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Are the Binding and Degradation of Low Density Lipoprotein Altered in Type 2 (Non-Insulin-Dependent) Diabetes Mellitus?

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Summary. Studies in vitro have shown that glycosylation of low density lipoprotein (LDL) will decrease its ability to bind to its receptor. We have evaluated the possibility that such an event might occur in vivo in diabetes by comparing the binding and degradation by normal fibroblasts and mouse peritoneal macrophages of LDL obtained from normal control subjects and patients with Type 2 (non-insulin-dependent) diabetes mellitus. When compared with control subjects, Type 2 diabetic patients had elevated fasting glucose (increased by 160%), haemoglobin A_{Ic} (increased by 75%), triglyceride (increased by 550%), and cholesterol (increased by 48%) levels. LDL from Type 2 diabetic patients displayed populations of particles with more heterogeneous hydrated densities than LDL from control subjects, with enrichment in the triglyceride content of the lighter population. ^{125}I-LDL from normal and Type 2 diabetic subjects bound to fibroblasts with similar binding affinities and binding capacities. The kinetics of degradation of LDL from normal and Type 2 diabetic subjects by fibroblasts were also similar. Furthermore, all populations of LDL particles from Type 2 diabetic patients were bound and degraded by normal fibroblasts in identical fashions. In addition, ^{125}I-LDL from normal and Type 2 diabetic subjects were not bound or degraded by mouse peritoneal macrophages. It is concluded that the LDL of patients with Type 2 diabetes with moderate hyperglycaemia are not modified sufficiently to alter their normal binding and degradation by human fibroblasts or to cause their uptake by mouse peritoneal macrophages.

Key words: Type 2 diabetes mellitus, low density lipoprotein, lipoprotein binding, lipoprotein degradation, fibroblast, macrophage.

Low density lipoprotein (LDL) catabolism takes place via a highly regulated process of receptor-mediated endocytosis [1]. The initial step in this pathway is the recognition of the B apoprotein of LDL by specific cell surface receptors. Lysine residues of the B apoprotein have been shown to have an important role in this recognition, since chemical modification of this amino acid will lead to a loss of normal binding behaviour [2, 3]. Recent studies have demonstrated that incubation of LDL in vitro with glucose can result in glycosylation of lysine residues of LDL, with a resultant decrease in the binding of LDL to receptors on cultured human fibroblasts [4, 5] and an increase in the binding of LDL to receptors on rat peritoneal macrophages [6]. These observations raised the possibility that a similar phenomenon could occur in uncontrolled diabetes in vivo. Indeed, Schleicher et al. have recently reported that the level of glycosylated B apoprotein was increased in diabetic patients [7]. In order to evaluate whether LDL from diabetic patients displays any changes in functional behaviour, we have isolated LDL from the plasma of patients with uncontrolled Type 2 (non-insulin-dependent) diabetes, and determined the ability of this LDL to be bound and degraded by cultured normal human fibroblasts and mouse peritoneal macrophages.

Subjects and Methods

Subjects

Ten healthy normal control subjects (aged 24–55 years, mean 46 years) and seven subjects with Type 2 diabetes (aged 45–61 years, mean 56 years) were studied. None of the subjects had evidence of renal or liver disease, or a family history of hyperlipoproteinaemia. All the diabetic subjects were treated with diet alone and/or sulphonylurea therapy at the time of the study. Informed consent was obtained in all cases.

Clinical Studies

Following an overnight fast, blood was drawn for measurement of glucose [8], insulin [9], haemoglobin A_{Ic} (HbA_{Ic}) [10], triglyceride [11] and cholesterol [12] concentrations. On a subsequent occasion, all

