Oxidized lipoproteins found in patients with NIDDM stimulate radical-induced monocyte chemoattractant protein-1 mRNA expression in cultured human endothelial cells

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Summary Although oxidized low density lipoprotein (LDL) exists in plasma from diabetic patients, there are few studies on its biological activity. Thus, we investigated the biological potency of LDL plus intermediate density lipoprotein fraction isolated from 12 non-diabetic and 24 non-insulin-dependent diabetic subjects of similar age and body mass index, in order to induce monocyte chemoattractant protein-1 (MCP-1) mRNA expression in cultured human endothelial cells. MCP-1 mRNA content in the cells exposed to the lipoproteins isolated from the diabetic patients was significantly higher than that from the control subjects ($p < 0.001$). The increment of MCP-1 mRNA content was positively correlated with not only HbA_{1c} ($r = 0.58$, $p < 0.0001$) but also lysophosphatidylcholine (LPC) content in the lipoprotein ($r = 0.46$, $p < 0.005$) and was negatively correlated with diene formation lag time as a marker of

oxidizability of the lipoprotein ($r = -0.33$, $p < 0.05$). Treatments of the cells with either 50 $\mu\text{mol/l}$ probucol, 50 $\mu\text{mol/l}$ α -tocopherol, or 0.1 mmol/l deferoxamine suppressed the increase in MCP-1 mRNA content induced by diabetic lipoproteins, respectively. Furthermore, the diabetic lipoproteins activated nuclear transcription factor NF- κ B in the cells, which was inhibited by pre-treatment of cells with 50 $\mu\text{mol/l}$ probucol. These data indicate that oxidatively modified lipoproteins found in diabetic plasma stimulate MCP-1 gene expression in endothelial cells. The LPC content which reflects oxidative modification of lipoprotein is at least a possible marker of biological activity to increase an atherogenic cytokine in endothelial cells. [Diabetologia (1997) 40: 662–670]

Keywords Oxidized lipoprotein, free radical, atherosclerosis, endothelial cells, antioxidants.

Diabetes mellitus is postulated as an independent risk factor for the development of atherosclerosis, although the accelerated atherosclerosis in patients with non-insulin-dependent diabetes mellitus (NIDDM) is the result of multiple risk factors [1].

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; HUVECs, human umbilical vein endothelial cells; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor kappa B; LPC, lysophosphatidylcholine; MEM, minimal essential medium; FCS, fetal calf serum.

Among many risk factors for atherogenesis in diabetes, recent interest has focused on the possible involvement of oxidative modification of low density lipoprotein (LDL) as a crucial factor in the atherogenic process, since diabetes is believed to be associated with increased oxidative stress [2–6]. Evidence indicates that circulating levels of several forms of modified LDL are elevated in diabetic plasma [7–10]. Furthermore, it has also been shown that LDL isolated from patients with poorly controlled IDDM has an enhanced susceptibility to oxidation [11, 12]. Together this evidence suggests, the lipoproteins may be modified oxidatively in vivo in the diabetic condition.

Oxidized LDL has several biological activities: for example, it enhances lipid accumulation in macrophages [13]; stimulates chemotaxis of circulating

monocytes [14]; inhibits vasodilatation [15]; and modulates gene expression of various growth factors, adhesion molecules and cytokines [16, 17]. Thus, it is clear that lipoproteins oxidized after being incubated with cells or a heavy metal ion *in vitro* have atherogenic potency. However, there have been few studies on the biological activity of lipoproteins oxidized in diabetes. Although their clinical relevance is not well established, it has already been shown that LDL from diabetic patients stimulates intracellular cholesterol accumulation in human macrophages [18] and intimal aortic cells [7].

We investigated the effect of the lipoproteins obtained from patients with NIDDM on modulation of gene expression in human umbilical vein endothelial cells (HUVECs) to address other potential roles for diabetic lipoproteins. Monocyte chemoattractant protein-1 (MCP-1) is a specific chemoattractant factor for monocytes and is strongly expressed in human atheromatous plaques [19, 20]. Therefore, MCP-1 is thought to be a crucial factor in early stages of atherogenesis. Moreover, it is suggested that transcription factor nuclear factor- κ B (NF- κ B), which is regulated by oxidative stress, may contribute to gene expression of atherogenic factors such as MCP-1 [21]. Furthermore, it has been reported that NF- κ B is regulated by the intracellular redox state [22]; thus, both activation of NF- κ B and increased MCP-1 mRNA content in the cells which are induced by oxidized lipoprotein may be normalized by treating the cells with antioxidants.

Therefore, in the present study we studied whether: 1) the lipoprotein fraction containing IDL and LDL isolated from patients with NIDDM had increased ability to induce production of MCP-1 mRNA as compared with that from non-diabetic subjects; 2) any characteristics of the lipoprotein as an oxidatively modified lipoprotein were correlated to its activity to increase MCP-1 mRNA content; and 3) the lipoprotein was able to induce activation of transcription factor NF- κ B through free radical-mediated mechanisms in endothelial cells. These results indicate that lipoprotein lysophosphatidylcholine (LPC) content is a good marker for the ability to induce gene expression of MCP-1 in endothelial cells.

