Small dense low density lipoprotein has increased affinity for LDL receptor-independent cell surface binding sites: a potential mechanism for increased atherogenicity[1]

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Abstract Small dense low density lipoprotein (LDL) particles have altered apolipoprotein (apo) B conformation and lowered affinity for the LDL receptor (*J. Biol. Chem.* 1994. 269: 511–519). Herein, we examine the interaction of small dense LDL with cell LDL receptor-independent binding sites. Compared to normal LDL, at low LDL cell media concentrations (<10 μg/ml), small dense LDL had decreased specific binding to the LDL receptor on normal fibroblasts at 4°C, but a 2-fold increased binding to LDL receptor-independent cell sites. At higher LDL concentration (100 μg/ml), LDL receptor-independent binding of small dense LDL was 4.5-fold that of normal LDL in normal fibroblasts, but greater (2- to 14- fold) in LDL receptor-negative fibroblasts. In LDL receptor-negative fibroblasts at 37°C, small dense LDL had higher (3-fold) cell association than normal size LDL but no effective LDL degradation. At high LDL concentrations (≥100 μg/ml), LDL binding to normal or LDL receptor-negative fibroblasts was not affected by several anti-apoB monoclonal antibodies or by cell pretreatment with proteases, chondroitinase, or neuraminidase. In contrast, pretreating normal and receptor-negative fibroblasts with heparinase and heparitinase decreased LDL cell binding by 35% and 50%, respectively. Similarly, preincubation of receptor-negative fibroblasts with sodium chlorate, an inhibitor of proteoglycan sulfation, decreased LDL binding by about 45%.■ We hypothesize that small dense LDL might be more atherogenic than normal size LDL due to decreased hepatic clearance by the LDL receptor, and enhanced anchoring to LDL receptor-independent binding sites in extrahepatic tissues (e.g., the arterial wall), a process mediated, in part, by cell surface proteoglycans.— Galeano, N. F., M. Al-Haideri, F. Keyserman, S. C. Rumsey, and R. J. Deckelbaum. **Small dense low density lipoprotein has increased affinity for LDL receptor-independent cell surface binding sites: a potential mechanism for increased atherogenicity.** *J. Lipid Res.* 1998. 39: 1263–1273.

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Low density lipoprotein (LDL) is the major carrier of cholesterol in human plasma and increased levels of LDL cholesterol are associated with higher risk for atherosclerosis (1). LDL is catabolized mainly in the liver through the high affinity binding of apoprotein B (apoB) to the LDL receptor followed by cellular endocytosis, lysosomal hydrolysis of the lipid moiety and degradation of apoB (2). Still, substantial amounts of LDL, 30–50%, are removed from plasma by LDL receptor-independent pathways that may be particularly important in non-hepatic tissues (3, 4). As the LDL receptor pathway saturates at relatively low LDL concentrations, Goldstein, Basu, and Brown (5) suggested that the relatively "normal" LDL concentrations in humans are, in fact, "unphysiologically high" and may be responsible for the accumulation of LDL in the arterial intima via LDL receptor-independent processes. In vitro studies have shown that, in addition to the LDL receptor, LDL can also bind, with low affinity, to LDL receptor-independent cell surface sites, especially at high LDL concentrations. This non-receptor-mediated LDL binding is classically measured as the LDL binding to the cell surface in the presence of 25–50 times excess of non-radiolabeled ligand (to occupy the high affinity LDL receptor binding sites) and accounts for up to 10% of the total LDL binding to the cell surface in fibroblasts (6). The LDL receptor-independent-mediated LDL cell uptake does not regulate intracellular cholesterol metabolism, suggesting a

different intracellular pathway for LDL internalized through the LDL receptor as compared to other sites on the cell surface (7).

The sites and mechanisms of LDL degradation via LDL receptor-independent pathways in vivo have not been well defined or characterized (3, 8). Studies in animals have shown that although the liver is the principal organ in clearing LDL mainly via the LDL receptor, 60–70% of the LDL clearance via LDL receptor-independent-mediated pathways occurs in extrahepatic tissues (4). Although the properties of the low affinity LDL receptor-independent-mediated binding sites have not been fully characterized, this metabolic LDL pathway is likely important in a number of non-hepatic tissues, such as the arterial wall.

Both normal and dyslipidemic individuals show heterogeneity in the size and density of LDL (9–13). Based on these differences, two distinct LDL phenotypes, A and B, have been described (14, 15), the former characterized by LDL particles of normal or large size, the latter by a predominance of small dense LDL particles. Genetic factors determine, in part, LDL size (16) although age, gender, and diet also can contribute (17). Plasma triglyceride levels demonstrate a major, inverse relationship with LDL size (13, 18).

Small dense LDL has been reported to be a risk factor for atherosclerosis (14, 15, 18, 19) and frequently associates with other atherogenic conditions, such as hyperbetalipoproteinemia (12), familial combined hyperlipidemia (20), and Syndrome X (including hypertriglyceridemia, low levels of HDL, insulin resistance, and hypertension) (21). Recently, we demonstrated that small dense LDL had lower affinity for the LDL receptor, and this correlated with changes in overall apoB conformation, as well as altered configuration of the apoB receptor recognition domain (22). We also observed that lower binding of small dense LDL to the LDL receptor was accompanied by an apparent decrease in the number of LDL receptors (B_{max}) available for the binding of small dense LDL (22). We now question whether small dense LDL, in addition to having a lower affinity for the LDL receptor, could also have an increased binding to low affinity, LDL receptor-independent cell binding and, thus, be more atherogenic. In this paper we demonstrate that lower affinity of small dense LDL for the LDL receptor is accompanied by increased binding to low affinity, high capacity LDL receptor-independent cell binding sites, a process mediated, in part, by cell surface heparan sulfate proteoglycans (HSPG).

MATERIAL AND METHODS

Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from HyClone Laboratories (Logan, UT). Penicillin, 100,000 units/ml, 10,000 μg/ml streptomycin, and 200 mm l-glutamine were bought from Hazelton (St. Seneca, KS). Aprotinin (A 1153), bovine serum albumin fraction V (A 2153), HEPES (H 9136), pronase (P5147), and trypsin (T9003) were obtained from Sigma (St. Louis, MO). Chondroitinase (100330), heparinase (100700), heparitinase (100703), and neuraminidase (120052) were purchased from Seikagaku Kogyo (Tokyo, Ja-

pan). Na^{125}I was obtained from Amersham Corporation (Arlington Heights, IL). Lipoprotein lipase isolated from bovine milk was a generous gift from Dr. Ira Goldberg, (Columbia University, NY).

