S. Braschi · M. Geoffrion · A. Nguyen ·
Y. Gaudreau · R. W. Milne

The expression of apolipoprotein B epitopes is normal in LDL of diabetic and end-stage renal disease patients

Received: 2 September 2005 / Accepted: 17 January 2006 / Published online: 4 April 2006
© Springer-Verlag 2006

Abstract *Aims/hypothesis:* When LDLs are exposed to glucose in vitro, glycation of apolipoprotein B100 (apoB) leads to a loss in its affinity for the LDL receptor and reproducible alterations in the immunoreactivity of specific apoB epitopes, including several epitopes close to the LDL receptor binding site. The aim of this work was to determine if similar immunological changes are observed in vivo in LDLs of diabetic and end-stage renal disease (ESRD) patients. *Subjects, materials and methods:* The immunoreactivity of LDLs isolated from 14 diabetic patients with normal renal function and 13 patients with ESRD was studied with a panel of 25 well-characterised anti-apoB monoclonal antibodies. *Results:* Although diabetic and ESRD LDLs showed evidence of glycation modification, none of the changes in the apoB immunoreactivity induced by glucose in vitro was observed in vivo, including those for epitopes close to the LDL receptor binding domain. *Conclusions/interpretation:* These results suggest that in vivo glycation of LDLs is a complex process that is not mimicked by in vitro exposure of LDLs to high concentrations of glucose. This questions the clinical significance of the in vitro glycation studies used to understand the pathophysiological consequences of LDL glycation in diabetes and ESRD.

Keywords AGE · Apolipoprotein B · Diabetes · End-stage renal disease · LDL

Abbreviations AGE-apoB: glycated apoB · AGE-LDL: AGE-modified LDL · apoB: apolipoprotein B100 · ESRD: end-stage renal disease · LDLr: LDL receptor · Mab: monoclonal antibody

Introduction

Cardiovascular disease is the leading cause of death in patients with diabetes and/or end-stage renal disease (ESRD) [1, 2]. Protein glycation is believed to play a causative role in both diabetic and ESRD vasculopathy, including atherosclerosis and microangiopathy [3]. Glycation is a heterogeneous process where the modification of lysine and arginine groups of proteins with glucose or dicarbonyls, such as glyceraldehyde, glyoxal, methylglyoxal and 3-deoxyglucosone, forms Schiff's base, Amadori rearrangements and AGEs [4]. AGEs are increased in diabetes mellitus in parallel with increased plasma glucose concentration, and in ESRD, where there is a decreased renal elimination of free AGE adducts [3].

Apolipoprotein B100 (apoB), a predominant component of VLDLs, intermediate density lipoproteins and LDLs, and a ligand for the LDL receptor (LDLr) is an in vivo target of AGE modification [5]. Glycation damage of LDLs leads to the loss of recognition by the cellular LDLr [5, 6] and allows their degradation through alternative macrophage receptors leading to the intracellular accumulation of cholesteryl esters [7–9]. AGE-modified LDLs (AGE-LDLs) are elevated in the circulation of diabetic and ESRD patients, and exhibit markedly impaired clearance kinetics compared with control LDLs when injected into transgenic mice expressing the human LDLr [10]. In addition, the administration of the advanced-glycation inhibitor, aminoguanidine, to diabetic patients decreases their circulating LDL levels by 28% over a 4-week period [10]. Together, this suggests that AGE modification of LDLs impairs LDLr-mediated clearance mechanisms in

S. Braschi (✉)
Endocrinology Division, Medicine Department,
University of Ottawa, Ottawa Hospital,
Riverside Campus, 1967 Riverside Drive,
Ottawa, ON, K1H 7W9, Canada
e-mail: sbraschi@ottawaheart.ca
Tel.: +1-613-7985254
Fax: +1-613-7615281

M. Geoffrion · A. Nguyen · R. W. Milne
Lipoprotein and Atherosclerosis Group,
University of Ottawa Heart Institute,
Ottawa, ON, Canada

Y. Gaudreau
Biochemistry Department, CSSSG, Hull Hospital,
Gatineau, QC, Canada

vivo and contributes to elevated LDL levels and atherosclerosis development in diabetic and ESRD patients.

