Interaction of Native and Cell-modified Low Density Lipoprotein with Collagen Gel

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We have examined the binding of native and cell-modified low density lipoprotein (LDL) to gels of Type I collagen. Diffusion of native $^{125}$I-LDL into the collagen gel was slow, reaching equilibrium after 24 to 48 hours, while $^1$H-glucose, a low molecular weight marker, equilibrated in 6 hours. Binding of $^{125}$I-LDL was measured at 48 hours as the amount associated with the collagen after extensive washing. Binding was saturable with an increasing concentration of LDL. Prior incubation with cell-free culture medium resulted in modest, but progressive, increases in electrophoretic mobility and binding to collagen. Incubation with cells produced a marked increase in electrophoretic mobility and a 5- to 10-fold increase in collagen binding; the presence of butylated hydroxytoluene during incubation prevented both effects. These changes in LDL were induced by porcine aortic endothelial cells, smooth muscle cells, human skin fibroblasts, and a variety of cell lines, as well as by acetylation. There was a curvilinear relationship between the amount of LDL protein bound and the net negative charge of the LDL; increasing net charge was associated with progressively greater increases in binding. These results suggest a potential role for collagen in trapping lipid in the extracellular matrix of arterial intima by slowing the diffusion of and by binding LDL. The data also demonstrate that binding of LDL to collagen is enhanced by modifications that increase its net negative charge. (Arteriosclerosis 8:525–534, September/October 1988)

Plasma lipoproteins containing apoprotein B are the principal source of lipids that accumulate in atherosclerosis. Recent in vitro studies suggest that low density lipoprotein (LDL) that has been oxidatively modified by exposure to cells (vascular endothelial cells [EC], smooth muscle cells [SMC], macrophages, monocytes, or neutrophils) or to transition metal ions may play a more important role in this process than native LDL. LDL modified in this way is characterized by an increase in density and net negative charge, a loss of esterified cholesterol, hydrolysis of phosphatidylincholine, and partial degradation of apoprotein B, all of which can result from lipid peroxidation by free radicals and can be prevented by antioxidants. In plasma, oxidatively modified LDL may contribute to atherogenesis by virtue of its high rate of uptake by endothelium and perhaps by damaging EC; furthermore, in the arterial wall, modified LDL may recruit circulating monocytes through a chemotactic process, turn macrophages into foam cells via its enhanced uptake by the scavenger receptor, and inhibit the egress of macrophages from the arterial wall.

While cellular metabolism accounts for part of the uptake of LDL by the arterial wall, a large part of the uptake remains in the extracellular space. Smith and Ashall have demonstrated that the concentration of LDL in interstitial fluid of normal intima is twice that of plasma and is even greater in advanced lesions. At least some of the interstitial LDL of intima carries an increase in net negative charge compared with plasma LDL and therefore may be oxidatively modified LDL.

The material surrounding arterial cells includes collagen, elastin, and proteoglycans. The latter, especially their constituent glycosaminoglycan (GAG) side chains, have received much attention because of their ability to bind LDL. Iverius has also suggested that, because of their molecular chain network, GAG may impede the flow of LDL by a molecular sieving effect or by steric interaction, which reduces the solubility of LDL. Several studies have demonstrated the binding of lipoproteins to elastin. Collagen, however, is the major connective tissue component of atherosclerotic arteries, comprising up to 40% of the total protein in fibrous plaques and 60% in advanced lesions. Most of the collagen (50% to 75%) in normal artery or diseased intima is type I. Various methods to extract lipids from aortic wall have not demonstrated a positive association of lipid or apoprotein B with collagen. Nevertheless, the concentration of immobilized LDL is highest in fibrous plaques, and pure collagenase releases a substantial portion of this. Furthermore, immunofluorescent microscopy has located LDL along collagen fibers in fatty streaks and fibromuscular caps. In vitro studies also suggest an ability of collagen to bind lipids derived from LDL and there is some evidence that this interaction is electrostatic in nature. Thus, LDL in which the charge is modified may react with collagen in a different manner than native LDL.