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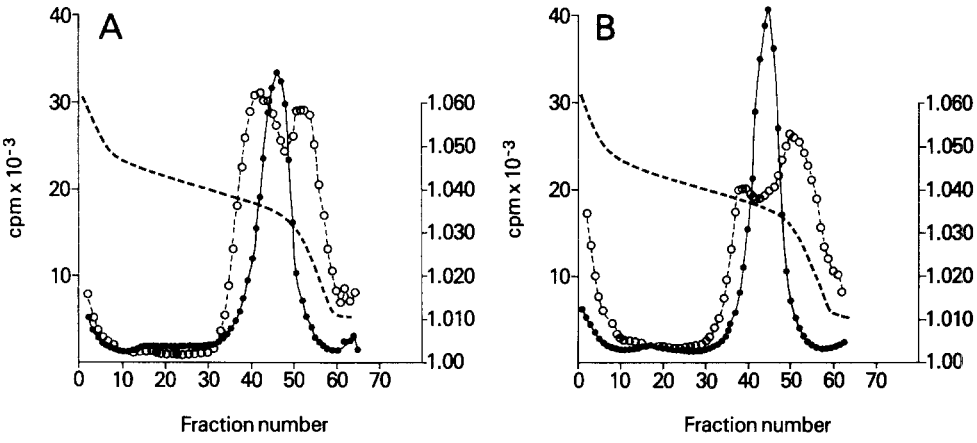

Fig. 1. Distribution of LDL from normal and diabetic subjects on a linear sucrose gradient. Panels *A* and *B* represent two individual diabetic subjects. LDL were isolated from normal and diabetic subjects. After radiolabelling normal LDL with ^{131}I and diabetic LDL with ^{125}I, the samples were ultracentrifuged on a linear sucrose gradient of 8.02%–12.85%. ●——●: normal LDL; O----O diabetic LDL; -----: hydrated density

Table 1. Fasting metabolic parameters in control and diabetic subjects.

	Glucose (mmol/l)	HbA_{1c} (%)	Triglyceride (mmol/l)	Cholesterol (mmol/l)
Control subjects (n = 10)	4.76 ± 0.17	6.8 ± 0.2	0.88 ± 0.05	4.46 ± 0.3
Diabetic subjects (n = 7)	12.56 ± 1.28	11.7 ± 0.9	2.94 ± 0.59	6.61 ± 0.61
p	< 0.001	< 0.001	< 0.001	< 0.01

Results are expressed as mean ± SEM

subjects were given a formula (containing: 150 protein, 420 carbohydrate and 430 fat; g/kg) at 12.00 h, and glucose and insulin responses were assessed before and at half-hourly intervals for 3 h after the meal.

Preparation of Lipoproteins

Blood was drawn after an overnight fast into tubes containing EDTA acid (1 mg/ml). LDL (d = 1.019–1.063) were isolated from plasma by ultracentrifugation, using solid KBr for density adjustment [13]. LDL were acetylated by the addition of acetic anhydride [2]. LDL were iodinated according to MacFarlane [14]. The lipoproteins were extensively dialyzed against 0.15 mol/l NaCl, 0.05 mol/l Tris-HCl (pH 7.4) and sterilized by filtration.

Sucrose Density Gradient

LDL from normal control subjects were iodinated with ^{131}I; LDL from diabetic patients with ^{125}I. Aliquots of normal ^{131}I-LDL and diabetic ^{125}I-LDL were then mixed and separated on a linear gradient of sucrose (8.02%–12.85%) by centrifugation at 40000 rev/min for 18 h and fractions collected by puncturing the bottom of the tubes. Radioactivity was determined in a two-channel gamma scintillation spectrophotometer and the density of the gradient followed by refractometry.

Cell Cultures

Human skin fibroblasts were grown from explants from the foreskin of a healthy newborn and maintained in culture as described previously [15]. Experiments were performed on dishes which were approximately 75%–95% confluent and contained 400–450 μg of cell protein. Mouse macrophages were harvested from peritoneal exudates of adult, male, Swiss-Webster mice (Simonsen Laboratories, Gilroy, California, USA) following the injection of 2 ml of thioglycollate medium (Bakte Bennett, Berkeley, California, USA) according to the procedure of Gallily and Feldman [16]. Each dish of adherent macrophages contained 200–250 μg of cell protein.

Assays

Binding was assessed by incubating the cells at 37 °C in a final volume of 1 ml containing 600 μl of media and 5% lipoprotein deficient serum, and various concentrations of ^{125}I-LDL (5–100 μg LDL protein). Non-specific binding was assessed by the addition of a 10–100-fold excess of radio-inert LDL to parallel dishes. After incubation (2 h for fibroblasts; 24 h for macrophages), the cells were placed on ice, the medium removed, and the dishes then washed four times with 1 ml of 0.15 mol/l NaCl, 0.05 mol/l Tris HCl (pH 7.4), 5 mmol/l $CaCl_2$, and 2 mg/ml of bovine serum albumin, followed by one wash with 2 ml of the same buffer without bovine serum albumin. The cells were dissolved in 1 ml of 0.5 N NaOH, and their radioactivity determined in a gamma scintillation spectrophotometer. An aliquot of the cells was taken for determination of cellular protein content [17]. Although total binding reflects both the ^{125}I-LDL that is surface bound, as well as that internalized, we have expressed the results as ng LDL protein bound/mg cell protein. The degradation of ^{125}I-LDL by the cells was determined by assessing the amount of radioactivity in the medium that is soluble after trichloroacetic acid (10% w/v final concentration) precipitation, and after extraction of free iodine with chloroform [15]. The results are expressed as ng LDL protein degraded/mg cell protein.