AGE modification of lipoproteins such as LDLs is particularly complex. In addition to apolipoproteins, phospholipids in LDLs can also undergo AGE modification both in vivo and in vitro and this can lead to increased lipoprotein uptake by macrophages [9, 11]. Furthermore, intramolecular oxidation–reduction reactions, which are known to occur during advanced glycation, may act within the hydrophobic environment of lipoproteins to initiate fatty acid oxidation [12, 13]. It has been demonstrated that when LDLs are exposed to glucose in vitro, lipid oxidation occurs concomitantly with AGE formation and can be blocked by an inhibitor of advanced glycation [14]. When LDL is exposed to glucose in vitro, glycation leads to loss in affinity of the LDL for the LDLr and reproducible alterations in the immunoreactivity of specific apoB epitopes [15]. However, given the different potential pathways for AGE formation in vivo, it remains unclear whether the immunochemical changes of apoB that result from the in vitro exposure of LDLs to glucose reflect those that occur in the circulation or tissues of patients with diabetes and ESRD. Likewise, it is not known if the AGE modification of LDLs that occurs in patients with diabetes and ESRD induces the same alterations in the expression of epitopes of apoB, or if patients within a disease group are homogeneous in terms of AGE-induced changes in apoB immunoreactivity. In the present study, we isolated LDLs from 14 diabetic and 13 ESRD patients and characterised their immunoreactivity with a panel of 25 well-characterised anti-apoB monoclonal antibodies (Mabs). We show that the alterations in the LDL immunoreactivity that are detected with in vitro-modified LDLs are not observed with the LDLs of patients with diabetes and ESRD.

Subjects, materials and methods

Subjects

Patients were recruited at the Hull Hospital (QC, Canada). The study was approved by the Ethics Committee of the hospital, and written consent was obtained from all the studied subjects. Fourteen patients with type 2 diabetes and normal renal function were recruited (five females, nine males, age 49 ± 11 years). Thirteen patients with ESRD and on dialysis were also recruited (four females, nine males, age 56 ± 19 years); six were on peritoneal dialysis and seven on haemodialysis. Diagnosis for ESRD was diabetic nephropathy in two patients, systemic lupus erythematosus in one, scleroderma in one, IgA nephropathy in three, renal artery stenosis in one, congenital nephropathy in two, Albright's disease in one, polycystic disease in one and undetermined in one. Smokers and patients with chronic hepatic disease, hypothyroidism, familial dyslipidaemia and taking hypolipidaemic drugs or insulin were excluded. Control LDLs were prepared from 16 normolipidaemic, non-diabetic, non-ESRD subjects recruited from the staff.

Blood sampling

Venous blood was collected after an overnight fast into tubes containing 0.1 mg/ml disodium EDTA and immediately placed on ice. Plasma was promptly separated by a 15-min centrifugation at 1,000 g at 4°C and supplemented with 1 mmol/l EDTA, 20 µmol/l butylated hydroxytoluene, 0.5 mmol/l phenylmethanesulfonyl fluoride and 0.02% sodium azide and stored at –20°C.

Analysis of plasma lipids and lipoprotein components

Plasma cholesterol and triglyceride concentrations were determined using a Fusion 5.1 automat (Ortho Clinical Diagnostic, Raritan, NJ, USA). Plasma HDL-cholesterol was directly measured with an enzymatic kit (Vitros DT Chemistry; Ortho Clinical Diagnostic). Plasma LDL levels were calculated using the formula of Friedewald et al. [16].

LDLs (density 1.019–1.063 g/ml) were isolated by sequential ultracentrifugation at 130,000 g for 18 h [17] and dialysed against PBS containing 1 mmol/l EDTA and 0.02% sodium azide. The electrostatic charge of LDL particles was evaluated by electrophoresis on pre-cast 0.5% agarose gels (Paragon Lipo Kit; Beckman, Fullerton, CA, USA). LDL triglyceride, cholesterol (Roche Diagnostics, Basel, Switzerland), phospholipids and non-esterified cholesterol (Wako Chemicals, Richmond, VA, USA) were measured with enzymatic kits. The esterified cholesterol mass content of each lipoprotein fraction was calculated as the difference between total cholesterol and non-esterified cholesterol and then multiplied by 1.67, to represent the sum of esterified cholesterol and fatty acid moieties. The protein concentration of the LDLs was measured by the modified Lowry method using BSA as a standard [18].

Mabs

Characterisation of the reactivity of the panel of 25 anti-apoB Mabs with in vitro-AGE-modified LDLs and their epitope specificities have been described previously [15, 19, 20].

Measurement of AGE-LDL

Glycated apoB (AGE-apoB) levels on isolated LDL fractions were determined using a competitive immunoassay with ES12 anti-glycated apoB Mab (Glycacor; Exocell, Philadelphia, PA, USA). Mab ES12 is specific for glycated apoB and does not cross-react with other AGE-modified plasma proteins [21, 22].