Materials and methods

Subjects. Twenty-four NIDDM patients and 12 non-diabetic control subjects were studied. Non-diabetic subjects were diagnosed according to the World Health Organization criteria of a 2-h plasma glucose level less than 11.1 mmol/l [23], and were matched for age, sex, and body mass index (Table 1). Duration of diabetes was 11.6 ± 1.6 years. Ten of the 24 diabetic patients were treated with oral hypoglycaemic agents (8 were treated with sulfonylurea and 2 with sulfonylurea plus an α -glucosidase inhibitor). Two of the diabetic subjects had

Table 1. Characteristics of the subjects

	Non-diabetic	NIDDM
<i>n</i>	12	24
Gender (male/female)	5/7	10/14
Age (years)	56.2 ± 3.0	58.7 ± 2.1
Body mass index (kg/m ²)	23.2 ± 0.8	25.5 ± 1.1
Fasting glucose (mmol/l)	5.28 ± 0.21	7.29 ± 0.44^a
Haemoglobin A _{1c} (%)	4.9 ± 0.2	7.7 ± 0.3^a
Total cholesterol (mmol/l)	5.24 ± 0.26	4.76 ± 0.16
Triglyceride (mmol/l)	1.50 ± 0.25	1.35 ± 0.16
HDL (mmol/l)	1.11 ± 0.08	1.10 ± 0.087
ApoB (mg/dl)	112.3 ± 9.4	98.6 ± 5.0
ApoAI (mg/dl)	115.3 ± 6.7	110.9 ± 5.01
Immunoreactive insulin (pmol/l)	46.9 ± 4.2	65.1 ± 4.9

Results are mean $\pm$ SEM.

^a $p < 0.001$ vs non-diabetic control subjects

macroalbuminuria (urinary albumin excretion rate greater than 200 μ g/min) and 10 had microalbuminuria (urinary albumin excretion rate between 15 and 200 μ g/min). Six of the diabetic subjects had background retinopathy and four had proliferative retinopathy. Five diabetic subjects had evidence of ischaemic heart disease on exercise ECG. Two control and 10 diabetic subjects had hypertension (based upon the criteria 140/90 mmHg) and were treated with either calcium channel blockers or beta-blockers, which had no antioxidant activity. Subjects who were cigarette smokers, or taking lipid-lowering agents, vitamins or antioxidative drugs were excluded from the study. Furthermore, subjects who had either hypercholesterolaemia (total cholesterol > 5.98 mmol/l) or renal insufficiency (serum creatinine > 106 μ mol/l) were also excluded from the present study. Fasting plasma concentrations of glucose, triglyceride and total cholesterol were assayed by standard enzymatic methods. Apolipoprotein (apo) AI, apo B and haemoglobin A_{1c} (HbA_{1c}) concentrations were measured by radioimmunoassay using a standard assay kit and HPLC (Daiichi Kagaku, Kyoto, Japan), respectively.

Materials. A PD-10 column was purchased from Pharmacia (Uppsala, Sweden) and Silica gel 60 was obtained from E. Merck (Darmstadt, Germany). Minimal essential medium (MEM) was obtained from Gibco Laboratories (Grand Island, N.Y., USA). Oligonucleotides for mobility shift assays were obtained from Promega (Madison, Wis., USA). Probuco was provided by Dai-ichi Pharmaco. Co. (Tokyo, Japan). All other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Lipoprotein isolation. Venous blood was collected after an overnight fast into tubes containing EDTA (final concentration: 4 mmol/l) for preparation of plasma. LDL plus IDL ($d = 1.006$ – 1.063 g/ml) were sequentially fractionated by ultracentrifugation of the plasma according to the method described previously [24] and the preparations were sterilized by filtration (pore size, 0.45 μ m). The lipoproteins were stored at 4°C under N₂ gas.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to the method described previously [25]. Cells were grown in MEM supplemented with 10% fetal calf serum (FCS; Gibco) 90 μ g/ml heparin (Novo Nordisk A/S, Copenhagen, Denmark) and 50 μ g/ml endothelial cell growth factor (Boehringer Mannheim GmbH, Mannheim, Germany), in a humidified atmosphere containing 5% CO₂. The cells were used prior to passage 6.

