of exposure at –70 °C. (Figure is reproduced here at 75% of its original size.)

(not shown), ^{125}I desialated LDL (not shown), and ^{125}I methyl LDL (not shown). Radioautographs for these focally accumulated modified LDLs were similar to the focal accumulation of ^{125}I native LDL seen in the aortas of probucol-fed ballooned rabbits, as shown in Figure 2D. Five weeks of probucol feeding had no detectable effect on the aortic accumulation of native LDL.

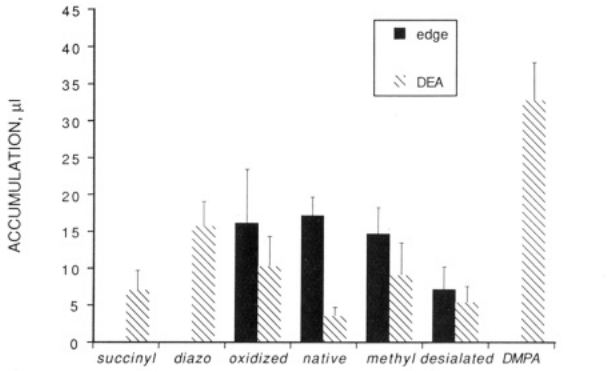

FIGURE 3: Aortic accumulation of ^{125}I-labeled lipoproteins. Accumulation (AC) of ^{125}I-labeled native or modified LDL by the individual compartments of the healing abdominal aorta represented the plasma volume equivalent amount (μL) of radioactivity in the entire edge or the entire DEA regions of the healing abdominal aorta. AC in edge (black bars) and DEA (hatched bars) regions was calculated as described under Experimental Procedures. Mean accumulation (±SD) was calculated by averaging 5 AC_{edge} or AC_{DEA} values from each rabbit for a total of 10–30 values for each animal (n = 2, 6, 5, 6, 3, and 5 rabbits for ^{125}I-labeled native, succinyl, diazobenzenearsonate, oxidized, methyl, and DMPA LDL, respectively.)

Quantitative Assessment of ^{125}I Lipoprotein Accumulation at Edges of Regenerating Endothelium and in Still-Deendothelialized Regions. The total ^{125}I activity in edges (R_{edge}) and in DEA (R_{DEA}) was computed on the basis of the ratio of radioactive intensity ($ratio_I$) at edges (I_{edge}) to that in DEA (I_{DEA}) and the absolute amount of radioactivity in the entire abdominal aorta (R_{AA}). R_{edge} and R_{DEA} then were normalized for mean plasma radioactivity to determine the total accumulation of ^{125}I lipoprotein in each of these compartments (AC_{edge} and AC_{DEA}). The results are shown in Figure 3.

For ^{125}I native LDL, AC_{edge} was 17.17 ± 2.44 μL. AC_{edge} was 0 for ^{125}I-labeled succinyl, diazobenzenearsonyl, and DMPA LDL because no focal ^{125}I activity was found at edges of healing islands with these lipoproteins. For ^{125}I-labeled desialated LDL, AC_{edge} was only 40% (7.17 ± 2.99 μL) of that for native LDL. ^{125}I-Labeled methyl LDL and oxidized LDL were the only modified lipoproteins which had AC_{edge} comparable to that of native LDL (14.69 ± 3.56 and 16.13 ± 7.32 μL, respectively).

For ^{125}I native LDL, AC_{DEA} was 3.55 ± 1.20 μL. AC_{DEA} values for ^{125}I succinyl LDL (7.06 ± 2.64 μL), ^{125}I diazobenzenearsonyl LDL (15.72 ± 3.36 μL), and ^{125}I DMPA LDL (32.74 ± 5.12 μL) were 2.0, 4.4, and 9.2 times greater, respectively, than that for native LDL. For ^{125}I-labeled desialated LDL, AC_{DEA} was 1.5 times higher (5.42 ± 2.11 μL) than for native LDL. For ^{125}I-labeled methyl and oxidized LDL, AC_{DEA} values were 9.07 ± 4.39 and 10.24 ± 4.11 μL, respectively, 2.6 and 2.9 times greater than that for native LDL. Thus, all of the modified lipoproteins accumulated to a greater degree than native LDL in the still-deendothelialized aorta.

DISCUSSION

We have shown previously that accumulation of LDL in experimental rabbit arterial lesions occurs differently at the edges of regenerating endothelial areas than it does in still-deendothelialized areas (Roberts et al., 1983; Chang et al., 1992). Where endothelium is actively regenerating, accumulation of radiolabeled LDL is focal, and the LDL is trapped, or sequestered (Chang et al., 1992), as it is in human ather-

osclerotic lesions (Lees et al., 1988). In contrast, LDL accumulation in deendothelialized areas is diffuse and labile, i.e., in free exchange with plasma LDL (Chang et al., 1992), and thus is less likely to be associated with atherogenesis. One of the major advantages of the rabbit model we have used is that we are able to examine arterial lesions at a very early stage, when LDL is bound focally, but there are as yet no lipid-laden foam cells present, as there are in more advanced arterial lesions. In an effort to determine some of the factors responsible for focal LDL accumulation, the effects of chemical and enzymatic modifications of LDL on *in vivo* accumulation in experimental rabbit lesions were examined.