Results

The results in Table 1 demonstrate that the patients with Type 2 diabetes had marked fasting hyperglycaemia, associated with a 75% increase in their HbA_{1c} levels. Postprandial excursions of plasma glucose were as high as 20–25 mmol/l, emphasizing the severity of diabetes in this problem. The insulin responses during feeding were not significantly different between diabetic and control subjects. Finally, patients with Type 2 diabetes had a sixfold higher value of fasting plasma triglyceride concentrations with a 50% elevation in their fasting serum cholesterol levels when compared with control subject (Table 1).

When LDL were isolated using conventional density adjusted ultracentrifugation methods, two popula-

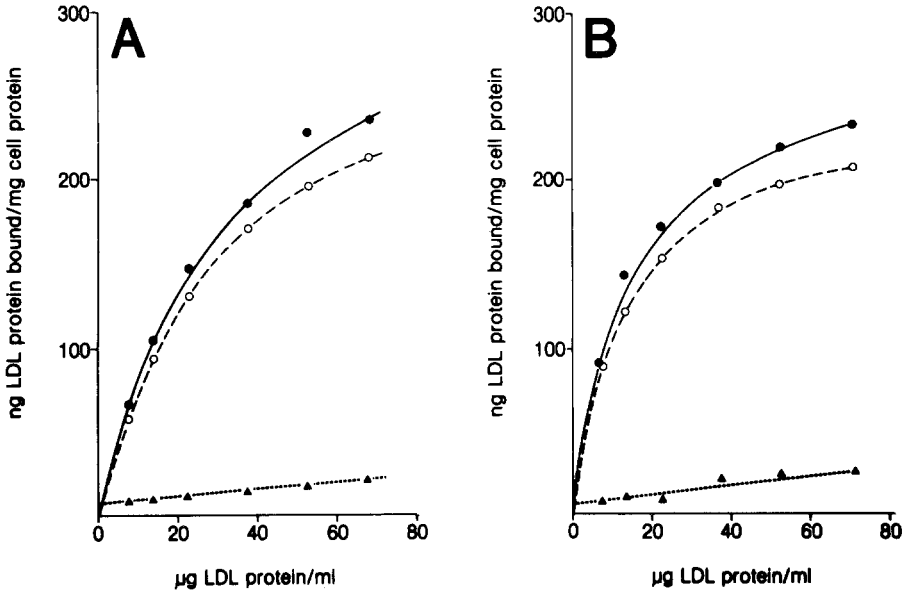

Fig. 2. Saturation curves of binding of LDL from a normal *(A)* and a diabetic *(B)* subject to normal human fibroblasts. The indicated concentrations of ^{125}I-LDL were incubated with monolayers of human fibroblasts at 37 °C for 2 h. Parallel dishes contained 2.5 mg/ml of radio-inert LDL. ●——●: total binding; ▲·····▲: non-specific binding; O----O: specific binding