Competitive immunoassay for apoB

The competitive immunoassay to determine the reactivity of the anti-apoB Mabs with LDLs from normal subjects

and patients was modified from that previously described [15]. Immulon 2 HB polystyrene microtitre strips (Thermo Labsystems, Franklin, MA, USA) were coated overnight with 50 µl of 25 µg/ml normal LDLs. Wells were washed three times with PBS containing 0.05% Tween 20 and three times with PBS and saturated with PBS containing 1% BSA (PBS-BSA). Anti-apoB Mabs, appropriately diluted in PBS-BSA (50 µl), were mixed with 50 µl of serial dilutions (in PBS-BSA) of test LDLs, incubated for 2 h and 50 µl of the mixture transferred to the LDL-coated, BSA-saturated microtitre wells. After an overnight incubation, the wells were washed as above and filled with 50 µl of horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences, Bucks, UK) or anti-rat IgG for antibodies B2 and B4 (Kirkegaard Perry Laboratories, Gaithersburg, MD, USA) and incubated for a further 2 h. After washing as above, 50 µl of tetramethylbenzidine substrate (Sigma, St Louis, MO, USA) was added to the wells and, after 20 min, the absorbance at 655 nm was read. ApoB epitope immunoreactivity of the test LDLs is expressed as ED₅₀, which represents the LDL protein concentration required for 50% inhibition of maximal binding (in the absence of competing antigen) of Mab to the plate. In each immunoassay, a control LDL formed with pooling LDLs from five normolipidaemic healthy controls was studied in parallel. In preliminary experiments we demonstrated that freezing the plasma did not affect the immunoreactivity of LDLs with the panel of anti-apoB Mabs used in the study (results not shown).

Western blotting analysis

LDL samples were subjected to SDS-PAGE (3–5% gradient) electrophoresis and transferred to nitrocellulose membranes. The membranes were probed with appropriately diluted anti-human apoB Mabs and then with horseradish peroxidase-conjugated anti-mouse IgG (Amersham). Immune complexes were revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Statistical analysis

Comparisons of means of the three groups (diabetic, ESRD and control groups) were performed using the ANOVA *F* test. Two-group comparisons of means using *t* tests with the Tukey multiple-comparison adjustment were done if the three group comparisons were statistically significant. Additional *t* tests were performed for prespecified comparisons between two groups. All tests used *p*<0.05 for the critical value of statistical significance. Analyses were performed using SAS version 9.1.3.

Results

Plasma parameters of diabetic and ESRD subjects

The diabetic population included 14 patients, five females and nine males, 49±11 years (mean ± SD). Seven were normoglycaemic with well-controlled diabetes (HbA_{1c}: 5.6±0.5%, normal range: 3.6–6.8%) and seven were chronically hyperglycaemic (HbA_{1c}: 10.5±1.4%). The ESRD population included 13 patients, four females and nine males, 56±19 years. Three ESRD patients were diabetic, but were not frankly hyperglycaemic (individual HbA_{1c} values: 5.9 and 5.8%). As recently published [23], HbA_{1c} of the non-diabetic ESRD population was slightly elevated (*n*=11, 5.8±0.3%), probably reflecting these patients' chronic anaemia, reduced erythrocyte life span and repetitive exposure to the high glucose level in the dialysate. In terms of renal function, as expected, ESRD patients had renal failure with elevated plasma creatinine (766±247 µmol/l, normal range: 45–110 µmol/l). None of the diabetic patients showed renal failure (plasma creatinine for the population was 74±10 µmol/l) or macroalbuminuria.

Plasma lipid and lipoprotein composition

As shown in Table 1, plasma total cholesterol was not significantly different between diabetic, ESRD and five control subjects analysed individually. Similarly, no

Table 1 Plasma lipid parameters (means±SD) in diabetic and ESRD patients

Subjects	Total cholesterol (mmol/l)	LDL-cholesterol (mmol/l)	HDL-cholesterol (mmol/l)	Total:HDL-cholesterol ratio	Triglycerides (mmol/l)
Diabetic (<i>n</i> =14)	5.1±0.9	3.2±0.8	1.0±0.2 ^a	5.3±1.3 ^a	2.0±1.1
ESRD (<i>n</i> =13)	4.5±1.2	2.6±0.9	0.8±0.3 ^b	6.1±1.4 ^b	2.5±1.1
Controls (<i>n</i> =5)	4.8±0.8	2.7±0.5	1.6±0.4	3.2±0.7	1.1±0.7

Three-group comparisons of means were performed using one-way ANOVA, and when statistically significant, two-group comparisons of means were performed

Significantly different: ^a*p*<0.05, diabetic vs controls; ^b*p*<0.05, ESRD vs controls

statistical difference was found between diabetic, ESRD and control subjects for LDL-cholesterol and triglyceride. In contrast, HDL-cholesterol was significantly different between the three groups, and subgroup analysis showed that HDL-cholesterol was significantly lower in diabetic patients compared with controls, and in ESRD patients compared with controls, but there was no statistical difference between the diabetic and the ESRD groups (Table 1). Analysis of the lipid and the protein content of isolated LDLs is given in Table 2 and shows that LDLs from ESRD patients were enriched in lipids compared with diabetic LDLs, with a significantly higher LDL lipid:total mass. In addition, LDLs from ESRD patients had an abnormal lipid core composition, with increased triglyceride content, resulting in a lower esterified cholesterol:triglyceride ratio compared with diabetic LDLs. The lipid composition of the lipoprotein surface, evaluated from the phospholipid:non-esterified cholesterol ratio, showed a lower phospholipid:non-esterified cholesterol ratio in ESRD LDLs compared with diabetic LDLs.