Cells

Normal human skin fibroblasts (HS 68) were obtained from the American Type Culture Collection (Rockville, MD); LDL receptor negative fibroblasts (GM2000 and 01915C) from individuals with homozygous familial hypercholesterolemia (FH), were purchased from Coriell (Camden, NJ). Cells were plated from a frozen stock of cells (6th to 12th passage) in 12-well plates and grown in monolayers in Dulbecco's modified Eagle's medium, 1% glutamine (v/v), 1% penicillin/streptomycin (v/v), and 10% fetal bovine serum (v/v). On day 5 of culture, the fetal bovine serum of the medium was changed to 5% (v/v) lipoprotein deficient serum (d < 1.21 g/ml). Experiments were performed when cells were near confluence on day 7 of culture.

Lipoproteins

Normal LDL. LDL was isolated by sequential centrifugation (1.025 < d < 1.050 g/ml) of fasting plasma from normolipidemic donors (plasma total cholesterol 153 ± 16 mg/dl, triglyceride 103 ± 40 mg/dl, mean ± SD, n = 6) to which an antiproteolytic mixture (25 μl/ml of plasma) was added to reach a final concentration of 1.2 g/l EDTA, 0.1 g/l NaN$_3$, and 100,000 kallikrein inhibitory units of aprotinin/l. Plasma density was adjusted with NaBr. Ultracentrifugation was performed in a Ti-50.3 rotor from Beckman Co. (Fullerton, CA) at 45,000 rpm for 20 h, after which the top fraction was washed by an additional centrifugation under the same conditions. Normal LDL was also isolated by non-equilibrium rate zonal ultracentrifugation with a discontinuous NaBr gradient of 1.0–1.3 g/ml in a Ti-14 zonal rotor from Beckman Co. (Fullerton, CA) for 170 min as described (22). Previous experiments revealed no differences in lipid composition, apoB structure, or binding to the LDL receptor comparing LDL isolated by either method (22). LDL was then dialyzed in 0.19 m NaCl, 0.25 mm EDTA, pH 7.4, and filtered using a 0.45 μm filter from Gelman, Sciences (Ann Arbor, MI). All LDL isolation procedures were done at 4°C and LDL, layered under argon gas, was used for experiments within 7 days.

Small dense LDL. Plasma from six chronic hypertriglyceridemic subjects (plasma triglyceride 731 ± 106 mg/dl, cholesterol 341 ± 71 mg/dl), prepared as above, was spun at 45,000 rpm for 18 h, at d 1.006 g/ml to remove triglyceride-rich particles. The plasma infranate was used for LDL isolation by non-equilibrium rate zonal ultracentrifugation. Different density fractions of the LDL effluent volume peak were collected and concentrated under vacuum, then dialyzed, filtered, and stored as described above. In addition, fractions of normal and small dense LDL from normolipidemic individuals were also isolated by zonal centrifugation, at rotor effluent volumes between 180–200 ml and 200–240 ml, respectively.

^{125}I-labeled LDL. LDL fractions were radiolabeled with ^{125}I as described by McFarlane (23) and modified by Bilheimer, Eisenberg, and Levy (24). To avoid oxidation of iodinated LDL (25), butylhydroxytoluene (20 μm) was added to ^{125}I-labeled LDL immediately after iodination. The specific activity of LDL varied between 100–300 cpm/ng LDL protein and the amount of free iodine after LDL precipitation with trichloroacetic acid was less than 2%. LDL, stored under argon at 4°C, was used within 1 week of radiolabeling.

Size and density of LDL. Size and density of LDL were determined by non-equilibrium zonal ultracentrifugation and elec-

tron microscopy. Our previous work has shown that the effluent volume of fractions of LDL isolated by zonal ultracentrifugation correlates inversely with LDL flotation constants and molecular weight (26), as well as with LDL size (13, 22). Electron microscopy was performed on a JEM 1200 EX electron microscope (JEDL) at an instrument magnification of 80,000. LDL at concentrations of 0.05–0.5 mg/ml were negatively stained with 2% sodium phosphotungstate, pH 7.45, on collodion carbon grids. LDL size was calculated by measuring the average diameter of over 100 particles per preparation.

LDL composition

LDL protein was measured according to Lowry et al. (27) and phospholipid by Bartlett's method (28). Total and unesterified cholesterol, as well as triglyceride, were determined by enzymatic kits: Boehringer Mannheim #236691, #310378 and #877557, respectively. Oxidation of LDL was assayed by the thiobarbituric acid reacting substances as described (29, 30) and by analysis of the electrophoretic mobility of LDL on agarose gels (31). Apolipoprotein composition of LDL and apoB integrity were assessed by PAGE using Phastgel gradient 4–15% (Pharmacia Biotech, Piscataway, NJ), as described by the manufacturer. Lp[a] measurement was performed, as described (32).

LDL cell binding uptake and degradation

Affinity of the different LDL for the LDL receptor was assayed by saturation binding assays of radiolabeled LDL performed in triplicate. Typically, cells were incubated at 4°C in modified Eagle's medium, 1% (v/v) albumin, 20 mm HEPES, pH 7.4, containing increasing amounts of experimental ^{125}I-labeled LDL in presence and absence of a 50-fold excess of unlabeled LDL. After incubations, cells were washed 3 times rapidly with 1 ml buffer of PBS, 0.2% albumin, pH 7.4, then incubated twice for 10 min with the same buffer, and finally washed twice more with PBS, pH 7.4. The total, specific, and receptor-independent (nonspecific) LDL cell binding was then calculated (5). In order to evaluate directly the contribution of the LDL receptor-independent-mediated cell binding, similar experiments were conducted on fibroblasts lacking the LDL receptor (GM2000 and 01915C).