The results showed that major increases in LDL surface charge in either direction inhibited focal accumulation of LDL at the edges of regenerating endothelial islands, although they did not impede LDL entry into deendothelialized areas. (Measurements were normalized for mean plasma radiolabeled lipoprotein concentrations to correct for differences in dose, plasma concentration, and residence time.) Thus, modified LDL that was either strongly anionized (succinyl and diazobenzenearsonyl LDL) or strongly cationized (DMPA LDL) did not accumulate focally, whereas modified LDL with a surface charge similar to that of native LDL (oxidized, desialated, and methyl LDL) did do so. In contrast, in deendothelialized areas, lipoprotein accumulation was generally related to net molecular charge. The lowest DEA accumulation occurred with succinyl LDL, the most highly anionized lipoprotein, while the highest DEA accumulation occurred with DMPA LDL, a highly cationized lipoprotein. These results suggest that the labile accumulation of LDL in DEA depends on electrostatic interactions with negatively charged extracellular matrix components such as glycosaminoglycans.

The short residence time in plasma of highly modified LDLs may have precluded their accumulation at the healing edges of lesions because edge accumulation occurs more slowly than DEA accumulation (Chang et al., 1992). Against this hypothesis, however, is the observation that with a synthetic peptide derived from apolipoprotein B of LDL, strong focal accumulation was present 24 h after injection, even though less than 10% remained in plasma 30 min after injection (Shih et al., 1990). Nevertheless, we attempted to rule out kinetic factors as reasons for lack of focal LDL accumulation by highly modified LDLs. In pilot experiments not presented here, an attempt was made to distinguish between the contribution of modified LDL plasma removal rates and that of charge change. We were able to prolong the plasma residence time of highly charge-modified LDL by performing short-term portacaval shunts (Yamaguchi et al., 1989) in several ballooned rabbits. No focal accumulation of the modified LDL occurred in these rabbits. However, native LDL did not accumulate focally in rabbits with portacaval shunts either, presumably because of the major systemic perturbations in the animal's physiology which resulted from the trauma of the portacaval shunt. Thus, we were unable to exclude rigorously the possibility that differences in residence time alone were responsible for the differences in arterial lesion accumulation of modified LDLs.

Another possible explanation for the negative effects of some LDL modifications on its accumulation in arterial lesions is that surface charge change may have led to changes in the secondary or tertiary structure of the molecule and that such changes may have inhibited focal LDL accumulation. However, immunoreactivity of LDL was maintained after modification (with the exception of DMPA-modified LDL), as detected by double-immunodiffusion, indicating that conformation was generally preserved.

The localized nature of focal LDL trapping is striking (Chang et al., 1992). Not only is trapping absent in deendothelialized areas, it is also absent where endothelium is undisturbed or completely regenerated. We considered the possible role of cell surface receptors in focal accumulation. The negative effects of chemical modification of lysine residues in LDL on its recognition by cellular receptors *in vitro* have been demonstrated by other investigators (Weisgraber et al., 1978; Mahley et al., 1979a; Haberland et al., 1984). However, in an earlier study, we found that focal accumulation of LDL in arterial lesions of the rabbit aorta occurred with both methylated LDL, which is not recognized by any known LDL receptor, and native LDL (Fischman et al., 1987). Those results were confirmed in the present study, which showed that focal accumulation of methyl LDL was quantitatively similar to that of native LDL. Furthermore, because receptor-bound LDL is rapidly metabolized by cells, it is most unlikely that focal LDL accumulation is intracellular. Thus, it is probable that such binding is to specific sites in extracellular matrix produced by (and perhaps still linked to) cells, rather than to the LDL or scavenger receptors, and that the effect of major charge change in either direction is to inhibit LDL binding to those matrix sites.

Finally, the behavior of copper-oxidized LDL and the effects of probucol feeding on the arterial accumulation of native LDL were of special interest because of recent speculation on the potential role of oxidized LDL in initiating arterial lesions (Jurgens et al., 1987; Yla-Herttuala et al., 1989). Focal accumulation of LDL was not enhanced by copper oxidation. In addition, ballooned rabbits that were fed probucol for 5 weeks after balloon catheterization had no decrease in focal accumulation of native LDL. These results support the conclusion that the initial trapping of LDL in arterial lesions is of native, not oxidized, LDL, although LDL may become oxidized after being trapped and thus help to propagate the disease process.

In summary, the data support the concept that interactions between native LDL and arterial extracellular matrix components associated with regenerating endothelium play a major role in focal LDL accumulation in early lesions. These interactions appear to be mediated by specific oxidation-independent patterns of charge and polarity on LDL which are disrupted by major changes in LDL surface charge.

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