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Glucose-Modified Low Density Lipoprotein Enhances Human Monocyte Chemotaxis

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In diabetes mellitus the progression of atherosclerosis is accelerated. The interaction of glucose with atherogenic lipoproteins may be relevant to the mechanisms responsible for this vascular damage. The aim of this study was to examine the effect of glucose-modified low density lipoprotein (LDL) on human monocyte chemotaxis and to investigate the roles of oxidation and glycation in the generation of chemotactic LDL. Cu(II)-mediated LDL oxidation was potentiated by glucose in a dose-dependent manner and increased its chemotactic activity. Incubation with glucose alone, under conditions where very little oxidation was observed, also increased the chemotactic property of LDL. Neither diethylenetriamine pentaacetic acid (DETAPAC) nor aminoguanidine, which both inhibited LDL oxidation, completely inhibited the chemotactic activity of glycated oxidised LDL. The results suggest that both oxidation and glycation contribute to increased chemotactic activity.

Keywords: Oxidation, glycation, low density lipoprotein, monocyte, chemotaxis

INTRODUCTION

The role of oxidative stress and oxidised lipoproteins in atherosclerosis is the subject of extensive research. Oxidised low density lipoprotein (oxLDL) has been proposed to play a role in several stages of the formation of atherosclerotic lesions and has various cellular effects which may contribute to the pathogenesis of the disease. OxLDL has been shown to be toxic to a number of cell types including macrophages.[1] The acellular core of advanced lesions is partly composed of dead macrophages,[2] leading to the suggestion that the toxic effects of oxLDL are important in the development of advanced lesions from clinically benign fatty streaks.[3] In addition, oxLDL has been reported to have a number of other properties which may contribute to atherogenesis, including stimulating cytokine secretion,[4] inducing the expression of adhesion molecules[5] and promotion of chemotaxis.[6]

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These effects have been attributed to oxidised lipid components of oxLDL.[7] The earliest events in the formation of an atherosclerotic lesion are the intimal accumulation of monocyte-derived macrophages and their subsequent development into foam cells.[8] OxLDL may influence this process and plays a crucial role in the development of vascular disease.

Diabetes mellitus is associated with the development of long-term vascular complications including accelerated atherosclerosis. Although the subject of controversy, diabetic complications have been attributed by some to hyperglycaemia, the primary clinical manifestation of diabetes.[9] Several mechanisms have been proposed to explain the vascular damage associated with diabetes.[10] One suggestion which has stimulated considerable interest in recent years is the participation of protein glycation.[11] This reaction begins with the spontaneous condensation of a sugar aldehyde with free amino groups and culminates in the irreversible cross-linking of proteins to form advanced glycation end products (AGEs). Glycated proteins have been shown to elicit a number of cellular effects which are believed to be due to the interaction between AGEs and specific receptors for them on a number of cell types.[11] In common with oxLDL, the cellular effects of glycated proteins include stimulation of cytokine release[12] and promotion of monocyte chemotaxis.[13]

The results of protein glycation are thought to be at least partly dependent upon oxidation.[14] Glycation chemistry is complex, oxidant species can be produced at various points along the glycation pathway in transition metal-dependent reactions,[15] and may result in oxidative damage to the protein being glycated or to other macromolecules. Therefore glycation may result in the production of oxidised proteins such as oxLDL.[16] However, the effect of glucose-oxidised LDL on monocyte chemotaxis, which may play an important role in accelerated atherosclerosis in diabetes mellitus, has not been previously examined. The aim of this study was to assess the effects of LDL glycation on chemotaxis for human monocytes.

METHODS AND MATERIALS

Radiochemicals were obtained from Amersham (Aylesbury, Bucks, UK). All biochemicals were obtained from Sigma (Poole, Dorset, UK) or Aldrich (Gillingham, Kent, UK). In order to remove transition metals contamination all buffers were treated with Chelex-100, as previously described.[17]

Lipoprotein Preparation

Human LDL was prepared from non-diabetic subjects as described previously.[18] Blood was centrifuged in the presence of 1 mg/ml EDTA to obtain plasma. Lipoprotein fractions were then obtained, from pooled plasma (from 4–6 healthy volunteers), by ultracentrifugation and flotation through KBr gradients. Centrifugations were performed in the presence of EDTA and the fraction that floated at a density of 1.019–1.063 g/ml was taken as LDL.