Northern blot analysis of MCP-1 mRNA content. The cells were cultured for 6 h in MEM containing 2% FCS and 100 µg/ml LDL plus IDL fraction obtained from either non-diabetic control or NIDDM subjects. To study the effects of anti-oxidants on MCP-1 mRNA content in endothelial cells, the cells were incubated in the absence or presence of the lipoprotein with or without pretreatment of cells with either 50 µmol/l probucol for 20 h, 50 µmol/l α -tocopherol for 20 h or 0.1 mmol/l deferoxamine for 1 h before exposing the cells to the lipoprotein. Then, MCP-1 mRNA content was measured by Northern blot analysis. In brief, total RNA was isolated from HUVECs by acid guanidium thiocyanate-phenol chloroform extraction [26]. Total RNA (20 µg) was separated by electrophoresis on 1.2% agarose gels containing formaldehyde, and transferred onto nytran membranes (NY13; Achleicher and Schuell, Dassel, Germany). The human MCP-1 cDNA obtained by the method described previously [27] was labelled with [α - 32 P] dCTP (New England Nuclear, Boston, Mass., USA), then hybridized at 65°C overnight in a buffer containing 0.5 mol/l Na_2HPO_4 , 1% bovine serum albumin, 1 mmol/l EDTA, and 7% SDS. After hybridization, filters were washed at a final stringency of $0.2 \times$ standard sodium citrate (SSC) ($1 \times$ is 150 mmol/l NaCl, 15 mmol/l sodium citrate) containing 5% SDS at 65°C. Autoradiography was performed with an intensifying screen at -70°C. Laser densitometry and digital analysis of scanned images were used for quantitative analysis of autoradiograms.

Measurements of phospholipid composition. Lipids were extracted from the lipoprotein fractions by the method of Bligh and Dyer [28] and then separated by two-dimensional thin-layer chromatography on Silica gel 60 plate with methyl acetate : 1-propanol : chloroform : methanol : 0.25% KCl (25 : 25 : 10 : 6), followed by chloroform : methanol : water (65 : 35 : 6). Separated lipids were visualized using iodine vapor, scraped off and analysed for inorganic phosphate by the method of Bartlett [29]. Each phospholipid was identified by co-migration with standards. The concentrations of LPC, phosphatidylcholine, phosphatidyl ethanolamine, and sphingomyelin in the fraction were expressed as nmol Pi/mg apoB protein.

Measurements of LDL oxidation. The lipoprotein fraction was passed through a PD-10 column to desalt and remove the EDTA just before measurement. The protein concentration was measured by the method described previously [30]. LDL oxidation was measured by the method of Esterbauer et al. [31] in which 20 µmol/l copper was added to a spectrophotometer cuvette (U-2000; Hitachi Instruments Inc., Tokyo, Japan) containing lipoprotein (25 µg protein/ml in phosphate buffered saline), and the kinetic study of conjugated diene formation was monitored by changes in absorbance at 234 nm at 25°C at 10-min intervals. Susceptibility of the lipoprotein to peroxidation was quantified by the duration of the lag phase before the propagation phase of diene conjugation.

Electrophoretic mobility shift assay of NF- κ B activity. HUVECs were incubated with the lipoprotein for 1 h and then rinsed with ice-cold phosphate buffered saline, scraped off and collected in tubes. To study the effect of probucol on NF- κ B activity, the cells were pre-incubated with or without probucol (50 µmol/l) for 20 h before exposing cells to the lipoproteins. Nuclear extracts were prepared as reported previously [32]. The double-strand DNA probe (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was labelled by using [γ - 32 P]-ATP and T4 polynucleotide kinase. The protein DNA binding reaction was performed at room temperature for 20 min in a 20 µl volume. The reaction mixture contained 2 µg

of nuclear extract, 100 µg/ml poly dI:dC, 10 mmol/l Tris/HCl (pH 7.5), 50 mmol/l NaCl, 0.5 mmol/l EDTA, 0.5 mmol/l dithiothreitol, 1 mmol/l MgCl_2 , 4% glycerol and 100000 cpm labelled DNA. Following the incubation, the samples were loaded onto a 6% polyacrylamide gel in $0.25 \times$ TBE ($1 \times$ TBE is 90 mmol/l Tris, 90 mmol/l boric acid, 2.5 mmol/l EDTA) and run at 150 V for 2 h. Gels were dried and visualized by autoradiography. For competition experiments, conditions were identical except that appropriate non-radiolabelled competitor oligonucleotides were added at a 100-fold molar excess to the reaction mixture before addition of nuclear extracts. The amount of nuclear protein was determined with Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif., USA).

Statistical analysis

Values presented are mean $\pm$ SEM. Statistical analyses were performed with the unpaired Student's *t*-test. Significance was determined at the 95% confidence level.

Results

Patient characteristics. Patients with NIDDM showed high levels of fasting blood glucose and HbA_{1c} as compared with control subjects (Table 1). No differences were observed in plasma lipid and lipoprotein levels between diabetic and control groups. The mean of both body mass index and fasting plasma insulin of the diabetic group tended to be higher than those of the control group, but those differences were not statistically significant.