Characterisation of LDL particles

LDL particles obtained from diabetic and ESRD patients were analysed by agarose gel electrophoresis. LDLs from hyperglycaemic diabetic patients showed a slight increase in electrophoretic mobility on agarose gel compared with LDLs from well-controlled diabetic patients (relative mobility of hyperglycaemic LDLs were 1.2 ± 0.1 and 1.1 ± 0.1 , respectively, vs control LDLs), indicating that they were generally more negatively charged in hyperglycaemic conditions. Similarly, LDLs from ESRD patients also showed an increase in electrophoretic mobility (relative mobility was 1.3 ± 0.2 vs 1.1 ± 0.1 for control LDLs), indicating that they were overall more negatively charged.

Plasma AGE-apoB levels were determined in LDLs from diabetic and ESRD patients and compared with those of a subgroup of five control subjects analysed individually. Using a competitive immunoassay to quantify AGE-apoB, a low level of AGE-apoB immunoreactivity was found to be present in control subjects (21.7 ± 2.9 mg/l), and was within the normal range given by the manufacturer. This verifies previous observations that measurable quantities of AGE-LDL also circulate in healthy, non-diabetic, indi-

viduals [10, 14, 24, 25]. Comparisons of AGE-apoB means between the diabetic, ESRD and control groups showed a significant difference between the three groups ($p=0.02$). Two-group comparisons showed that AGE-apoB levels were higher in diabetic patients compared with controls (29.4 ± 5.4 vs 21.7 ± 2.9 mg/l, respectively, $p=0.03$). In addition, AGE-apoB levels were higher in ESRD patients compared with controls (29.8 ± 6.5 vs 21.7 ± 2.9 mg/l, respectively, $p=0.02$). In contrast, AGE-apoB levels were not statistically different between the diabetic and ESRD groups. AGE-apoB levels tended to be higher in hyperglycaemic diabetic patients compared with well-controlled diabetic patients, but the difference did not reach statistical difference (31.5 ± 5.1 vs 27.3 ± 5.2 mg/l, respectively). Among ESRD patients, AGE-apoB levels tended to be higher in patients on peritoneal dialysis compared with those on haemodialysis, but the difference did not reach statistical significance (33.6 ± 4.5 vs 26.5 ± 6.3 mg/ml, respectively).

Immunoreactivity of LDLs with anti-apoB Mabs

To determine the immunological changes of apoB occurring in LDLs in vivo, LDLs from diabetic and ESRD patients were tested for immunoreactivity against 25 anti-apoB Mabs. We have previously demonstrated that the immunoreactivities of these Mabs are minimally influenced by the particle size and lipid composition of the LDLs [26]. The mean ED_{50} value for each Mab was determined in diabetic and ESRD patients and normalised for the ED_{50} obtained with the control LDL pool, which was assigned a value of 1 for each antibody (Fig. 1). Overall, no change in the immunoreactivity of LDLs was observed for most Mabs in the diabetic and ESRD patients, with the exception of Mabs 376 (epitope situated between apoB residues 474 and 582), 746 (epitope situated between apoB residues 474 and 582) and 3A5 (epitope situated between apoB residues 1,880 and 2,100) [26].

Individual analysis of LDL immunoreactivity showed that 3 out of 14 (15%) diabetic LDLs and 4 out of 13 (30%) ESRD LDLs displayed a high ED_{50} value for Mabs 376 and 746, indicating that the corresponding epitope(s) is poorly expressed in these LDLs. To determine if the poor reactivity of these LDLs with Mabs 376 and 746 is due to

Table 2 LDL composition (means $\pm$ SD) in diabetic and ESRD patients

Subjects	Composition of total lipoprotein mass (%)						EC: TG ratio	PL: FC ratio
	PL	NEC	TG	EC	Protein	Lipid		
Diabetic	21 $\pm$ 1	7 $\pm$ 1	7 $\pm$ 3	39 $\pm$ 3	26 $\pm$ 3	74 $\pm$ 3	7 $\pm$ 3	7 $\pm$ 3
ESRD	24 $\pm$ 1 ^a	8 $\pm$ 1	11 $\pm$ 3 ^a	37 $\pm$ 6	21 $\pm$ 3 ^a	79 $\pm$ 3 ^a	4 $\pm$ 2 ^a	3 $\pm$ 0 ^a
Control pool	24	8	6	40	22	78	7	3

Data are from 14 diabetic and 13 ESRD patients and pooled LDLs from five control subjects. LDLs were isolated by ultracentrifugation and lipid and protein composition was measured as described in the [Subjects, materials and methods](#) section