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The present study was designed to examine the in vitro interaction of native and cellularly modified LDL with type I collagen. We have used a collagen gel system to evaluate LDL diffusion into a collagen matrix and to measure its binding to the collagen fibers.

Methods

Cell Culture

The procedure of Gimbrone et al. for releasing EC from umbilical cord veins was adapted for obtaining EC from descending thoracic aortas of young pigs. Porcine aortic SMC were grown from explants of the middle layers of media from the arch region. Cultures of human skin fibroblasts (HSF) were established from single cell suspensions obtained by enzymatic dispersion of normal foreskin dermis of patients ages 7 months to 90 years. Stock cultures of all cell types were grown in 100 mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Incorporated, Mississauga, Ontario) supplemented with 1 mM nonessential amino acids (GIBCO Canada, Burlington, Ontario) and sodium bicarbonate (2.25 g/l). For EC and HSF, the growth medium contained 10% (vol/vol) defined calf bovine serum (supplemented) (HyClone Laboratories, Incorporated, Logan, Utah) heat inactivated at 56°C for 30 minutes. SMC received 10% (vol/vol) fetal bovine serum (FBS) instead of the special calf serum. In addition, after the first day in primary culture, EC received EC growth factor in the form of a crude extract of bovine brain at 150 μg protein/ml medium. For EC and HSF, culture dishes were coated with human plasma fibronectin as previously described. All cell types were passaged at confluence once weekly by exposure to 0.05% trypsin/0.02% disodium EDTA in Earle's salt solution (GIBCO), followed by a wash in serum-containing medium. They were redistributed at a 1:10 (SMC) or 1:20 (HSF, EC) split ratio and given fresh medium every 2 days.

To prepare cultures for incubating with LDL, cells between the second and fifteenth passages were seeded in 60 mm tissue culture dishes 1 week before use and grown to confluence under conditions described above for stock cultures.

Madin-Darby canine kidney cells (MDCK) and rat hepatoma cells (H4-II-E-C3) were from the American Type Culture Collection (ATCC, Rockville, Maryland) and were maintained for other purposes in this laboratory. They were cultured under conditions recommended by the ATCC. Human nongenital skin fibroblasts (from a 0.22 μm membrane, were stored in the dark at 4°C, and were used within 3 weeks.

Preparation of Low Density Lipoprotein

Samples of normolipemic serum from the diagnostic laboratories of the Jewish General Hospital were pooled. LDL was isolated from this serum after addition of 0.01% disodium EDTA by precipitation with heparin and MnCl₂. To remove the very low density lipoprotein (VLDL) that coprecipitated, the pellet was redissolved in cold NaCl at density 1.084 g/ml and was pipetted into ultracentrifuge tubes under a layer of warm NaCl at density 1.006 g/ml; after centrifugation in an SW40 rotor at 35 000 rpm for 24 hours at 15°C, the VLDL formed a layer at the top and the golden LDL layer was removed from the center of each tube by puncture with a needle and syringe. The pooled LDL layers were dialyzed exhaustively against 0.9% NaCl:0.01% EDTA (pH 8.6). LDL isolated by this process retained its normal density range of 1.019 to 1.063 g/ml. For certain experiments as noted, LDL was prepared by centrifugation in KBr solutions as the fraction of density between 1.019 and 1.063 g/ml. Protein concentration was determined by the method of Lowry et al. and the purity of the LDL preparation was confirmed by electrophoresis on agarose gel stained for lipid and by electrophoresis on cellulose acetate stained for protein and quantitated by scanning densitometry.

Freshly prepared LDL was iodinated (¹²⁵I-LDL) with carrier-free Na¹²⁵I (Amersham Canada Limited, Oakville, Ontario) according to the iodine monochloride procedure of McFarlane as modified by Shepherd et al. After dialysis against the NaCl: EDTA buffer, less than 3% was extractable in chloroform/methanol (2:1). The final specific activities ranged from 90 to 150 cpm/ng LDL protein.