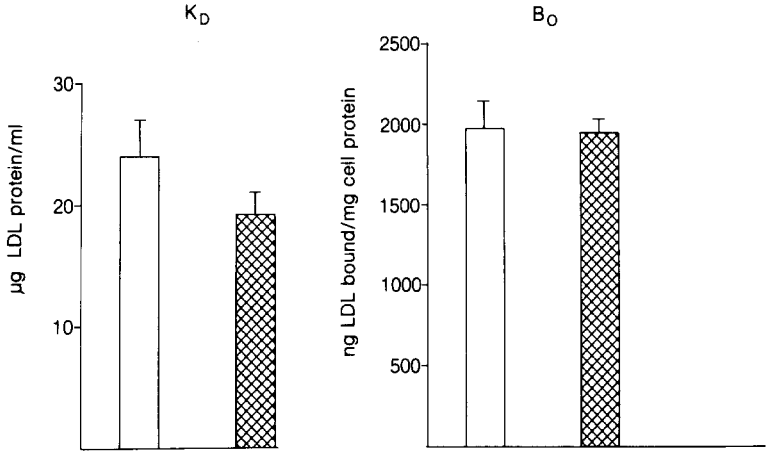

Fig. 3. Binding affinity (K_D) and binding capacity (B_O) of LDL from normal control and diabetic subjects. K_D and B_O were derived from Scatchard plots made from saturation curves of binding to normal human fibroblasts of ^{125}I-LDL isolated from individual subjects. The mean ± SEM of K_D and B_O for the control and diabetic groups were then calculated. □: control subjects; ▨: diabetic patients

tions of LDL were noted in several patients with Type 2 diabetes. This was in contrast to the apparent homogeneity of LDL from normal subjects. When the isolated LDL were differentially radiolabelled and separated in a linear gradient of sucrose, the differences between the LDL from normal and diabetic subjects became clearer (Fig. 1). The normal LDL displayed a homogeneous pattern, with a mean hydrated density of 1.035. In contrast, LDL from diabetic patients were more heterogeneous, with peaks occurring at mean hydrated densities of 1.033 and 1.0375. A comparison of the lipid compositions of normal LDL and the two populations of diabetic LDL is shown in Table 2. The diabetic LDL of greater density (peak 1) have a similar triglyceride/protein ratio and a slightly lower cholesterol/protein ratio when compared with controls; while the lighter diabetic LDL (peak 2) are enriched in triglyceride content with comparable or small increases in cholesterol/protein ratios.

The binding of LDL to cultured human fibroblasts is illustrated in Figures 2 and 3. Figure 2 displays typical curves of the binding of LDL from a normal and a diabetic subject when incubated with normal human fibroblasts in culture. LDL from normal and diabetic subjects showed high affinity, saturable binding of a similar degree. The binding results of LDL obtained from all subjects were analyzed by Scatchard plots (Fig. 3). It is apparent that there are no differences between either the apparent binding affinities or capacities of LDL isolated from normal and diabetic subjects.

In addition, cultured fibroblasts degraded LDL obtained from normal and diabetic subjects to the same extent (Fig. 4). The results of the degradation studies of all subjects were analyzed by Lineweaver-Burk plots, and there were no differences between either the apparent K_m or V_{max} for degradation of LDL obtained from normal and diabetic subjects (Fig. 5).

In order to test the possibility that the different peaks of LDL identified in these patients might behave differently, studies were carried out comparing the two different classes of LDL seen in the plasma of patients with Type 2 diabetes. These results indicated that the

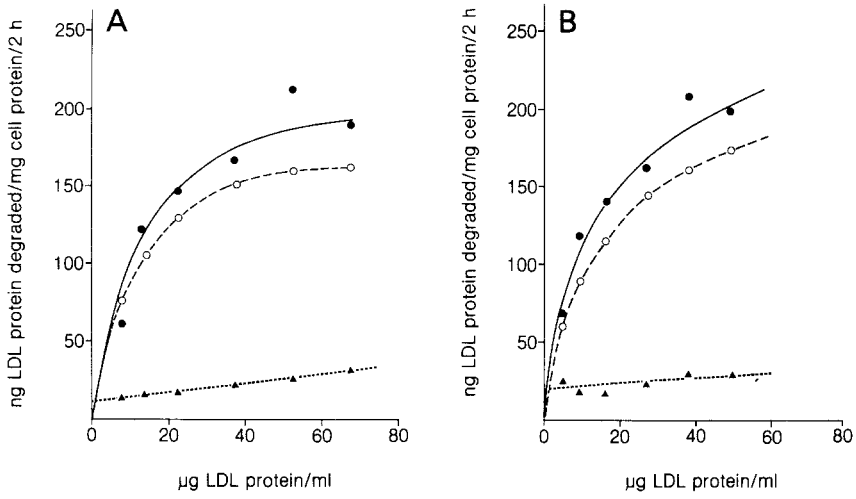

Fig. 4. Saturation curves of degradation of LDL from a normal *(A)* and a diabetic subject *(B)* by normal human fibroblasts. The indicated concentrations of ^{125}I-LDL were incubated with monolayers of human fibroblasts at 37 °C for 2 h. Parallel dishes contained 2.5 mg/ml of radio-inert LDL. Media were assayed for trichloroacetic acid soluble, non-free iodine, degradative products. ●——●: total degradation; ▲ · · · · · · ▲: non-specific degradation; ○----○: specific degradation