Two-group comparison of means between diabetic and ESRD were performed using a *t* test

EC Esterified cholesterol, NEC non-esterified cholesterol, PL phospholipid, TG triglyceride

^aSignificantly different: $p < 0.05$, ESRD vs diabetics

Fig. 1 The immunoreactivity of LDLs from diabetic and ESRD patients. **a** LDLs were isolated from 13 ESRD and 14 diabetic subjects and evaluated for their immunoreactivity with 25 anti-apoB Mabs. The immunoreactivity of the LDL particle was expressed as ED₅₀, which represents the LDL concentration required for 50% inhibition of maximal binding of Mab to the plate. The mean ED₅₀ value for each Mab was determined for diabetic and ESRD LDLs

and normalised to the ED₅₀ obtained with the control LDL pool, which was assigned a value of 1 for each antibody. The *inset* (**a**) represents the same figure with a different scale for the ordinate to better show the mean and SD for the ED₅₀ for Mab 376, 746 and 3A5. *Filled bars*, ESRD patients; *open bars*, diabetic patients. **b** The position of the epitopes recognised by the Mabs within the apoB primary structure

inherent differences in the apoB (genetic polymorphism, post-translational modifications including AGE modification) or secondary to altered physical and chemical properties of the LDL particles (particle diameter, lipid composition etc.) we determined the reactivities of the LDLs by western blotting after SDS-PAGE. Results for Mab 746 are shown in Fig. 2. LDLs that reacted poorly with Mab 746 in the competitive ELISA also did not react with these antibodies after SDS-PAGE. Similar results were obtained for Mab 376 (not shown), and LDLs that failed to react with Mab 746 also failed to react with Mab 376. The immunoreactivity of LDLs with Mabs 746 and 376 was not related to the glycaemic control in the diabetic population, or to the presence of diabetes in the ESRD population. Likewise, Mab 376 and Mab 746 immunoreactivity was independent of the type of dialysis treatment (haemodialysis vs peritoneal dialysis) in ESRD patients and renal function (plasma creatinine) and LDL lipid composition in the whole population. We then determined the Mab 376 and Mab 746 immunoreactivity of LDLs isolated from 16 normal subjects. Competition curves for Mab 746 with LDLs from the 16 normal subjects and from the ESRD patients are shown in Fig. 3. The LDLs of 3 out of 16 (19%) control subjects lacked immunoreactivity for both Mabs 376 and 746. Together this indicates that Mabs 376 and 746 detect a common genetic polymorphism of apoB. In the case of Mab 3A5, LDLs from 10 of 14 (70%) diabetic patients and 9 of 13 (65%) ESRD patients showed decreased immunoreactivity compared with the pooled

LDLs from normal subjects. The reactivity of the LDLs with 3A5 by western blotting reflected that observed in the ELISA (not shown). There was no correlation of 3A5 immunoreactivity with clinical or biochemical parameters and the LDLs of 13 out of 16 (80%) of normal subjects showed reduced 3A5 immunoreactivity (not shown). As was the case for Mabs 376 and 746, Mab 3A5 appears to recognise a common apoB genetic polymorphism.

Discussion

Many of our concepts concerning the pathophysiological consequences of AGE modification of LDLs have come

Fig. 2 Immunoreactivity of ESRD and control LDLs for Mab 746 after SDS-PAGE in denaturing conditions. ESRD LDLs: lanes 1–13; control LDLs: control lane. LDL samples were subjected to SDS-PAGE (3–5% gradient) and transferred to nitrocellulose membranes. The membranes were subsequently exposed to Mab 746 and horseradish peroxidase-conjugated anti-mouse IgG. Bound IgG was detected using a chromogenic substrate

Fig. 3 Competition inhibition curve obtained with Mab 746 for ESRD and control subjects. The immunoreactivity of LDLs isolated from 13 ESRD patients (*closed circles*) and 16 control subjects (*open circles*) analysed individually with Mab 746 was determined by ELISA. The competition curves are expressed as B/B_0 , where B represents binding in the presence of competitor and B_0 binding in the absence of competitor as a function of LDL concentration. The *upper line* represents the average competition curve for four ESRD and three control LDLs that poorly expressed the epitope for Mab 746. The *lower curve* represents the average competition curve for nine ESRD and 13 control LDLs that expressed the epitope for Mab 746 at their surface