Effect of the lipoproteins on MCP-1 gene expression in HUVECs. To investigate the difference in biological activity of the lipoproteins containing IDL and LDL obtained from the diabetic and non-diabetic subjects, we used Northern blot analysis to measure the increase of MCP-1 mRNA content in HUVECs exposed to the lipoproteins. The accumulation of MCP-1 mRNA content peaked at 4 h and sustained the maximal level until 8 h after the stimulation. MCP-1 mRNA content in cells exposed to the lipoproteins obtained from patients with NIDDM was significantly ($p < 0.001$) higher than that from control subjects (non-diabetic control vs diabetic: 102.2 ± 12.0 vs $182.0 \pm 12.4\%$, when expressed as a percentage of the constitutional level) (Fig. 1).

Relationship between characteristics of the IDL plus LDL fraction and its effect on MCP-1 mRNA content. We characterized the oxidative modification of the lipoproteins, which had a biological ability to increase MCP-1 mRNA content. As shown in Table 2, total phospholipid content in the lipoprotein fraction was similar between non-diabetic and diabetic subjects. However, the mean value of LPC content in the fraction obtained from diabetic patients was a 2.8 times

Fig. 1 a, b. Effect of lipoprotein on MCP-1 gene expression in HUVECs. Cells were cultured for 6 h in MEM containing 2% fetal calf serum and 100 $\mu\text{g/ml}$ LDL + IDL fraction obtained from either non-diabetic control ($n = 12$) or NIDDM subjects ($n = 24$). Total RNA was isolated from those cells and MCP-1 mRNA was measured by Northern blot analysis. **(a)** Autoradiograph showing mRNA levels for MCP-1 or GAPDH in HUVECs induced by the lipoprotein. Results for four control subjects and four NIDDM patients are shown. **(b)** Densitometric analysis showing MCP-1 mRNA levels in HUVECs induced by the lipoproteins. MCP-1 mRNA is expressed as percent of the constitutional level in each experiment. Data are expressed as mean $\pm$ SEM. * $p < 0.001$ vs control

higher than that from control subjects ($p < 0.01$). On the other hand, the other phospholipid contents were not significantly different between the two groups. To evaluate the susceptibility of the lipoproteins to oxidative stress, we measured the duration of lag phase of conjugated diene formation during copper-induced oxidation. The mean value of lag time showed no difference between non-diabetic control and NIDDM subjects (non-diabetic: 113.6 ± 18.0 min, NIDDM: 105.5 ± 15.9 min), although the value in patients with poorly controlled diabetes was significantly ($p < 0.01$) shortened (67 ± 5.6 min, $n = 12$, at HbA_{1c} $9.0 \pm 0.3\%$), when compared with that of diabetic patients in good glycaemic control (144 ± 28 min, $n = 12$, at HbA_{1c} $6.4 \pm 0.2\%$).

We observed that the increase in MCP-1 mRNA content induced by the lipoproteins of both control and diabetic subjects was positively correlated with HbA_{1c} level ($r = 0.58$, $p < 0.0001$) (Fig. 2a). Furthermore, LPC content in the lipoprotein was increased

Table 2. Analysis of phospholipid composition in the lipoprotein fraction

(nmol/mg apoB)	Non-diabetic	NIDDM
Lysophosphatidylcholine	69.8 ± 20.0	195.6 ± 27.3^a
Sphingomyelin	334.1 ± 8.1	328.1 ± 52.4
Phosphatidylcholine	681.4 ± 80.7	729.3 ± 70.1
Phosphatidylethanolamine	262.9 ± 62.1	426.6 ± 55.9
Total	1348.1 ± 199.9	1679.3 ± 149.2

Data are mean $\pm$ SEM. ^a $p < 0.01$ vs non-diabetic

The lipoproteins were fractionated between d 1.006 and 1.063

according to elevated plasma HbA_{1c} concentrations ($r = 0.60$, $p < 0.0001$) (Fig. 2b) and a weak negative correlation between diene formation lag time as a marker of oxidizability of the lipoprotein and HbA_{1c} concentrations was found ($r = -0.34$, $p < 0.05$) (Fig. 2c). Consistent with those results, the increased MCP-1 mRNA content in endothelial cells exposed to the lipoproteins was positively correlated with LPC content in the lipoprotein ($r = 0.46$, $p < 0.01$) (Fig. 3a) and negatively correlated with diene formation lag time ($r = -0.33$, $p < 0.05$) (Fig. 3b).