To examine the LDL receptor-independent cell binding, uptake, and degradation of LDL, both normal and receptor-negative fibroblasts were incubated at 37°C for 5 h and 18 h in presence of different concentrations of ^{125}I-labeled LDL. After incubation, the medium was collected and the proteolytic degradation of ^{125}I-labeled LDL was measured as described (5). The spontaneous degradation of ^{125}I-labeled LDL was measured in blank dishes containing non-cells and subtracted from that obtained in dishes containing cells. Cells were washed 3 times with PBS, 0.2% albumin, pH 7.4, followed by two incubations of 10 min each with the same buffer, and subsequently incubated with 1 ml of PBS buffer containing either dextran sulfate (4 µg/ml) or heparin 1400 units/ml, pH 7.4, for 1 h. The buffer was collected and the radioactivity of ^{125}I-labeled LDL (cell binding) was measured. Cells were then washed twice with PBS buffer, harvested in 0.1 N NaOH, and the amount of ^{125}I-labeled LDL radioactivity was counted (cell uptake). Data are expressed as the mean ± SD of experimental determinations performed in triplicate.

Statistics

Statistical differences between series were established by paired and non-paired Student's t-test. Results are expressed as mean ± standard deviation of experimental determinations. When appropriate, statistical differences were determined by analysis of variance.

RESULTS

LDL size and composition

A zonal ultracentrifugation profile of normal size LDL as compared to small dense LDL is shown in **Fig. 1**. Fractions of normal size LDL from normolipidemic subjects typically appeared at a rotor effluent volume of 187 ± 4 ml and at a relative (non-equilibrium) density of 1.12 ± 0.01 g/ml, while fractions of small dense LDL from hypertriglyceridemic subjects eluted later (215 ± 16 ml) at a relative density of 1.35 ± 0.01 g/ml. Sizes of the collected LDL fractions were confirmed by electron microscopy. Diameters of a normal size LDL were 24–25 nm, whereas that of small dense LDL varied between 18–20 nm, as previously reported (22). Compared to normal LDL, small dense LDL was relatively enriched in protein and triglyceride and depleted in both free and esterified cholesterol (**Table 1**). No apoB degradation or changes in apolipoprotein composition or LDL oxidation were detected in the different LDL preparations (data not shown).

LDL cell binding

As previously reported (22), in contrast to normal LDL, small dense LDL had decreased total cell and specific binding to the LDL receptor in cultured fibroblasts at 4°C at LDL concentrations between 0.25–10 µg of protein/ml (**Fig. 2**). Consistently, LDL receptor-independent (or nonspecific) binding was always greater for small dense LDL at different LDL concentrations when compared to normal size LDL (Fig. 2). Compared to normal LDL, small dense LDL also exhibited decreased specific LDL receptor-mediated degradation in normal fibroblasts incubated
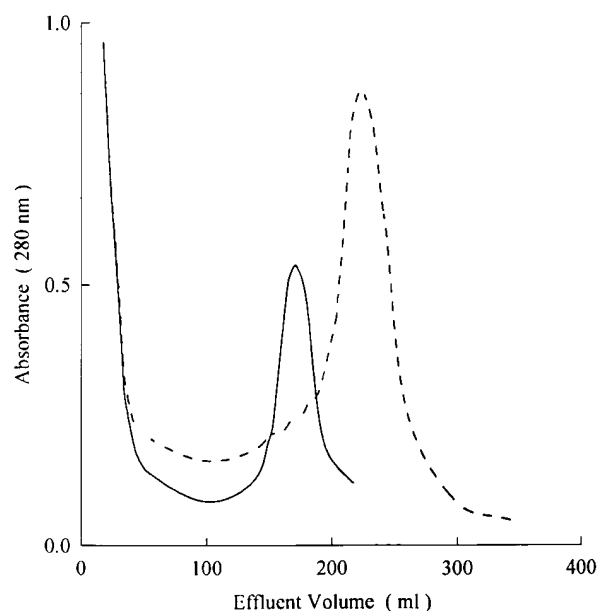

Fig. 1. Non-equilibrium zonal ultracentrifugation elution profile of normal LDL (solid line) and small dense LDL (dashed line). LDL were isolated by zonal ultracentrifugation from plasma d > 1.006 g/ml using a discontinuous NaBr gradient of 1.0–1.3 g/ml in a T1-14 zonal rotor for 170 min. Elution is from left to right with larger and lighter particles eluting first in the rotor effluent.

TABLE 1. Relative weight compositions of LDL

	Normal Size LDL n = 6	Small Dense LDL n = 6
Protein (Pr)	20.79 ± 3.37	26.38 ± 3.40[a]
Triglyceride (TG)	5.73 ± 1.57	13.58 ± 12.76
Free cholesterol (FC)	8.36 ± 1.57	6.89 ± 1.45
Esterified cholesterol (EC)	45.00 ± 6.65	33.28 ± 8.42[a]
Phospholipid (PL)	20.25 ± 4.16	19.82 ± 4.03
TG/Pr	0.29 ± 0.11	0.56 ± 0.61
FC/Pr	0.41 ± 0.08	0.26 ± 0.03[b]
EC/Pr	2.20 ± 0.63	1.27 ± 0.34[a]
PL/Pr	1.02 ± 0.29	0.75 ± 0.13[a]

Results are expressed as the mean of the percent of total lipoprotein mass ± SD.

[a] $P < 0.05$.

[b] $P < 0.01$.

with 10 μg/ml of ^{125}I-labeled LDL for 5 h at 37°C (2584 ± 41 and 875 ± 184 ng LDL/mg cell protein, respectively).

To further examine the role of LDL size on LDL receptor-independent-mediated cell binding, normal size (rotor effluent volume: 170–200 ml) and small dense (rotor effluent volume: 200–240 ml) LDL fractions from the same subjects were isolated by zonal ultracentrifugation. LDL receptor-independent-mediated binding of normal LDL isolated from three normolipidemic subjects, as well as that of normal and small size LDL fractions isolated from three hypertriglyceridemic subjects, was analyzed by incubating normal fibroblasts with LDL at concentrations between 0.25–10 μg of protein/ml at 4°C, in the presence of 50 times excess of normal control LDL (**Fig. 3**). As previously reported (5), at low concentrations of LDL, LDL receptor-independent low affinity binding of normal control LDL was less than 10% of the total LDL cell binding (mean ± SD = 8.0 ± 4%). In contrast, small dense LDL demonstrated over 2-fold higher LDL receptor-independent binding (19.5 ± 6% of total LDL binding, $P < 0.0001$). However, normal-size LDL fractions isolated from the same dyslipidemic donors, although also relatively enriched in triglyceride (triglyceride/protein ratio 0.7 ± 0.3), had LDL receptor-independent binding similar to that of control LDL (11.6 ± 3.7% of total LDL binding, $P = 0.086$). Increased LDL receptor-independent binding was also evident in samples of small dense LDL (isolated from normolipidemic subjects) that did not have elevated triglyceride content (data not shown).