from experiments using LDLs that had been exposed *in vitro* to supra-physiological concentrations of glucose for periods that greatly exceed the intravascular life span of LDLs. A major site of AGE modification in apoB that was predicted to have important consequences in apoB function was identified using LDLs that had been exposed to 200 mmol/l glucose for up to 2 weeks [10]. It is unknown if this site is also a target of AGE modification *in vivo*. We have reported that a similar *in vitro* exposure of LDLs to glucose resulted in AGE modification of the LDLs, a loss of affinity for the LDLr and reproducible alterations in the immunoreactivity of a subset of apoB epitopes that are located between apoB residues 1,438 and 1,481 (2D8), 1,854 and 1,878 (B4), 1,880 and 2,100 (3A5), 2,980 and 3,084 (4G3), 3,687 and 4,081 (588) and 4,342 and 4,536 (234) [15]. In the present study, we have used the same panel of Mabs to compare the immunoreactivity of LDLs from diabetic and ESRD patients. Of the six Mabs that showed reduced reactivity with *in vitro*-AGE-modified LDLs, only 3A5 was less reactive with some LDLs from both diabetic and ESRD patients. However, these differences in 3A5 immunoreactivity did not result from AGE modification of the LDLs but probably reflect a genetic polymorphism of apoB. In fact, no differences in immunoreactivity were detected that could be attributed to AGE modification in LDLs from diabetic and ESRD patients with any of the 25 anti-apoB Mabs that were tested.

The high glucose concentrations and the extended period of exposure of the LDLs to glucose used in *in vitro* AGE modification may induce changes of greater magnitude than those that occur *in vivo*. LDLs glycated *in vitro* with a

physiologically relevant concentration of glucose (10 mmol/l) are taken up by the LDLr pathway, in contrast to LDLs glycated with 50 mmol/l of glucose that are mostly taken up by the scavenger receptor, illustrating that different concentrations of glucose induce different changes in LDL particle properties [27].

We have also observed that *in vitro* modification of LDLs with glucose leads to a uniform increase in the electrophoretic mobility of LDL particles (X. Wang and R. W. Milne, unpublished observations). In contrast, fractionation of LDL particles from diabetic and control subjects on the basis of their charge showed that only a subpopulation of plasma LDL particles, ranging from 5 to 20% of total LDLs, have an increased negative electrostatic charge [7, 28]. Using an AGE-apoB immunoassay with ES12 Mab, which recognises a fructosyl adduct uniquely in the context of glycated apoB [22], it was determined that 2–5% of total apoB in control subjects and 3–15% in diabetic patients is glycated *in vivo* [21, 29]. Using the same assay, AGE-apoB levels were significantly higher in diabetic and ESRD subjects than in control subjects in our study. In addition, AGE-apoB levels tended to be higher in poorly controlled than in well-controlled diabetic subjects, but the difference did not reach the significance level. This contrasts with the HbA_{1c} levels that were markedly different between poorly and well-controlled diabetic subjects. It is of note that HbA_{1c} levels are poorly correlated with AGE-apoB levels, as determined with the ES12 antibody, with HbA_{1c} levels lower than 12–13% [21], which was the case for most of the hyperglycaemic patients studied here. It is nevertheless possible that Mabs specific for other AGE adducts or a polyclonal antiserum against a range of adducts may have detected significant differences between well-controlled and poorly controlled diabetic subjects. The lack of significance may also relate to the limited number of patients in each group (seven patients in each group).

Since the negatively charged LDL subfraction, from both non-diabetic and diabetic subjects, induces a greater esterified cholesterol accumulation when incubated with macrophages *in vitro* compared with the rest of the LDLs [7, 30], it was suggested that only a subset of negatively charged AGE-LDLs would specifically contribute to atherosclerosis development. In this study, we were unable to identify apoB epitopes whose immunoreactivities are altered in LDLs from diabetic and ESRD patients. However, subpopulations of LDL particles, such as negatively charged LDLs or immunoaffinity-purified AGE-LDLs isolated from plasma of diabetic or ESRD patients, may show altered apoB epitope expression compared with non-modified LDLs. However, using GC-MS, Jenkins et al. did not find significant differences in the content of two major AGE species, carboxymethyllysine and carboxyethyllysine, between the negatively charged LDL subfraction and the bulk of the LDLs isolated from diabetic patients, although fructosyl-lysine, an early glycation end product, was significantly increased in more negatively charged LDLs [25].

Our inability to identify apoB epitopes whose immunoreactivities are altered in LDLs from diabetic and ESRD patients may also reflect the experimental design. In our previous study [15], the immunoreactivity of LDLs that were incubated in the presence of an elevated concentration of glucose was compared with that of the same LDLs incubated in the absence of glucose. In the present study we have compared LDLs from diabetic and ESRD patients. In this case, inter-individual heterogeneity in the expression of individual epitopes may have masked changes in the immunoreactivity that result from AGE modification. In addition, extensively AGE-modified LDLs may also be rapidly cleared in vivo by the reticuloendothelial system through scavenger receptor-mediated uptake. It is also possible that AGE modification of LDLs particles occurs mostly after transcytosis in the subendothelium of the artery walls [31–33] and exposure to subendothelial AGEs [34] and not during their circulation in plasma.