Effect of antioxidants on MCP-1 mRNA content induced by the lipoprotein. To clarify the mechanism by which the diabetic lipoproteins stimulate MCP-1 gene expression in the cells, we investigated the effect of antioxidants. Cells were pre-treated with either 50 $\mu\text{mol/l}$ probucol or 50 $\mu\text{mol/l}$ α -tocopherol for 20 h and then incubated in MEM containing 2% fetal bovine serum with or without 100 $\mu\text{g/ml}$ lipoprotein for 6 h. The increase of MCP-1 mRNA content induced by the lipoprotein isolated from NIDDM was significantly ($p < 0.05$) reduced after antioxidant treatment (probucol-treated: $99.5 \pm 8.3\%$ and α -tocopherol-treated: $100 \pm 15.3\%$ of the constitutional level), when compared with the values of the non-treated group ($336.7 \pm 44.8\%$ of the constitutional level) (Fig. 4a, b). However, MCP-1 mRNA content in the cells exposed to the lipoprotein isolated from control subjects was not affected by either treatment (Fig. 4a, b). Moreover, deferoxamine (0.1 mmol/l), an iron-chelating agent, also significantly ($p < 0.05$) suppressed MCP-1 expression induced by the diabetic lipoproteins (deferoxamine-treated: $86.0 \pm 4.9\%$ of basal vs the non-treated: $192 \pm 9.9\%$ of constitutional level). (Fig. 5a, b). However, the level in the cells exposed to control lipoprotein was not affected by the deferoxamine treatment.

NF- κ B binding activity in HUVECs exposed to the lipoprotein. To further clarify the role of cellular oxidative signalling induced by the lipoproteins, we investigated the activity of DNA binding of NF- κ B in HUVECs exposed to the lipoproteins. In the present study, incubation of cells with only 2% fetal bovine serum did not activate NF- κ B activity. However, two

Fig 2a, b. Relationship between glycaemic control expressed as HbA_{1c} concentrations (%) and biological potency to increase MCP-1 mRNA in the cultured endothelial cells (% increase over basal) (a) ($r = 0.58, n = 36, p < 0.0001$), LPC content of the lipoprotein (b) ($r = 0.60, n = 36, p < 0.0001$), or diene formation lag time (c) ($r = -0.34, n = 36, p < 0.05$)

distinct retardation bands shown in arrows (Fig. 6) were found in cells treated with 2 % fetal bovine serum containing either control or diabetic lipoproteins. Density of these bands was increased by the treatment of cells with the lipoprotein obtained from

Fig 3a, b. Relationship between the increment of MCP-1 mRNA in endothelial cells exposed to the lipoprotein and either LPC content in the lipoprotein fraction containing IDL and LDL (a) ($r = 0.46, n = 36, p < 0.01$) or diene formation lag time as characteristics of the oxidizability of the lipoprotein (b) ($r = -0.33, n = 36, p < 0.05$)

three patients with NIDDM (HbA_{1c} level was 9.2 ± 0.5 %), when compared with those from a control subject (HbA_{1c} level was 5.4 %) (Fig. 6a). The activation of endothelial cell NF- κ B induced by the diabetic lipoproteins was inhibited by pretreatment with 50 μ mol/l probucol for 20 h (Fig. 6b). A NF- κ B-specific complex with higher mobility (in the lower band) was increased to a greater extent than that of a slower band in the cells exposed to the diabetic lipoprotein, which was completely inhibited by the probucol treatment.

Discussion

The aim of our study was to determine whether lipoproteins from NIDDM patients have a biological effect on gene expression of an atherogenic cytokine in cultured HUVECs. Previous studies indicate that lipoproteins oxidized in vitro have atherogenic ability [13–17], but little is known about the biological

Fig. 4a, b. Effect of antioxidants on MCP-1 mRNA induced by the lipoprotein. **(a)** MCP-1 mRNA content in endothelial cells incubated in the absence (basal) or presence of the lipoprotein obtained from either a control subject (control) or NIDDM patient (NIDDM) with or without pretreatment of cells with either 50 μ mol/l probucol or 50 μ mol/l α -tocopherol for 20 h. **(b)** Densitometric analysis showing the effect of antioxidants on MCP-1 mRNA levels in the cells exposed to the lipoproteins obtained from three control subjects (control) and three NIDDM patients. MCP-1 mRNA contents are expressed as percent of the constitutional level in each experiment. Data are expressed as means $\pm$ SEM. * p < 0.05 vs control without treatment with either antioxidant, # p < 0.05 vs NIDDM without treatment with either antioxidant

activity of the lipoproteins modified in vivo. The present study showed that lipoproteins obtained from NIDDM patients increased MCP-1 mRNA content to a greater extent than that from control subjects. These results indicate that diabetic lipoprotein is possibly more atherogenic than that from control subjects.