Acetylated LDL was prepared as described by Basu. All LDL preparations were sterilized by filtration through a 0.22 μm filter and stored in the dark at 4°C. For purposes of electrophoresis, cold LDL (200 μg protein/ml) was incubated for 24 hours with or without cells, and the conditioned medium was concentrated to
of total GAG was 500 μg/ml.

The final concentration in barbitral buffer with a Panagel Electrophoresis Reagent (Princeton Separations Incorporated, Adelphia, NJ) as specified by the manufacturer for lipoprotein electrophoresis. Of the concentrated medium containing modified LDL, 20 μl was applied to the agarose gel slide. Native (unincubated) LDL was diluted to the same concentration and was applied as a control in each electrophoretic run. Dried gels were stained with Sudan Black B dye according to Johansson. Distance traveled by each sample was measured in millimeters from the origin to the front of the band. Relative mobility was expressed as a ratio of the migration distance of sample to that of native LDL.

For most experiments, EC were used to modify LDL. 125I-LDL incubated with EC was verified by its increased buoyant density on a sucrose gradient to a peak density of 1.0754 g/ml and by its increased electrophoretic mobility on agarose gel to an average of 2.5 times the distance travelled by native LDL. This will be referred to as EC-modified LDL. The peak buoyant density of CF-modified LDL was slightly increased (1.0537 g/ml); its electrophoretic mobility also increased slightly to an average of 1.5 times that of native LDL.

**Preparation of Collagen Gels**

Type I collagen was extracted from rat-tail tendon as described by Eisdale and Bard but with dialysis against water adjusted with acetic acid to pH 4.0 instead of against dilute medium. All reagents and equipment for the extraction were sterilized. The final collagen concentration of individual preparations as determined by dry weight was 2 to 2.5 mg/ml.

Collagen gels were prepared by mixing rapidly on ice the collagen solution, the concentrated DMEM, and water to achieve the desired final concentration of collagen (generally 1 mg/ml) in 1 x DMEM. One milliliter aliquots of this solution were pipetted into 35 mm culture dishes and incubated at 37°C in 5% CO2/95% air to allow the collagen to gel completely. After 1 hour, the gels were covered with the overlying medium with radioactive markers. The percent of total radioactivity that was contained within the gel was measured at intervals up to 48 hours. The presence of 500 μg/ml of mixed GAG had no additional effect on the rate of 125I-LDL diffusion.

**Results**

**Diffusion of Low Density Lipoprotein Into Collagen Gel**

The collagen gel system consisted of two compartments: the gel, containing 1 ml total volume of collagen fibers and interstitial medium and a 1 ml volume of overlying medium with radioactive markers. The percentage of total radioactivity that was contained within the gel compartment was determined at intervals up to 48 hours after addition of markers to the overlying medium (Figure 1). Although the diffusion of L-3H-glucose from the overlying medium into the interstitial medium of the gel matrix had reached equilibrium by 6 hours, native 125I-LDL diffused into the gel more slowly, reaching equilibrium only after 24 to 48 hours. The presence of 500 μg/ml of mixed GAG had no additional effect on the rate of 125I-LDL diffusion.

EC-modified 125I-LDL initially entered the gel at the same rate as native 125I-LDL up to 4 hours but was retained within the gel matrix; after 24 hours, 60% was in the gel compartment (Figure 2). CF-modified 125I-LDL behaved similarly to native LDL.
Time (h)

Figure 1. Diffusion of 125I-LDL and L-3H-glucose into collagen gel. Collagen gels (1 mg/ml/dish) were prepared in 35 mm culture dishes. DMEM (1 ml) containing L-3H-glucose (broken line) or 125I-LDL (solid line) was layered on top of the gel at time 0 and the dishes were incubated at 37°C. Half of the dishes contained 500 μg/ml mixed GAG in the gel and overlying medium (■) and half were without GAG (□). At the times indicated, replicate dishes were analyzed for the amount of radioactivity in the overlying medium and gel compartments as described in Methods. Each point represents the average percent of total radioactivity in the gel compartment of three experiments (coefficient of variation among experiments was less than 10%).