Effects of LDL concentration on LDL receptor-independent cell binding

As LDL receptor-independent cell binding of LDL has been shown to be linearly related to LDL concentration (7), we next questioned whether differences between normal and small dense LDL might be even greater at higher physiological LDL concentrations. **Table 2** compares the total LDL cell binding at 4°C on normal fibroblasts at a low LDL concentration (5 μg/ml), where LDL receptor binding typically saturates (Fig. 2), to that of LDL cell binding at a high LDL concentration (100 μg/ml). Again, at low concentrations of LDL (5 μg/ml) small dense LDL showed decreased affinity for the LDL receptor compared
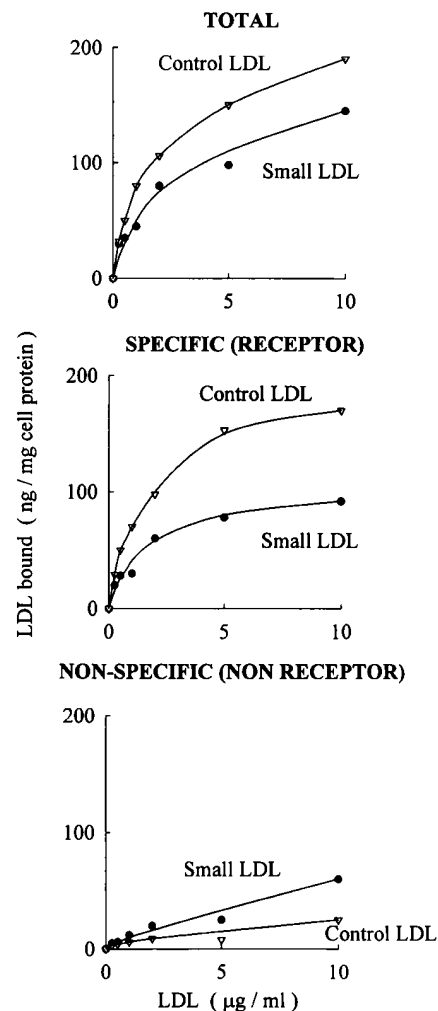

Fig. 2. LDL cell binding of control, normal size LDL (open triangles), and small dense LDL (solid circles) to normal human skin fibroblasts. After 5 days of growth, cells were up-regulated for the LDL receptor by incubation in cholesterol-depleted medium for 48 h. Monolayers were incubated for 2.5 h at 4°C with increasing amounts of ^{125}I-labeled experimental LDL in the presence and absence of 50-fold excess unlabeled LDL. The total specific LDL binding (to the LDL receptor) and LDL receptor-independent binding (to non-LDL receptor sites) were measured as described in Material and Methods.

to normal LDL (K_d, 0.6 and 0.9 μg/ml, respectively) but 2-fold greater LDL receptor-independent binding. Approaching physiologic LDL concentrations at 100 μg/ml, total cell binding of small LDL was over 2-fold higher than that of normal LDL; but after subtracting the contribution of specific LDL receptor binding, small LDL had over 4-fold greater LDL receptor-independent cell binding compared to normal LDL.

We directly assessed the magnitude of LDL receptor-independent binding in LDL receptor-negative fibroblasts (**Fig. 4**). This precluded potential confounding effects of both the LDL receptor and of the excess of unlabeled LDL used to calculate LDL receptor-independent binding (which might also compete for these binding sites). In these experiments, performed at low LDL concentrations
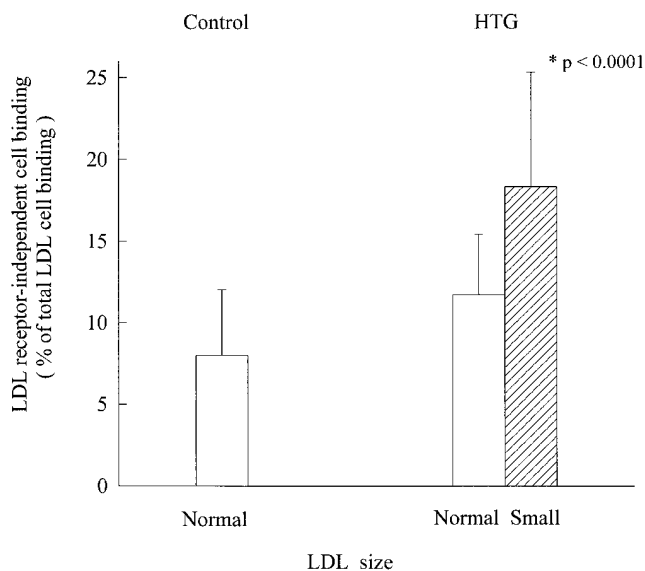
Fig. 3. LDL receptor-independent cell binding of normal size (open bars) and small dense LDL (hatched bar). Control LDL of normal size (N-LDL, n = 3) and two different fractions (normal and small size) of LDL were isolated from three typertriglyceridemic individuals (HTG-LDL) using non-equilibrium zonal ultracentrifugation. LDL were radiolabeled with ^{125}I and monolayers of normal fibroblasts were incubateed with experimental LDL (0.25, 0.5, 1, 2, 5, and 10 μg/ml), in the absence or presence of 50-fold excess unlabeled LDL at 4°C for 25 h. The total cell-associated radioactivity and LDL receptor-independent cell binding were measured in triplicate as described in Material and Methods. The LDL receptor-independent cell binding was calculated at each LDL concentration; the data were pooled and expressed as a percentage (mean ± SD) of total LDL binding; *different from both normal control LDL and normal size HTG-LDL; *P* < 0.0001.