Evidence indicates that circulating levels of several forms of modified-LDL including oxidative modified LDL are elevated in diabetic plasma [7–10]. Therefore, lipoproteins may be oxidatively modified in vivo in diabetes and have potent biological ability to increase MCP-1 mRNA content in endothelial cells, since previous studies indicate that oxidized LDL can induce MCP-1 mRNA expression in cultured endothelial cells [17, 33]. LDL isolated from

Fig. 5a, b. Effect of deferoxamine on MCP-1 mRNA induced by the lipoprotein. **(a)** MCP-1 mRNA in endothelial cells incubated in the absence (basal) or presence of the lipoprotein obtained from either a control subject (control) or NIDDM patient (NIDDM) with or without pretreatment of cells with deferoxamine (0.1 mmol/l) for 1 h. **(b)** Densitometric analysis showing effect of deferoxamine on MCP-1 mRNA in the cells exposed to the lipoproteins obtained from three control subjects (control) and three NIDDM patients. MCP-1 mRNA contents are expressed as the percent of the constitutional levels in each experiment. Data are expressed as means $\pm$ SEM. * p < 0.05 vs control without antioxidant treatment, # p < 0.05 vs NIDDM without antioxidant treatment

patients with NIDDM had increased levels of LPC [7, 8]. Consistent with these studies, LPC content isolated from patients with NIDDM was increased when compared with that from control subjects. Furthermore, in the present study, we also demonstrated that LPC content increased according to impaired glycaemic control. LPC is known to be a major phospholipid component of oxidized LDL and it has been reported that almost 40% of phosphatidylcholine is converted to LPC during in vitro oxidation [15]. Moreover, previous studies suggest that LPC itself has several biological functions of oxidized LDL, i.e. inhibits endothelial-dependent relaxation [15]; stimulates smooth muscle cell proliferation [34, 35]; stimulates production and release of heparin-binding growth factor from macrophages [36]; and induces gene expression of several growth factors and adhesion molecules in endothelial cells [37]. We have already reported that LPC, in the pathophysiological range stimulates MCP-1 gene expression in

Fig 6. a) NF- κ B transcription factor binding activity in HUVECs exposed to lipoproteins obtained from control and diabetic subjects was detected in nuclear extracts of HUVECs by using electrophoretic mobility shift assays as described in Materials and methods. Lane 1, non-diabetic control subjects (HbA_{1c} 5.4 %); lane 2–4, patients with NIDDM (HbA_{1c} 9.2 ± 0.5 %); lane 5, a 100-fold excess of unlabelled NF- κ B oligomer was incubated with the nuclear extract before the binding reaction. Arrows indicate activated NF- κ B. Two studies using different subjects showed similar results. **b)** Effect of probucol on NF- κ B activation was measured by gel mobility shift assay. HUVECs were pre-incubated with or without probucol (50 $\mu\text{mol/l}$) for 20 h and then incubated with either control or diabetic lipoproteins for 1 h. Lane 1, non-diabetic control subjects (–probuco); lane 2, control subjects (+probuco); lane 3, diabetic patients (–probuco); lane 4, diabetic patients (+probuco). Arrows indicate activated NF- κ B. These experiments were repeated three times with similar results

HUVECs [27]. Therefore, it may be speculated that the increased LPC content in the lipoprotein, which is oxidized in vivo in diabetes, may play a crucial role in induction of MCP-1 mRNA expression in endothelial cells.

Oxidizability of LDL is known to be associated with the severity of coronary atherosclerosis [38] and is believed to be determined by not only antioxidants as inhibitory factors, but also lipid peroxides as stimulatory factors. In the present study, we could not find a significant difference in diene formation lag time between non-diabetic and diabetic subjects, although the lag time was shorter in patients with poorly controlled diabetes when compared with patients in good glycaemic control, as previously reported [11, 12]. Furthermore, there was only a weak negative correlation between diene formation lag time and ability to induce MCP-1 mRNA expression (Fig. 3b). These results indicate that shortening of diene formation lag time might be a weak indicator of its biological activity.

MCP-1 mRNA content induced by the lipoproteins from NIDDM patients was significantly decreased by treatment with probucol, α -tocopherol or deferoxamine. Oxygen radicals have been postulated as a second messenger for oxidized LDL-mediated gene expression of atherogenic cytokines including

MCP-1 [39–41]. Therefore, antioxidants may interfere with intracellular radical signalling. Probuco and α -tocopherol act as free radical-trapping antioxidants and deferoxamine also inhibits hydroxyl radical formation through the activity of an iron-chelating agent. It has been shown that these antioxidants inhibit high cholesterol diet-induced atherosclerosis [42], endothelial dysfunction [43], and monocyte adhesion [44]. The present results are also consistent with minimally modified LDL effectively stimulating MCP-1 mRNA expression in endothelial cells through oxygen free radical-mediated mechanisms [45]. Thus, the oxidatively modified lipoproteins found in diabetic plasma may activate intracellular oxidative signalling which may contribute to MCP-1 gene expression in the cells.

NF- κ B has been suggested to play an important role in atherogenesis [46]. NF- κ B regulates gene expression of several growth factors or cytokines including MCP-1 [47–49]. We showed that lipoprotein isolated from NIDDM patients in poor glycaemic control significantly stimulated NF- κ B binding activity in HUVECs to a greater degree than that from control subjects. Although we did not identify active components for the activation, it was almost completely inhibited by the pretreatment of cells with probucol. Therefore, these results also indicate that the oxidatively modified lipoproteins found in plasma of diabetic subjects with poor glycaemic control can activate radical-induced biological process in endothelial cells.