Time (h)

Figure 3. Time course of binding of native and EC-modified 125I-LDL to collagen. Collagen gels (1 mg/ml/dish) were prepared in DMEM and incubated for 48 hours at 37°C with 1 ml overlying medium containing EC-modified (■) or CF-modified (□) 125I-LDL (50 μg protein). Radioactivity was measured in washed collagen gels. Points are means ± SD of two experiments, each with four replicates.

Binding of Low Density Lipoprotein to Collagen Gel

The time course of binding of 125I-LDL to collagen (Figure 3) indicates that binding was not complete by 48 hours; however, binding was routinely measured at 48 hours because at this time the amount bound was directly proportional to the concentration of the collagen gel over the range of 0.1 to 2 mg/ml of collagen (Figure 4). The amount of native or modified 125I-LDL bound increased with increasing concentration of 125I-LDL in the medium and in each case appeared to approach a saturation level (Figure 5). The amount of modified LDL bound was considerably greater than that of native LDL.

To determine if the increased capacity of EC-modified LDL to bind to collagen depended on the prior exposure of LDL to EC themselves or to factors secreted by EC into the medium, native 125I-LDL was incubated with F-12 medium previously exposed to an EC monolayer for 24 hours; when incubated with collagen gel (1 mg/ml), LDL (33 μg LDL protein/ml) bound to the same extent as 125I-LDL previously incubated in fresh medium (0.68 μg vs. 0.62 μg of LDL protein/mg collagen, respectively). The amount of CF-modified 125I-LDL bound to collagen could be increased by longer incubation times in F-12

binding of native and modified 125I-LDL into collagen gel. Collagen gels (1 mg/ml/dish) were prepared in 35 mm culture dishes. DMEM (1 ml) containing L-3H-glucose (broken line) or 125I-LDL (solid line) was layered on top of the gel at time 0 and the dishes were incubated at 37°C. Half of the dishes contained 500 μg/ml mixed GAG in the gel and overlying medium (■) and half were without GAG (□). At the times indicated, replicate dishes were analyzed for the amount of radioactivity in the overlying medium and gel compartments as described in Methods. Each point represents the average percent of total radioactivity in the gel compartment of three experiments (coefficient of variation among experiments was less than 10%).

Time (h)

Figure 2. Diffusion of native and modified 125I-LDL into collagen gel. Collagen gels were prepared as described in the legend for Figure 1. At time 0, 1 ml of DMEM containing 20 μg protein of native (△), CF-modified (□), or EC-modified (■) 125I-LDL was layered over each gel. After incubation at 37°C, replicate dishes were analyzed for the percentage of total radioactivity that was in the gel compartment at the times indicated. Each point represents the mean of four replicates (coefficient of variation, 10% or less). The data are from one experiment which was representative of three separate experiments.

Figure 4. Effect of collagen concentration on binding of EC-modified 125I-LDL to collagen. Collagen gels were prepared in DMEM at the indicated concentrations and incubated for 48 hours at 37°C with 1 ml overlying medium containing EC-modified 125I-LDL (40 μg protein). Radioactivity was measured in washed collagen gels. Points are the means ± SD of four replicates.

The time course of binding of 125I-LDL to collagen (Figure 3) indicates that binding was not complete by 48 hours; however, binding was routinely measured at 48 hours because at this time the amount bound was directly proportional to the concentration of the collagen gel over the range of 0.1 to 2 mg/ml of collagen (Figure 4). The amount of native or modified 125I-LDL bound increased with increasing concentration of 125I-LDL in the medium and in each case appeared to approach a saturation level (Figure 5). The amount of modified LDL bound was considerably greater than that of native LDL.