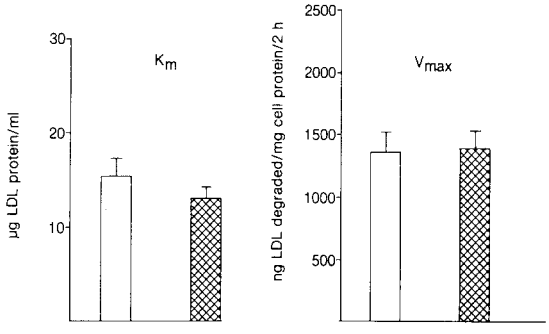

Fig. 5. Kinetics of degradation of LDL from normal control and diabetic subjects. Apparent K_m and V_{max} were derived from Lineweaver-Burk plots made from saturation curves of degradation by normal human fibroblasts of ^{125}I-LDL isolated from individual subjects. The mean ± SEM of apparent K_m and V_{max} for the control and diabetic groups were then calculated: □: control subjects: ▩: diabetic subjects

two different forms of LDL interacted in identical fashion with LDL receptors of cultured fibroblasts (data not shown).

Although LDL isolated from patients with Type 2 diabetes interacted normally with the LDL receptor on fibroblasts, it was possible that these LDL were modified in a manner that now would permit them to be processed by an alternate or 'scavenger' pathway, as proposed by Goldstein et al. [18, 19]. This possibility was evaluated by examining the binding and degradation of LDL by macrophages isolated from the mouse peritoneum. As reported previously [19], chemical modification of LDL by acetylation resulted in a particle which was avidly taken up and degraded by a high affinity saturable process by mouse peritoneal macrophages (Fig. 6). In contrast, LDL isolated from patients with Type 2 diabetes failed to display any saturable binding

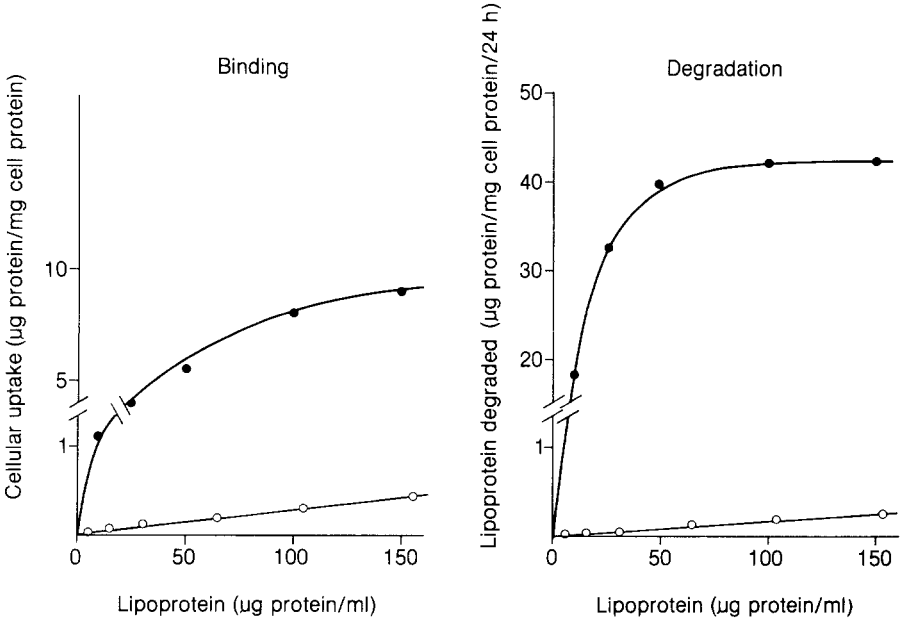

Fig. 6. Saturation curves of binding and degradation of acetylated LDL (●——●) and LDL from a diabetic subject (○——○) by mouse peritoneal macrophages. The indicated concentrations of ^{125}I-lipoproteins were incubated with monolayers of mouse peritoneal macrophages at 37 °C for 24 h. The curves represent the specific binding and degradation after correction for the amount of lipoproteins bound and degraded in the presence of 2 mg/ml of radio-inert lipoproteins

and degradation by the mouse macrophages. In addition, LDL from normal controls behaved identically (not shown). Therefore, LDL from patients with Type 2 diabetes interacted normally with receptors on both fibroblasts and cells of the scavenger pathway.