Nevertheless, it is well documented that LDLs from diabetic and ESRD patients have altered functional properties. Compared with LDLs from normal subjects, they show a reduced fractional clearance rate when injected into transgenic mice expressing a human LDLr transgene [10]. Total LDL [35] and the isolated AGE-LDL subfraction [7] from diabetic patients show increased binding to macrophage scavenger receptors compared with LDLs from normal subjects. A number of functional parameters are also altered in cultured endothelial cells that are exposed to diabetic LDLs [36, 37]. Assuming that these functional differences in LDLs from diabetic and ESRD patients reflect AGE modification of apoB, unlike the situation with in vitro-modified LDLs, the modifications are not manifested in changes in immunoreactivity that can be detected by our panel of anti-apoB Mabs. Recent studies conducted with human serum albumin showed that glycation of the molecule with methylglyoxal affects not only residues located at the surface of the molecule, but also specific residues with low surface exposure [38]. It is therefore possible that apoB modification in vivo involves residues with low surface exposure that are not detected by our panel of Mabs. These modifications may also induce conformational changes in the secondary and tertiary structure of the apoB molecule that affect its functional properties [39, 40].

In summary, we show here that the changes in LDL immunoreactivity induced by the in vitro glycation of LDL with glucose are not observed in vivo in diabetic and ESRD patients. This questions the clinical significance of in vitro glycation studies. Moreover, the in vivo AGE modification of LDLs is a complex process that involves different compounds, such as free glycation adducts [3], and cannot be reproduced by the co-incubation of LDLs with glucose in vitro.

Acknowledgements This work was supported by a grant from the Canadian Diabetes Association in honour of the late Ian M. McLean. We are grateful to R. N. N. Fillion, M. Cyr and D. Boivert for their expert clinical assistance and to K. Williams for statistical analysis. The antibodies B2 and B4 were kindly provided by J. C. Fruchart (Institut Pasteur, Lille, France).

References

1. Koivisto VA, Stevens LK, Mattock M et al (1996) Cardiovascular disease and its risk factors in IDDM in Europe. EURODIAB IDDM Complications Study Group. *Diabetes Care* 19:689–697
2. Schwedler SB, Metzger T, Schinzel R, Wanner C (2002) Advanced glycation end products and mortality in hemodialysis patients. *Kidney Int* 62:301–310
3. Thornalley PJ, Battah S, Ahmed N et al (2003) Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* 375:581–592
4. Thornalley PJ, Langborg A, Minhas HS (1999) Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* 344:109–116
5. Sasaki J, Arora V, Cottam GL (1982) Nonenzymatic galactosylation of human LDL decreases its metabolism by human skin fibroblasts. *Biochem Biophys Res Commun* 108:791–796
6. Lorenzi M, Cagliero E, Markey B, Henriksen T, Witztum JL, Sampietro T (1984) Interaction of human endothelial cells with elevated glucose concentrations and native and glycosylated low density lipoproteins. *Diabetologia* 26:218–222
7. Klein RL, Laimins M, Lopes-Virella MF (1995) Isolation, characterization, and metabolism of the glycosylated and non-glycosylated subfractions of low-density lipoproteins isolated from type I diabetic patients and nondiabetic subjects. *Diabetes* 44:1093–1098
8. Lam MC, Tan KC, Lam KS (2004) Glycosylated low-density lipoprotein regulates the expression of scavenger receptors in THP-1 macrophages. *Atherosclerosis* 177:313–320
9. Ravandi A, Kuksis A, Shaikh NA (1999) Glycated phosphatidylethanolamine promotes macrophage uptake of low density lipoprotein and accumulation of cholesteryl esters and triacylglycerols. *J Biol Chem* 274:16494–16500
10. Bucala R, Makita Z, Vega GL, Grundy SM, Koschinsky T, Cerami A (1994) Modification of low density lipoprotein by advanced glycation end products contributes to the dyslipidemia of diabetes and renal insufficiency. *Proc Natl Acad Sci USA* 91:9441–9445
11. Ravandi A, Kuksis A, Shaikh NA (2000) Glycosylated glycerophosphoethanolamines are the major LDL glycation products and increase LDL susceptibility to oxidation: evidence of their presence in atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 20:467–477
12. Baynes JW (1991) Role of oxidative stress in development of complications of diabetes. *Diabetes* 40:405–412
13. Bucala R (1997) Lipid and lipoprotein modification by advanced glycosylation end-products: role in atherosclerosis. *Exp Physiol* 82:327–337
14. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H (1994) Lipid advanced glycosylation: pathway for lipid oxidation in vitro. *Proc Natl Acad Sci USA* 90:6434–6438
15. Wang X, Bucala R, Milne R (1998) Epitopes close to the apolipoprotein B low density lipoprotein receptor-binding site are modified by advanced glycation end products. *Proc Natl Acad Sci USA* 95:7643–7647

16. Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18:499–502
17. Havel RJ (1998) Receptor and non-receptor mediated uptake of chylomicron remnants by the liver. *Atherosclerosis* 141:S1–S7
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
19. Pease RJ, Milne RW, Jessup WK et al (1989) Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B100. *J Biol Chem* 265:553–568
20. Wang XY, Chauhan V, Nguyen AT et al (2003) Immunochemical evidence that human apoB differs when expressed in rodent versus human cells. *J Lipid Res* 44:547–553
21. Cohen MP, Lautenslager G, Shea E (1993) Glycated LDL concentrations in non-diabetic and diabetic subjects measured with monoclonal antibodies reactive with glycated apolipoprotein B epitopes. *Eur J Clin Chem Clin Biochem* 31:707–713
22. Shea EA, Cohen MP (1993) Immunologic detection and measurement of glycated apolipoprotein B with site specific monoclonal antibodies. *J Immunol Methods* 162:85–95
23. Wang X, Peesapati SK, Renedo MF, Moktan S (2004) Hemoglobin A1c levels in non-diabetic patients with end-stage renal disease (ESRD) receiving hemodialysis. *J Endocrinol Invest* 27:733–735
24. Bucala R (1996) Lipid and lipoprotein oxidation: basic mechanisms and unresolved questions in vivo. *Redox Rep* 2:291–307
25. Jenkins AJ, Thorpe SR, Alderson NL et al (2004) In vivo glycated low-density lipoprotein is not more susceptible to oxidation than nonglycated low-density lipoprotein in type 1 diabetes. *Metabolism* 53:969–976
26. Wang XY, Pease R, Bertinato J, Milne RW (2000) Well-defined regions of apolipoprotein B-100 undergo conformational change during its intravascular metabolism. *Arterioscler Thromb Vasc Biol* 20:1301–1308
27. Gugliucci CA, Dumont S, Siffert JC, Stahl AJ (1992) In vitro glycated low-density lipoprotein interaction with human monocyte-derived macrophages. *Res Immunol* 143:17–23
28. Shimano H, Yamada N, Ishibashi S et al (1991) Oxidation-labile subfraction of human plasma low density lipoprotein isolated by ion-exchange chromatography. *J Lipid Res* 32:763–773
29. Stahl AJ, Rima A, Blickle JF, Brogard JM (1998) Short-term variations of serum glycated apolipoprotein B. *Diabetes Metab* 24:151–155
30. Avogaro P, Bon GB, Cazzolato G (1988) Presence of a modified low density lipoprotein in humans. *Arteriosclerosis* 8:79–87
31. Kaplan M, Aviram M (2001) Retention of oxidized LDL by extracellular matrix proteoglycans leads to its uptake by macrophages: an alternative approach to study lipoproteins cellular uptake. *Arterioscler Thromb Vasc Biol* 21:386–393
32. Proctor SD, Mamo JC (2003) Intimal retention of cholesterol derived from apolipoprotein. *Arterioscler Thromb Vasc Biol* 23:1595–1600
33. Tabas I (1999) Nonoxidative modifications of lipoproteins in atherogenesis. *Annu Rev Nutr* 19:123–139
34. Kirshtein M, Brett J, Radoff S, Ogawa S, Stern D, Vlassara H (1990) Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: role in vascular disease of diabetes and aging. *Proc Natl Acad Sci USA* 87:9010–9014
35. Dobrian A, Lazar V, Tirziu D, Simionescu M (1996) Increased macrophage uptake of irreversibly glycated albumin modified low density lipoproteins of normal and diabetic subjects is mediated by non-saturable mechanisms. *Biochim Biophys Acta* 1317:5–14
36. Brizzi MF, Dentelli P, Gambino R et al (2002) STAT5 activation induced by diabetic LDL depends on LDL glycation and occurs via src kinase activity. *Diabetes* 51:3311–3317
37. Rabini RA, Cester N, Staffolani R et al (1999) Modifications induced by LDL from type 1 diabetic patients on endothelial cells obtained from human umbilical vein. *Diabetes* 48:2221–2228
38. Ahmed N, Dobler D, Dean M, Thornalley PJ (2005) Peptide mapping identifies hotspot site of modification in human serum albumin by methylglyoxal involved in ligand binding and esterase activity. *J Biol Chem* 280:5724–5732
39. Gallego-Nicasio J, López-Rodríguez G, Martínez R, Tarancón MJ, Fraile MV, Carmona P (2003) Structural changes of low density lipoproteins with Cu²⁺ and glucose induced oxidation. *Biopolymers* 72:514–520
40. Kramer-Guth A, Quaschnig T, Galle J et al (1997) Structural and compositional modifications of diabetic low-density lipoproteins influence their receptor-mediated uptake by hepatocytes. *Eur J Clin Invest* 27:460–468