To determine if the increased capacity of EC-modified LDL to bind to collagen depended on the prior exposure of LDL to EC themselves or to factors secreted by EC into the medium, native 125I-LDL was incubated with F-12 medium previously exposed to an EC monolayer for 24 hours; when incubated with collagen gel (1 mg/ml), LDL (33 μg LDL protein/ml) bound to the same extent as 125I-LDL previously incubated in fresh medium (0.68 μg vs. 0.62 μg of LDL protein/mg collagen, respectively). The amount of CF-modified 125I-LDL bound to collagen could be increased by longer incubation times in F-12.
medium (Table 1). This increase was accompanied by a progressive increase in LDL mobility on agarose gel. Nevertheless, the electrophoretic mobility and amount bound to collagen of 125I-LDL incubated with EC for 24 hours in the same medium were greater than those of 125I-LDL incubated without cells for up to 72 hours. Changes in binding and in electrophoretic mobility by EC modification were similar for LDL prepared by heparin-manganese precipitation and by ultracentrifugation in a density gradient (Table 1). These effects on LDL of exposure to EC or to cell-free medium could be prevented by the addition of butylated hydroxytoluene (BHT) to the medium during incubation (Table 2) and, therefore, were probably due to oxidation.

To establish whether the cell-induced changes in binding of LDL to collagen were specific to EC, we compared binding of 125I-LDL previously incubated with EC, SMC, or HSF. All cell types modified LDL as indicated by LDL's increased electrophoretic mobility on agarose gel (Figure 6) and by increased binding to collagen gel matrix. Because others have reported that skin fibroblasts incubated in F-10 medium do not modify LDL, we compared LDL modification by the three cell types in F-10 and F-12 media. Although the effects were smaller in F-10 than in F-12, both media permitted modification of LDL by all cell types, as indicated by electrophoretic mobility. Furthermore, collagen binding of HSF-modified 125I-LDL in both media was greater than that of SMC- and EC-modified 125I-LDL (Figure 7). We tested several strains of human foreskin fibroblasts, as well as human nongenital skin fibroblasts, with similar results. We also measured the electrophoretic mobility and collagen binding of 125I-LDL incubated with several cell lines unrelated to the vascular system. In all cases, incubation of 125I-LDL with these cells resulted in an increase in its electrophoretic migration on agarose gel and in its binding to collagen matrix to a degree similar to or greater than EC (Table 3).

The changes in oxidatively modified LDL are similar to those in acetylated LDL in terms of uptake by specific cell receptors on macrophages. To determine if acetylation of LDL also changed its capacity to bind to collagen in a manner similar to oxidative modification, we measured the binding of various concentrations of acetylated 125I-LDL incubated in KBr, density 1.019 to 1.063 g/ml. Qualitatively, the binding resembled that of EC-modified 125I-LDL in that the amount of acetylated 125I-LDL bound per mg collagen was dependent on its concentration (Figure 8). The modification by acetylation was greater than that induced by incubation with EC for both binding to collagen and relative electrophoretic mobility (3.8x native for acetylated LDL, 2.9x native for EC-modified LDL in this experiment).

For all types of modified LDL combined, the relationship between the electrophoretic migration relative to that of

### Table 1. Effect of Incubation Time on Relative Electrophoretic Mobility and Binding to Collagen Gel of Modified Low Density Lipoprotein

<table>
<thead>
<tr>
<th>LDL preparation</th>
<th>Incubation time (hrs)</th>
<th>Relative electrophoretic mobility</th>
<th>125I-LDL bound (µg protein/mg gel protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0</td>
<td>1.0</td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td>CF-modified</td>
<td>6</td>
<td>1.1</td>
<td>0.84 ± 0.10</td>
</tr>
<tr>
<td>CF-modified</td>
<td>12</td>
<td>1.1</td>
<td>0.82 ± 0.05</td>
</tr>
<tr>
<td>CF-modified</td>
<td>24</td>
<td>1.3</td>
<td>1.09 ± 0.12</td>
</tr>
<tr>
<td>CF-modified</td>
<td>48</td>
<td>1.7</td>
<td>2.17 ± 0.14</td>
</tr>
<tr>
<td>CF-modified</td>
<td>72</td>
<td>2.0</td>
<td>4.95 ± 0.27</td>
</tr>
<tr>
<td>EC-modified</td>
<td>24</td>
<td>2.4</td>
<td>10.47 ± 0.55</td>
</tr>
<tr>
<td>Native (KBr)</td>
<td>0</td>
<td>1.0</td>
<td>0.63 ± 0.13</td>
</tr>
<tr>
<td>CF-modified (KBr)</td>
<td>24</td>
<td>1.2</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>EC-modified (KBr)</td>
<td>24</td>
<td>2.5</td>
<td>14.81 ± 1.24</td>
</tr>
</tbody>
</table>