Discussion

The results of these studies have indicated that LDL isolated from patients with Type 2 diabetes are bound and degraded normally by both human fibroblasts and mouse peritoneal macrophages. Thus, we could find no evidence that hyperglycaemia in vivo led to a change in the functional behaviour of isolated LDL. These findings are in contrast with recent reports in which glycosylation in vitro markedly altered the binding of LDL to cultured human fibroblasts [5] and rat peritoneal macrophages [6]. The fact that glycosylation in vitro altered the binding and degradation of LDL is not unexpected in light of the current knowledge. It is known that the recognition site of LDL for its receptor involves arginine and lysine residues. By blocking the amino groups of arginine with ^{1,2}cyclohexanedione, or the ε-amino groups of lysine by reductive methylation in vitro, the high affinity binding and subsequent degradation can be abolished [3, 20]. Hyperglycaemia has been shown to lead to abnormal glycosylation of haemoglobin, as well as other serum proteins, by forming a Schiff base with the ε-amino groups of lysine and subsequently undergoing the non-enzymatic Amadori rearrangement to form a more stable compound [21]. Recently, LDL from diabetic patients has been reported to be similarly glycosylated [7]. If the lysine residues of the apoprotein of LDL were glycosylated to a significant degree, one might expect to observe an alteration in the manner with which LDL interacts with its receptor. Therefore, it is not immediately obvious why we could not document any change in the binding or degradation of LDL isolated from patients with Type 2 diabetes.

One possible explanation for the disparity of results in vitro and in vivo relates to the condition of the two kinds of studies. For example, the concentrations of glucose utilized in studies in vitro (up to 100 mmol/l) were much higher than those observed in our patients, and the incubations were maintained for a period of time greater than the normal half-life of LDL in plasma. Therefore, the fact that LDL isolated from patients with Type 2 diabetes did not express the same abnormality should not be too surprising. Although our results do not prove that glycosylation of LDL could never lead to a lipoprotein which demonstrated abnormal binding and degradation, the metabolic characteristics of our patient population suggest that this phenomenon must be limited to patients with extremely severe hyperglycaemia. The diabetes in our patients was clearly under poor control – they had significant fasting hyperglycaemia, HbA_{Ic} levels were aproximately twice-normal, and post-prandial plasma glucose concentrations were as high as 25 mmol/l. Unfortunately, our data provide no insight as to the level of hyperglycaemia that might be necessary to achieve the degree of glycosylation required to lead to abnormal LDL binding, but preliminary observations by Lopes-Virella et al. [22] lend support to the concept that subtle changes in LDL catabolism may exist in diabetes. They stated that the ability of fibroblasts to degrade LDL isolated from poorly controlled patients with Type 1 (insulin-dependent) diabetes was decreased, and that improved metabolic control remedied this defect. However, no changes were noted in LDL binding, and the information available does not permit a detailed comparison with our results. Nonetheless, their observations are consistent with the possibility that the LDL of at least some patients with diabetes might be altered (glycosylated) enough to lead to abnormal catabolism.

A second possible reason for the disparity between binding in vitro and in vivo relates to the catabolic rate of LDL in vivo. Thus, it is possible that a subset of LDL particles are generated in vivo which would demonstrate abnormal binding and/or degradation, but these particles may be cleared so rapidly in vivo that their isolation from plasma is impossible. Therefore, it is imperative that our results not be over-interpreted. All we can legitimately conclude is that LDL isolated from patients with a severe form of Type 2 diabetes interact normally with the LDL receptor on cultured human fibroblasts and do not interact abnormally with mouse peritoneal macrophages.

Finally, the observation that the hydrated densities of LDL from patients with Type 2 diabetes were more heterogeneous is worthy of some comment. Neither the origin nor the catabolic fate of these particles is apparent. For example, do they arise during the process of conversion of very low density lipoproteins to LDL, or subsequent to the formation of LDL? Do they only interact with the normal LDL receptor, or can they also be catabolized by alternative pathways? Answers to these questions are issues which we are currently addressing.

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