(5 μg/ml) at 4°C, total LDL binding of both normal size and small LDL was low, but that of small dense LDL was about 8-fold that of normal LDL. Compared to LDL cell binding at low LDL concentrations, binding of LDL at high LDL concentrations (100 μg/ml) was substantially increased, 9-fold for normal LDL, but even more for small dense LDL, 14-fold. Thus, LDL cell binding of small LDL at high LDL concentrations also exceeded that of normal LDL by up to 14-fold. Similar experiments at high LDL concentrations were performed using subfractions of nor-

mal and of small dense LDL isolated from the *same* individuals (three hypertriglyceridemic subjects). Compared to normal size LDL, small dense LDL had 2.9 ± 1.4 (mean ± SD) higher binding to LDL receptor-negative fibroblasts.

In pilot experiments, we noted that the peak of Lp[a] elution occurs, beyond the resolution of the LDL peak, at rotor effluent volumes higher than 240 cc (while small dense LDL from hypertriglyceridemic subjects typically eluted at 215 cc). To analyze the effect of LDL size in normolipidemic individuals, as well as the potential role of Lp[a] in LDL receptor-independent binding, we examined the LDL cell binding of normal and small size LDL isolated from a normolipidemic subject (plasma triglyceride 91 mg/dl; plasma Lp[a] 11.0 mg/dl). Lp[a] was not detected in any LDL subfraction. Compared to normal size LDL, small dense LDL from this subject had close to 3-fold higher LDL binding to LDL receptor-negative fibroblasts. Values for binding of normal size and of small dense LDL (at 4°C) were 123 ± 7 and 349 ± 50 ng LDL/mg cell protein, respectively. Thus, the presence of Lp[a] is not the explanation for increased cell binding of small dense LDL.

To examine the possible contribution of LDL receptor-independent binding sites to LDL cell metabolism, we determined cell binding and degradation of normal and small dense LDL, at 37°C, in receptor negative fibroblasts. **Figure 5** shows that, at high LDL concentrations (200 μg/ml), small dense LDL had up to 3-fold higher LDL cell association and that the amount of LDL cell binding released by heparin varied between 45% (small dense LDL) and 60% (normal LDL) of the total cell associated LDL. In receptor-negative fibroblasts, little or no apoB degradation was observed with either normal or small dense LDL (data not shown).

Role of apoB and cell surface heparan sulfate proteoglycans in LDL receptor-independent cell binding

To investigate whether particular regions of apoB were involved in LDL receptor-independent-mediated cell binding, normal and receptor negative fibroblasts (GM2000) were incubated at 4°C and 37°C in the presence of 5 and 100 μg/ml of LDL, previously incubated overnight at 4°C with 3-fold excess (w/w) of purified monoclonal apoB antibodies. The following antibodies, which recognize differ-

TABLE 2. Effects of LDL concentration on LDL receptor-independent-mediated cell binding

	Normal LDL		Small Dense LDL	
	5 μg/ml	100 μg/ml	5 μg/ml	100 μg/ml
Total LDL binding (ng/mg cell protein)	190 ± 8	292 ± 21	183 ± 9	696 ± 10
LDL (ng/ml cell protein) receptor independent binding	11 ± 0.4	113[a]	22 ± 0.5	505[a]

Experiments were performed in triplicate on normal human skin fibroblasts at 4°C for 2.5 h, as described in Material and Methods. Data are expressed as mean ± SD.

[a] Values of LDL receptor independent binding at high LDL concentrations (100 μg/ml) were calculated by subtracting the LDL receptor specific binding at 5 μg/ml (where maximum occupancy of the LDL receptor occurred) from total LDL binding; these values were 179 ± 8 and 161 ± 9 ng LDL protein/mg cell protein from normal and small dense LDL, respectively. K_d values of both normal and small dense LDL were 0.6 and 0.9 μg/ml, respectively, and were obtained after Scatchard analysis of LDL specific binding at 0.5, 1, 1.5, 3, and 5 μg/ml of ^{125}I-labeled LDL.
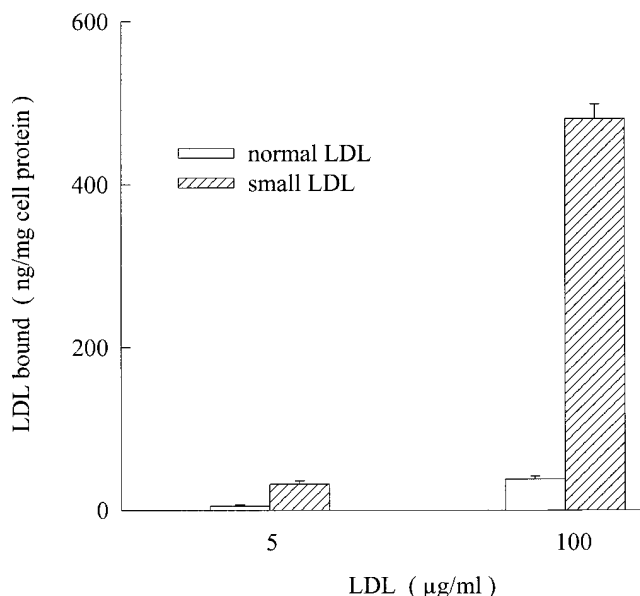
Fig. 4. LDL receptor-independent cell binding at 4°C of normal size and small dense LDL. Monolayers of fibroblasts lacking the LDL receptor (GM 2000) were incubated with normal size LDL isolated from a normolipidemic subject (open bars) or small dense LDL isolated from a hypertriglyceridemic individual (hatched bars). Incubations with each LDL were performed at low (5 μg/ml) and high (100 μg/ml) LDL concentrations for 2.5 h, and the total cell-associated radioactivity was measured as described in Material and Methods.