LDL (200 µg protein/ml) was incubated in 2 ml F-12 medium for various lengths of time without cells (CF) or for 24 hours with endothelial cells (EC). Mobility of modified LDL, relative to native LDL on agarose gel electrophoresis and the amount of radioactivity associated with washed collagen gels (1 mg/ml) 48 hours after the addition of 125I-LDL (50 µg protein) to the overlying medium were assessed as described in Methods. Binding data are the means ± SD of four replicates. (KBr) indicates that the LDL was prepared by ultracentrifugation in KBr, density 1.019 to 1.063 g/ml.
Table 2. Effect of BHT on Relative Electrophoretic Mobility and Binding to Collagen Gel of Low Density Lipoprotein Preparations

<table>
<thead>
<tr>
<th>LDL preparation</th>
<th>BHT during incubation</th>
<th>BHT added after incubation</th>
<th>Relative electrophoretic mobility</th>
<th>$^{125}$I-LDL bound (µg protein/mg gel protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
<td>0.82 ± 0.06</td>
</tr>
<tr>
<td>CF-incubated</td>
<td>-</td>
<td>-</td>
<td>1.4</td>
<td>1.08 ± 0.13</td>
</tr>
<tr>
<td>CF-incubated</td>
<td>+</td>
<td>-</td>
<td>1.2</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>CF-incubated</td>
<td>-</td>
<td>+</td>
<td>1.3</td>
<td>1.18 ± 0.02</td>
</tr>
<tr>
<td>EC-incubated</td>
<td>-</td>
<td>-</td>
<td>2.4</td>
<td>9.92 ± 0.11</td>
</tr>
<tr>
<td>EC-incubated</td>
<td>+</td>
<td>-</td>
<td>1.4</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>EC-incubated</td>
<td>-</td>
<td>+</td>
<td>2.5</td>
<td>9.80 ± 0.08</td>
</tr>
</tbody>
</table>

LDL (200 µg protein/ml) was incubated for 24 hours in 2 ml F-12 medium, with endothelial cells (EC) or without cells (CF); butylated hydroxytoluene (BHT) was added to a concentration of 20 µM during or after the incubation. Mobility of modified LDL relative to native LDL on agarose gel electrophoresis and the amount of radioactivity associated with washed collagen gels (1 mg/ml) 48 hours after the addition of $^{125}$I-LDL (50 µg protein) to the overlying medium were assessed as described in Methods. Binding data are the means ± SD of four replicates.

Figure 6. Agarose gel electrophoresis of native and modified LDL. LDL (200 µg protein/ml) was incubated for 24 hours in F-12 medium with porcine aortic endothelial cells (EC), porcine aortic smooth muscle cells (SMC), human foreskin fibroblasts (HSF), or without cells (CF) and prepared for electrophoresis as described in Methods. Native, unincubated LDL (N) served as the control. The arrow indicates the origin. The amount of modified $^{125}$I-LDL bound to collagen gel protein in this experiment (50 µg $^{125}$I-LDL protein/dish) was for CF, 2.00 ± 0.07 µg/mg, for HSF, 15.58 ± 0.35, for SMC, 11.25 ± 0.27, for EC, 12.02 ± 0.44 (means ± SD, n = 4).

Figure 7. Binding to collagen gel of $^{125}$I-LDL modified by incubation with various cell types in Ham's F-10 and F-12 media. $^{125}$I-LDL (200 µg protein/ml) was incubated in 2 ml of F-10 or F-12 medium without cells (CF) or with human foreskin fibroblasts (HSF), porcine aortic smooth muscle cells (SMC), or porcine aortic endothelial cells (EC) for 24 hours. Binding to collagen gels (1 mg/ml) was measured 48 hours after addition of modified $^{125}$I-LDL (50 µg protein/dish). Means ± SD of four replicates are shown. Numbers above bars are the migration distance of modified LDL on agarose gel electrophoresis relative to native LDL.