The present study suggests that LPC levels in the lipoprotein fraction which reflect oxidative modification of the lipoprotein are at least a possible atherogenic marker for biological activity of the lipoprotein found in diabetic patients. However, it should be remembered that other components of lipoproteins found in diabetic subjects, such as oxidized phospholipids [34, 50] or glycated lipoproteins [7, 10, 18] may not be excluded as candidate factors for inducing present gene expression in endothelial cells. Furthermore, biological lipoprotein activity should be further evaluated using aortic endothelial cells to evaluate its clinical relevance in the atherogenic process. Further investigation may provide new insights into the molecular mechanisms of atherogenesis in diabetes.

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References

1. Kannel WB, McGee DL (1979) Diabetes and cardiovascular disease: the Framingham Study. *JAMA* 241: 2035–2038
2. Baynes JW (1991) The role of oxidative stress in the development of complications in diabetes. *Diabetes* 40: 405–412

3. Chisolm GM, Irwin KC, Penn MS (1992) Lipoprotein oxidation and lipoprotein-induced cell injury in diabetes. *Diabetes* 41[Suppl 2]:61–66
4. Hunt JV, Smith CCT, Wolf SP (1990) Autooxidative glycation and possible involvement of peroxides and free radicals in LDL modification by glucose. *Diabetes* 39: 1420–1424
5. Lopes-Virella MF, Virella G (1996) Cytokines, modified lipoproteins, and arteriosclerosis in diabetes. *Diabetes* 45[Suppl 3]:S40–S44
6. Giugliano D, Paolisso G, Ceriello A (1996) Oxidative stress and diabetic vascular complications. *Diabetes Care* 19: 257–267
7. Sobenin IA, Tertov VV, Koschinsky T et al. (1993) Modified low density lipoprotein from diabetic patients causes cholesterol accumulation in human intimal aortic cells. *Atherosclerosis* 100: 41–54
8. Bagdade JD, Buchana WE, Kuusi T, Taskinen MR (1990) Persistent abnormalities in lipoprotein composition in non insulin-dependent diabetes after intensive insulin therapy. *Arteriosclerosis* 10: 232–239
9. Palinski W, Rosenfeld ME, Yla-Herttuala S et al. (1989) Low density lipoprotein undergoes oxidative modification in vivo. *Proc Natl Acad Sci USA* 86: 1372–1376
10. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H (1993) Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci USA* 90: 6434–6438
11. Tsai EC, Hirsch IB, Brunzell JD, Chait A (1994) Reduced plasma peroxyl radical trapping capacity and increased susceptibility of LDL to oxidation in poorly controlled IDDM. *Diabetes* 43: 1010–1014
12. Haffner SM, Agil A, Mykkanen L, Stern, MP, Jialal I (1995) Plasma oxidizability in subjects with normal glucose tolerance, impaired glucose tolerance, and NIDDM. *Diabetes Care* 18: 646–653
13. Sparow CP, Parthasarathy S, Steinberg D (1989) A macrophage receptor that recognizes oxidized LDL but not acetylated LDL. *J Biol Chem* 264: 2599–2604
14. Berliner JA, Territo MC, Sevanian A et al. (1990) Minimally modified low density lipoproteins stimulates monocyte endothelial interactions. *J Clin Invest* 85: 1260–1266
15. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD (1990) Impairment of endothelial-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature (London)* 344: 160–162
16. Rajavashisth TB, Andalbi A, Territo MC et al. (1990) Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low density lipoproteins. *Nature (London)* 344: 254–257
17. Cushing SD, Berliner JA, Valente AJ et al. (1990) Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci USA* 87: 5134–5138
18. Lyons TJ, Klein RL, Baynes JW, Stevenson HC, Lopes-Virella MF (1987) Stimulation of CE synthesis in human monocyte-derived macrophages by LDL from type 1 diabetic patients: the influence on non-enzymatic glycosylation of LDL. *Diabetologia* 30: 916–923
19. Ylä-Herttuala S, Lipton BA, Rosenfeld ME et al. (1991) Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc Natl Acad Sci USA* 88: 5252–5256
20. Nelken NA, Coughlin SR, Gordon D, Wilcox JN (1991) Monocyte chemoattractant protein-1 in human atherosclerotic plaques. *J Clin Invest* 88: 1121–1127
21. Collins T (1993) Endothelial nuclear factor- κ B and initiation of the atherosclerotic lesion. *Lab Invest* 68: 499–508
22. Sen CK, Packer L (1996) Antioxidant and redox regulation of gene transcription. *FASEB J* 10: 709–720
23. World Health Organization (1985) Diabetes mellitus: report of a WHO study group. World Health Organization Technical Report Series 727, Geneva
24. Havel RJ, Eder HA, Bragdon JH (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 34: 1345–1353
25. Ikebuchi M, Kashiwagi A, Asahina T et al. (1993) Effect of medium pH on glutathione redox cycle in cultured human umbilical vein endothelial cells. *Metabolism* 42: 1121–1126
26. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159
27. Takahara N, Kashiwagi A, Maegawa H, Shigeta Y (1996) Lysophosphatidylcholine stimulates the expression and production of MCP-1 by human vascular endothelial cells. *Metabolism* 45: 559–564
28. Bligh EG, Dyer J (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37: 911–917
29. Bartlett GR (1959) Phosphorus assay in column chromatography. *J Biol Chem* 234: 466–468
30. Lowry OH, Rosebrough MJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin Phenol reagent. *J Biol Chem* 193: 265–275
31. Esterbauer H, Striegl G, Puhl H, Rotheneder M (1989) Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Res Com* 6: 67–75
32. Compton JL, Bellard M, Chambon P (1976) Biochemical evidence of variability in the DNA repeat length in the chromatin of higher eukaryotes. *Proc Natl Acad Sci USA* 73: 4382–4386
33. Liao F, Berliner JA, Mehrabian M, Navab M, Demer LL, Lusis AJ, Fogelman AM (1991) Minimally modified low density lipoprotein is biologically active in vivo in mice. *J Clin Invest* 87: 2253–2257
34. Heery JM, Kozak M, Stafforini DM et al. (1995) Oxidatively modified LDL contains phospholipids with platelet-activating factor-like activity and stimulates the growth of smooth muscle cells. *J Clin Invest* 96: 2322–2330
35. Stiko A, Regnstrom J, Shah PK, Cercek B, Nilsson J (1995) Active oxygen species and lysophosphatidylcholine are involved in oxidized low density lipoprotein activation of smooth muscle cell DNA synthesis. *Arterioscler Thromb Vasc Biol* 16: 194–200
36. Nakano T, Raines EW, Abraham JA, Klagsbrun M, Ross R (1994) Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. *Proc Natl Acad Sci USA* 91: 1069–1073
37. Kume N, Gimbrone MA (1994) Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. *J Clin Invest* 93: 907–911
38. Regnstrom J, Nilsson J, Tornvall P, Landou C, Hamste A (1992) Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* 339: 1183–1186
39. Satriano JA, Shuldiner M, Kazuhiko H, Xing Y, Shan Z, Schlondorff D (1993) Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, M-CSF, in response to tumor necrosis factor- α and immunoglobulin G. *J Clin Invest* 92: 1564–1571
40. Khan BV, Parthasarathy SS, Alexander RW, Medford RM (1995) Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion

- molecule-1 gene expression in human vascular endothelial cells. *J Clin Invest* 95: 1262–1270
41. Coffey MD, Cole RA, Colles SM, Chisolm GM (1995) In vitro cell injury by oxidized low density lipoprotein involves lipid hydroperoxide-induced formation of alkoxyl, lipid and peroxy radicals. *J Clin Invest* 96: 1866–1873
 42. Sasahara M, Raines EW, Chait A et al. (1995) Inhibition of hypercholesterolemia-induced atherosclerosis in the non-human primate by probucol. *J Clin Invest* 94: 155–164
 43. Keaney JF, Xu A, Cunningham D, Jackson T, Frei B, Vita JA (1995) Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. *J Clin Invest* 95: 2520–2529
 44. Faruqi R, Motte C, Dicorleto PE (1994) α -tocopherol inhibits agonist-induced monocytic cell adhesion to cultured human endothelial cells. *J Clin Invest* 94: 592–600
 45. Navab M, Imes SS, Hama SY et al. (1991) Monocyte transmigration induced by modification of low density lipoprotein in culture of human aortic wall cells is due to induction of monocyte chemotactic protein 1 and is abolished by high density lipoprotein. *J Clin Invest* 88: 2039–2046
 46. Brand K, Page S, Rogler G et al. (1996) Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest* 97: 1715–1722
 47. Rajavashisth TB, Yamada H, Mishra NK (1995) Transcriptional activation of the macrophage-colony stimulating factor gene by minimally modified LDL. Involvement of nuclear factor-kappa B. *Arterioscler Thromb Vasc Biol* 15: 1591–1598
 48. Liao F, Andalibi A, DeBeer FC, Fogelman AM, Lusis AJ (1993) Genetic control of inflammatory gene induction and NF- κ B-like transcription factor activation in response to an atherogenic diet in mice. *J Clin Invest* 91: 2572–2579
 49. Liao F, Andalibi A, Qiao JH, Allayee H, Fogelman AM, Lusis AJ (1994) Genetic evidence for a common pathway mediating oxidative stress, inflammatory gene induction, and aortic fatty streak formation in mice. *J Clin Invest* 95: 877–884
 50. Watson A, Navab M, Hama S et al. (1995) Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J Clin Invest* 95: 774–782