Fig. 5. Effect of heparin of LDL cell binding at 37°C in LDL receptor negative fibroblasts (GM 2000). Cells were incubated for 18 h at 37°C at high LDL concentrations (100 μg/ml) as explained in Material and Methods. After 5 washes with PBS buffer, cells were incubated with 1 ml of PBS buffer containing heparin 1400 units/ml, pH 7.4, for 1 h at 4°C, and the ^{125}I-labeled LDL was measured. Cells were washed twice with PBS buffer and the radioactivity of the cell lysates (with 0.1 N NaOH) was measured (LDL cell uptake). The total LDL cell association represents the sum of the heparin-releasable fractions (LDL cell binding, hatched bars) and the LDL cell uptake.

ent apoB epitopes, were used: Mb_3 (apoB residues 1022–1031), Mb_{19} (recognizing a not yet defined epitope in the amino terminal region), Mb_{47} (residues 3429–3453), all kindly provided by Dr. Linda Curtiss, (Scripps Clinic: La Jolla, CA) and monoclonal antibodies 1D1 (residues 474–539), 2D8 (1438–1480), 3F5 (2835–2922), 4G3 (2980–3084), and 3A10 (3441–3569) generously given by Drs. Yves Marcel and Ross Milne (University of Ottawa). As expected, variable reduction (40–80% depending on the Mab used) of LDL binding to normal fibroblasts was seen at low LDL concentrations (5 μg/ml) in the presence of Mabs Mb_{47}, $3F_5$, $3A_{10}$ and $4G_3$, which recognize epitopes at or near the LDL receptor binding region of apoB (33). However, at low (5 μg/ml) or high LDL concentration (100 μg/ml), no consistent differences in LDL binding to receptor-negative fibroblasts were observed in the presence of different apoB monoclonal antibodies, indicating that the apoB epitopes examined are not directly involved in LDL receptor-independent cell interactions (data not shown).

To define better the characteristics of the LDL receptor-independent cell binding sites, receptor-negative fibroblasts (GM2000) were preincubated at 37°C for 15 min with 250 μg/ml trypsin (34), or for 1 h with either 3 μg/ml pronase (5), 2 U/ml chondroitinase, or 0.05 U/ml neuraminidase (35). Cells were then incubated with high concentrations of ^{125}I-labeled LDL (100–200 μg/ml) for 2.5 h at 4°C. No consistent decrease in LDL cell binding was observed in cells pretreated with trypsin, pronase, or neuraminidase, and variable decrease (0–20%) was ob-
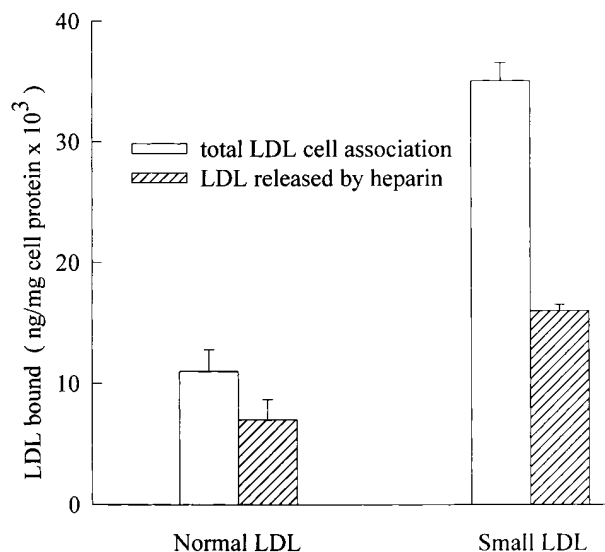
served in cells pretreated with chondroitinase (data not shown).

Because heparin addition to media releases a substantial fraction of LDL receptor-independent bound particles, we questioned whether the removal of cell surface heparan sulfate proteoglycans (HSPG) by a mixture of 5 U/ml each of heparinase and heparitinase (H/H), would decrease LDL cell surface binding. In experiments at 37°C, using high LDL concentrations (500 μg/ml), H/H treatment decreased total LDL cell binding by 35 ± 21% (n = 3) in receptor-negative fibroblasts. In incubations at 4°C and at high LDL concentrations (100 μg/ml), a significant decrease in total LDL cell binding (50 ± 14%, $P <$ 0.002) was also observed in LDL receptor-negative fibroblasts pretreated with H/H (**Fig. 6**).

To assess the efficacy of H/H treatment in removing cell surface proteoglycans, in parallel experiments, we examined the effect of these enzymes on blocking the well-described increase in LDL cell binding mediated by lipoprotein lipase (36, 37). H/H treatment blocked the increased (>5-fold) LDL binding of 5–20 μg/ml of LDL mediated by lipoprotein lipase (10 μg/ml) by 40–100% (data not shown).

We next examined the effect of pretreating LDL receptor-negative fibroblasts with H/H on LDL binding, at 4°C, on two fractions of normal and small dense LDL (200 μg/ml) isolated from the same subjects. No differences in the relative decreases of LDL cell binding were observed between normal and small size LDL after cell pretreatment with
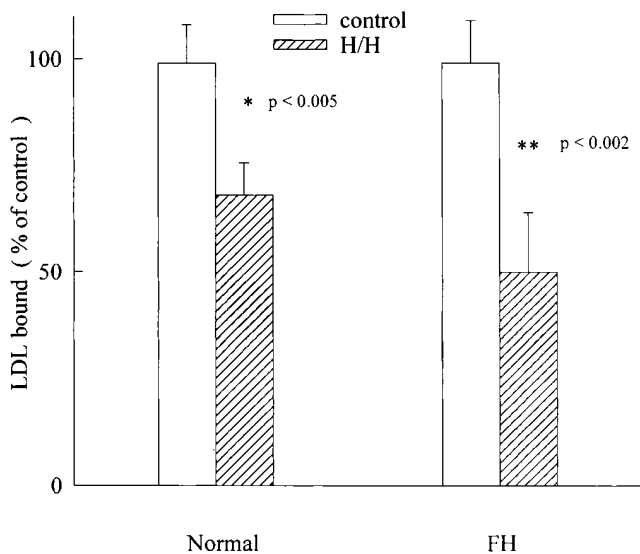
Fig. 6. Effect of heparinase and heparitinase on ^{125}I-labeled LDL cell binding to LDL receptor-independent cell surface sites. Normal skin fibroblasts and LDL receptor-negative fibroblasts (GM 2000) were grown for 7 days, as described in Material and Methods. Cells were incubated in the absence (open bar) or presence (hatched bar) of 5 U/ml each of heparinase and heparitinase (H/H) for 60 min at 37°C. Cells were then incubated in triplicate at 4°C for 2.5 h with 100 μg/ml of normal size ^{125}I-labeled LDL isolated from a normolipidemic individual, and the total cell associated radioactivity was analyzed, as described in Material and Methods. The data are presented as the percentage of LDL cell binding of treated cells compared to untreated cell and show the results of three different experiments with normal fibroblasts and three different experiments with LDL receptor negative fibroblasts (GM 2000), *$P < 0.005$ and **$P < 0.002$ compared to control LDL. Range values for LDL cell binding in control (untreated cells) varied as follows: 456 to 631 ng LDL/mg cell protein in normal fibroblasts and 239 to 503 ng LDL/mg cell protein in LDL receptor-negative fibroblasts.