Discussion

The concentration of LDL in arterial intima, especially in fibrous plaques, is higher than in plasma.22 One of the proposed mechanisms by which LDL may accumulate in the extracellular space of arterial intimas during atherogenesis is that GAG may act as a molecular sieve, thus increasing the intimal LDL concentration relative to plasma LDL.30,31 This hypothesis is based on the observation that hyaluronic acid at concentrations of 0.3% (wt/vol) or less slows the flotation rates of serum lipoproteins during CsCl ultracentrifugation.30 In the present study, the diffusion of $^{125}$I-LDL, both native and oxidatively modified, from overlying medium into the interstitial medium of a 0.1% (wt/vol) native collagen gel was retarded compared with that of small molecules measured by the diffusion of L-3H-glucose. Thus, the collagen matrix exerted a molecular sieving effect. The effective "pore" size of the gel was large enough that the diffusion of albumin was not retarded.91 We found that 0.05% (wt/vol) mixed GAG added to the collagen gel and overlying medium had no further effect on the diffusion rate of $^{125}$I-LDL. This ratio of GAG/collagen (0.5:1) was greater than that found by Smith37 in normal intima and fatty streaks (0.11), fibrous plaques (0.06), or calcified plaques (0.02). Therefore,
Table 3. Relative Electrophoretic Mobility and Binding to Collagen Gel of Low Density Lipoprotein Incubated with Various Cell Types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Relative electrophoretic mobility</th>
<th>125I-LDL bound (μg protein/mg gel protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free</td>
<td>1.3</td>
<td>0.64 ± 0.09</td>
</tr>
<tr>
<td>Porcine aortic endothelial cells</td>
<td>2.5</td>
<td>6.87 ± 0.45</td>
</tr>
<tr>
<td>Rat hepatoma</td>
<td>2.6</td>
<td>6.56 ± 0.62</td>
</tr>
<tr>
<td>Rabbit VX2 carcinoma</td>
<td>2.8</td>
<td>9.47 ± 0.73</td>
</tr>
<tr>
<td>Chinese hamster lung fibroblasts</td>
<td>2.9</td>
<td>11.40 ± 0.87</td>
</tr>
<tr>
<td>Madin-Darby canine kidney</td>
<td>2.9</td>
<td>(not tested)</td>
</tr>
</tbody>
</table>

LDL (200 μg protein/ml) was incubated in 2 ml F-12 medium for 24 hours without cells or with the type of cell indicated. Mobility of modified LDL relative to native LDL on agarose gel electrophoresis and the amount of radioactivity associated with washed collagen gels (1 mg/ml) 48 hours after the addition of 125I-LDL (50 μg protein) to overlying medium were assessed as described in Methods. Binding data are the means ± SD of four replicates.

Figure 8. Binding to collagen gel of acetylated 125I-LDL (x), 125I-LDL incubated with EC (□) or without cells (○), and native 125I-LDL (△). Radioactivity remaining with washed collagen gels (1 mg/ml) was measured 48 hours after addition to 1 ml of overlying medium of modified or native 125I-LDL at the indicated concentrations. Points represent the means of four replicates (coefficient of variation, less than 10%).

Figure 9. Relationship of relative electrophoretic mobility on agarose gel to collagen binding of all types of modified LDL. Data are from Tables 1 and 2, Figures 6, 7, and 8, as well as other experiments in which 125I-LDL (50 μg protein/dish) was added to medium overlying collagen gels (1 mg/ml). Radioactivity associated with washed collagen gels was measured 48 hours after addition of LDL. The curve was fitted by the method of least squares. For all points combined, y = 0.6655x^{0.9663}, r^2 = 0.92, where y = 125I-LDL bound, x = relative migration, for all points except that for acetylated LDL, y = 0.6656x^{1.0248}, r^2 = 0.91.

LDL interaction with collagen gel

We conclude from the results reported here that type I collagen may play a direct role in trapping LDL in the arterial intima, both by slowing its passage through the extracellular matrix and by binding it.

Binding was greatly increased by oxidative modification of the LDL molecule by cultured cells. The changes necessary for the increase in binding were mediated by free radicals because the increase did not occur when BHT was present during LDL processing. Furthermore, the effects produced by exposure to cultured cells required exposure to the cells themselves, rather than to products secreted by the cells because LDL was not modified by cell-conditioned medium. The extent of binding was also increased by incubation of native LDL in cell-free medium, but to a much smaller degree than by incubation with cells. It is possible that binding of native LDL resulted from a mild degree of oxidation occurring in vivo (in the donors) or subsequently during experimental procedures; it is likely that the subsequent increase in binding ability after exposure to cell-free medium was due to further oxidation.

Cell-mediated changes in the LDL molecule include lipid peroxidation, blocking of lysine ε-amino groups by lipid decomposition products, loss of lipids, and fragmentation of the apoprotein.17,18,63,64 These are reflected in an increase in net negative charge and density and in more rapid degradation by macrophages.5,6,7,9,17,18,63,64 To these consequences of LDL oxidation may be added an...
increased ability to bind to type I collagen. The precise mechanistic relationships between the structural changes and the various consequences are not known. A significant degree of lipid peroxidation may occur in LDL incubated with EC in F-10 medium containing EDTA without a change in LDL degradation by macrophages,17 while an increase in degradation by macrophages may occur without lipid peroxidation.69 At the present time, we have no explanation for the mechanism of the increase in binding to collagen by modified LDL. Haberland et al.66 have shown that the increase in LDL uptake by macrophages can be induced by malondialdehyde modification of a specific group of lysine residues, which constitute only 16% of the total lysine of the LDL. This is associated with a minimal change in electrophoretic mobility. Further modification of lysines causes a progressive linear increase in LDL mobility with no further change in macrophage uptake.66 The relationship between electrophoretic mobility and binding to collagen was quite different, as increasing mobility was associated with progressively greater changes in binding. Thus, the sites involved in collagen binding are apparently unrelated to the binding sites for the macrophage receptor. The nature of the curve relating collagen binding to mobility suggests positive cooperativity of binding; this might be due to progressive binding-induced changes in collagen conformation that expose an increasing number of reactive sites or, more likely, to progressive modification of lysine residues that leads to an increasing change in conformation and exposure of reactive sites on the LDL.

Henriksen et al.5,6,7 reported that human skin fibroblasts were unable to increase the density of LDL (presumably the result of removal of lipid) or to increase its rate of degradation by macrophages. Morel et al.8 found no difference in electrophoretic mobility of LDL incubated with fibroblasts. However, we observed that a wide variety of cell types, including HSF, were capable of altering the electrophoretic mobility of LDL and its binding to collagen. The discrepancies may be related to the culture media used during exposure of LDL to the cells. Transition metals which facilitate free radical reactions play an important role in cell-mediated modification of LDL: copper and iron have been shown to promote modification by EC and SMC,12,13 and, when the concentration is high enough, copper alone, in the absence of cells, can bring about the same modification.64 Morel et al.8 used medium 199, which contains no copper or zinc and a lower concentration of iron than F-10 and F-12; Henriksen et al.5,6,7 used F-10, which has a much lower zinc content than F-12 and which we found to be less effective than F-12 and which we found to be less effective than 

render it highly susceptible to entrapment in the extracellular matrix by its binding to collagen. Furthermore, this study illustrates the potential importance of type I collagen in contributing to the retention of lipid in the arterial wall. By slowing the passage of LDL, collagen may increase LDL concentration, thus favoring its binding to collagen, as suggested here, and possibly to other extracellular elements. The binding of LDL, in turn, would increase the amount of extracellular lipid, contributing to the development of atherosclerotic plaques.

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