H/H (data not shown). In normal fibroblasts at high LDL concentrations (100 μg/ml), H/H treatment significantly decreased total LDL cell binding by 32 ± 8% ($P < 0.005$) at 4°C (Fig. 6), but no reduction in cell binding was observed at low LDL concentrations (5 μg/ml) (data not shown). In parallel experiments, the LDL receptor-independent binding of LDL, measured in the presence of 50 times excess unlabeled LDL, was 43 ± 6% of the total LDL cell binding in these cells. Therefore, in normal fibroblasts, removing HSPG reduced LDL receptor-independent binding by approximately 70% but did not affect LDL binding to the LDL receptor. To assess further the role of proteoglycans in receptor-independent LDL binding, we preincubated two different lines of LDL receptor-negative fibroblasts (GM2000 and 01915C) with 50 mm of sodium chlorate, an inhibitor of proteoglycan sulfation (38). After 48 h, cells were washed and incubated (in quadruplicate) with 100 μg/ml of ^{125}I-labeled LDL for 5 h. Compared to untreated cells, LDL cell binding in treated cells was inhibited by about 45%. LDL binding was 1377 ± 139 and 1261 ± 54 ng LDL/mg cell protein in untreated GM2000 and 01915C cells, respectively, and 777 ± 107 and 597 ± 54 ng LDL/mg cell protein in treated cells.

These results suggest that, at high physiological LDL concentrations, cell surface HSPG are important contributors to LDL receptor-independent LDL cell binding.

DISCUSSION

We previously reported that small dense LDL isolated from hypertriglyceridemic individuals has decreased affinity for the LDL receptor compared to normal size LDL (22). In this paper, we demonstrate that differences in small dense LDL receptor binding are also accompanied by substantially higher LDL receptor-independent binding to the cell surface. We provide direct evidence that increased binding of small dense LDL to LDL receptor-independent-mediated cell binding sites was present at low LDL concentrations (<10 μg/ml) where the LDL receptor is responsible for most LDL cell binding. Further, binding differences were substantially greater at higher more physiologic LDL concentrations (100–500 μg/ml) where LDL receptor-independent sites on the cell surface account for an increasing proportion of cell surface binding. Finally, our data indicate that HSPG contribute to receptor-independent anchoring of LDL particles on cell surfaces.

Previously, we provided evidence that changes in LDL size and lipid composition correlated with plasma triglyceride levels (13, 39). We proposed that higher circulating triglyceride-rich lipoprotein levels provide an increased acceptor pool for LDL cholesteryl esters and increased transfer of triglycerides into LDL, processes mediated by plasma cholesteryl ester transfer protein (40–42). Concomitant hydrolysis of newly acquired triglyceride in LDL (by lipase) shrinks LDL particle size after reduction of the particle core size because of lost core cholesteryl esters (13, 41, 43). As lipoprotein lipases generally do not remove all of newly acquired triglyceride, LDL also becomes enriched with triglyceride, especially at plasma triglyceride levels over 200 mg/dl (13).

Different investigators have suggested that in hypertriglyceridemic individuals the increased triglyceride content of LDL decreases the interaction of LDL with the LDL receptor (44–46). Our previous work, however, demonstrated that depleting LDL free or esterified cholesterol, increasing phospholipid content, or enriching LDL in triglyceride without decreasing LDL particle size does not affect LDL binding to the LDL receptor (22). The present work also demonstrates that the increase in triglyceride content in normal-size LDL does not affect LDL binding to LDL receptor-independent cell binding. It is possible that factors other than size such as differences in phospholipid or sugar composition in small dense LDL, e.g., decreased sialic acid (47) could be responsible for the increased LDL receptor-independent cell binding of small dense LDL.

Likely, neither apoE nor apoC accounted for the differences in the LDL receptor-independent cell binding observed between LDL subfractions, as these apoproteins were not visualized in samples analyzed by PAGE. Although

we did not directly measure the contents of apoE or of apoC in the fractions used in the experiments described in this paper, in other studies we have directly measured (by ELISA) the apoE and apoC contents of the LDL subfractions taken from five different hyperlipidemic individuals and found only minimal presence of apoE or of apoC-III (<2 and <3 molecules/100 LDL particles, respectively). Furthermore, there were no differences in these apoproteins between normal and small dense LDL (data not shown). Thus, our data indicate that small LDL size, rather than lipid or apoprotein composition, is also a major predictor for LDL receptor independent cell binding.

The molecular mechanisms of the LDL binding to low affinity cell binding sites are poorly understood. Initial studies by Goldstein and Brown (7) showed that the low affinity LDL receptor-independent-mediated LDL cell binding was not only non-saturable, but, unlike LDL receptor binding, was also calcium–independent and resistant to cell treatment with pronase. As well, LDL receptor-independent-mediated particle uptake does not regulate cell cholesterol synthesis and appears to occur mainly by adsorptive endocytosis, with small amounts by fluid phase endocytosis (7, 48, 49).

Initial investigation of the role of apoB in LDL binding to LDL receptor-independent binding sites demonstrated that cationized (50) or trypsinized (51) LDL, but not methylated LDL (52), had increased LDL degradation by receptor-negative fibroblasts, suggesting that changes in apoB integrity or charge may modify LDL receptor-independent binding. Our results using intact LDL demonstrated that blocking several epitopes of apoB with different monoclonal antibodies spanning the apoB molecule did not alter LDL binding to LDL receptor-independent cell surface sites.

An important role for cell surface proteoglycans in binding of apoE lipoproteins has been shown (53, 54). In agreement with Ji et al. (53), we found that H/H did not affect LDL cell binding mediated via the LDL receptor. However, at high LDL concentrations (100–500 μg/ml), HSPG contribute to LDL receptor-independent cell binding. Two lines of evidence support this conclusion. First, LDL receptor-negative cells pretreated with heparinase and heparitinase, or with sodium chlorate, significantly decreased LDL cell binding at 4°C and 37°C. Second, at 37°C, in these cells, heparin, a competitor for proteoglycan cell binding sites released 45–60% of the total LDL cell bound. Further evidence that cell surface proteoglycans are involved in LDL cell uptake at high LDL concentrations comes from two recent publications. Olsson et al. (55) reported that pretreating fibroblasts with a mixture of chondroitinase and heparitinase or sodium chlorate decreased LDL binding to low affinity binding sites in normal fibroblasts. Further, Seo and St. Clair (38) found that treating pigeon peritoneal macrophages with heparinase alone decreased cell uptake of pigeon β-VLDL (an apoB lipoprotein lacking apoE) by a low affinity, high capacity mechanism, while the high affinity LDL receptor-like pathway was unaffected.

LDL can interact in vitro with HSPG with variable affinities depending upon the experimental methodology used (56, 57). Although the interaction between LDL and arterial wall matrix and its potential role in atherogenesis has been established (58), less is known about the interaction of LDL with the endothelial cell surface proteoglycans (59–61), the first barrier for the LDL influx in the arterial wall. This interaction may be especially important in the endothelium where cell injury and shear forces increase synthesis of HSPG (62). Of interest, in preliminary experiments (at 37°C) in endothelial cells, we have observed that small dense LDL also has higher cell binding and uptake, which were not accompanied by an increase in LDL cell degradation (data not shown). It is tempting to speculate that in the arterial wall, at physiological LDL concentrations, recruitment of LDL by endothelial cell surface HSPG may facilitate interaction of LDL with other molecules, such as lipoprotein lipases or apoE, which then could mediate uptake or degradation of LDL via the lipoprotein receptor related protein (LRP) or other pathways. We have now described similar pathways for the clearance of apoE-model triglyceride-rich particles in non-hepatic cells, where, at low concentration, particles were cleared via the LDL receptor, while, at high particle concentration, uptake was mediated by cell surface proteoglycans (54).

Compared to normal LDL, small dense LDL has different apoB overall structure and immunoreactivity at the apoB receptor recognition domain (22). In our studies, none of the apoB monoclonal antibodies used recognized the described heparin binding sites of apoB (63, 64), the most probable binding sites for HSPG. Thus, it is possible that changes in the conformation of apoB epitopes different from those examined herein (e.g., heparin binding domains) might increase the affinity of small LDL for certain LDL receptor-independent cell binding sites (e.g., cell surface proteoglycans). In this regard it has also been demonstrated that the affinity of LDL for other vascular proteoglycans is mediated by clusters of basic epitopes also located in the LDL receptor binding region (65, 66), and that small dense LDL has increased affinity for chondroitin sulfate proteoglycans in the arterial wall matrix (67, 68).

LDL cell binding to LDL receptor-independent binding sites might be related to other cell surface binding sites such as the scavenger receptor (69), VLDL receptor (70), or the LRP (71). This seems unlikely because, in our experiments, we did not observe a decrease in LDL receptor-independent cell LDL binding after pretreating the cell surface with trypsin or pronase. In addition, our studies were conducted on fibroblasts, which appear not to express the scavenger or the VLDL receptor. Using fibroblasts rather than other cell lines such as macrophages or hepatocyte-like cell lines, we avoided potentially confounding variables such as cell-secreted apoprotein E or lipases, which would also contribute to LDL receptor-independent-mediated uptake. Under the conditions of our studies, LDL was not significantly oxidized, precluding contributions of other receptors recognizing oxidized LDL. LRP is also not a binding site for

small dense LDL, as apoB does not bind to this receptor. Furthermore, we did not find decreases in LDL cell binding in receptor-negative fibroblasts in the presence of the 39 kDa receptor-associated protein (RAP), a known inhibitor of binding to LRP (71) (data not shown). Fogelman, Haberland, and Edwards (8) have proposed that lipid–lipid interaction might be involved in LDL cell binding to LDL receptor-independent binding sites. Our experiments did not provide data relevant to this latter possibility.

Differences in cross-sectional surface areas of normal and small LDL may also contribute, in part, to higher LDL receptor-independent cell binding. Assuming an average diameter of 18–20 nm for small LDL particles and 22–25 nm for normal LDL, we estimate 1.5-fold more small LDL particles would be necessary to cover a similar surface as compared to normal size LDL.

We speculate that although the LDL receptor, especially in the liver, is the major mechanism regulating plasma LDL levels, differences in LDL receptor independent uptake by different non-hepatic tissues will contribute significantly to atherogenic processes. For example, transcytosis of LDL across the endothelial cell occurs by a LDL receptor-independent-mediated mechanism, which is also non-saturable, concentration-dependent, and not associated with effective LDL degradation (72).

Retention of LDL in the subendothelial space in the arterial wall by proteoglycans has been proposed as an important mechanism in atherogenesis (73). Our results allow us to hypothesize that in the arterial wall, the increased anchoring of small dense LDL to LDL receptor-independent cell sites (e.g., in macrophages, endothelial and muscle cells) as well as to proteoglycans of the arterial matrix would increase the probability of small dense LDL to be modified (e.g., oxidation, aggregation). This mechanism could contribute to the reported decrease in intravascular residency time (74, 75) and enhanced arterial wall clearance of small dense LDL (76). Small dense LDL compared to normal size LDL has intrinsically increased susceptibility to LDL oxidation and aggregation (77, 78). Of relevant interest, Aviram and Rosenblat (79) have shown that binding of LDL to the cell surface (e.g., to the LDL receptor) is one mechanism leading to increased LDL cell-mediated oxidation, and Camejo et al. (80) and Hurt-Camejo et al. (81) have reported increased susceptibility to oxidation of LDL exposed to arterial wall chondroitin sulfate proteoglycans and glycosaminoglycans.

In summary, our studies provide evidence that, compared to normal LDL, small dense LDL has decreased binding to the LDL receptor but increased cell binding to LDL receptor-independent sites. We have demonstrated that cell surface proteoglycans play a significant role in LDL cell binding at physiological LDL concentrations. Thus, we hypothesize that high affinities of small dense LDL for arterial proteoglycans, or other cell surface sites, may enhance small dense LDL trapping and modification in the vascular wall, processes that will contribute to the development of atherosclerosis.

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