Modification of LDL

The LDL was stored for up to 1 week at 4°C in 1 mM EDTA prior to use. It was then dialysed extensively against phosphate-buffered saline to remove EDTA. The protein concentration was determined by the Lowry assay and the LDL diluted to 1 mg/ml protein in 100 mM sodium phosphate buffer (pH 7.4). LDL was oxidised by the addition of copper sulphate (0.5 μM) and glycated by the addition of glucose (0–500 mM) in the presence or absence of copper sulphate (0.5 μM). The LDL preparations were filter-sterilised and samples were incubated for 2 weeks at 37°C. Following incubation samples were extensively dialysed against phosphate buffer to remove unbound glucose and other reagents.

The electrophoretic mobility of the LDL was determined by agarose gel electrophoresis as previously described[19] using Beckman Paragon 'LIPO' lipoprotein electrophoresis gels (Beckman, Brea, CA, USA). The formation of fluorophores, which is often taken as a measure of advanced glycation end products, was monitored at an excitation wavelength of 340 nm and an emission wavelength of 425 nm.[20] Lipid peroxides in LDL were measured using the xylenol orange assay, as described previously.[21] To monitor the extent of glucose attachment the incorporation of D-[U-^{14}C]-glucose into LDL was assessed as previously described.[22]

Cell Culture

Human peripheral blood monocytes were isolated from healthy volunteers as previously described[23] and were suspended in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies Ltd., Paisley, Scotland, UK) containing 0.25% BSA (essentially fatty acid free) at a concentration of 1×10^6 cells/ml.

Chemotaxis Assay

Chemotaxis was measured in a 48-well modified Boyden microchemotaxis chamber (Neuroprobe Inc., Cabin John, MD, USA) as described previously.[24] Assays were carried out in triplicate. N-formylmethionylleucylphenylalanine (FMLP), a synthetic peptide highly chemotactic for monocytes, served as a positive control and DMEM as the negative control. Human monocytes were incubated in the chamber at 37°C in a 5% CO_2 atmosphere for 2 h and chemotaxis through a 5 μm pore size PVP-free polycarbonate membrane (Poretics Corp., Livermore, CA, USA) was determined. The membrane was then dehydrated, stained (Diff-Quik, Baxter Diagnostics) and mounted onto glass slides. The cells that had migrated onto the underside of the membrane were counted and the mean from 4 high powered fields (100×) taken for each sample. Chemotactic activity is expressed as a chemotactic index (CI) defined as the number of cells migrating in response to test substance divided by the number when control medium (DMEM + 0.25% BSA) was present in both upper and lower chambers.

Statistical Analysis

Statistical differences were assessed using the paired Student's *t*-test.

RESULTS

Effect of Glucose on the Oxidation of LDL

Glucose potentiated the oxidation of LDL (1 mg/ml) in a dose-dependent manner when 0.5 μM Cu(II) was present in the incubation, judged by the electrophoretic mobility of LDL (Figure 1(A)) and lipid hydroperoxide content (Figure 1(B)). Cu(II) alone increased the electrophoretic mobility and lipid hydroperoxide content of LDL by *ca.* 2-fold, but glucose alone had little effect. However, in the presence of both Cu(II) and glucose the electrophoretic mobility and hydroperoxide content was increased above the additive effect of the two agents. With the highest concentration of glucose used (500 mM) this constituted a 2.5-fold increase in electrophoretic mobility and hydroperoxide content compared with Cu(II) alone.

The concentration of LDL used in this study (1 mg/ml) was higher than that used in other studies investigating oxidative glucose chemistry. Reduction of LDL concentration to 200 μg/ml facilitated the oxidation by 25 mM glucose, as assessed by electrophoretic mobility (Table I), and therefore it is conceivable that the level of oxidation achieved with 500 mM glucose could be attained *in vivo*. Table I also shows the effect of diethylenetriamine pentaacetic acid (DETAPAC), a transition metal chelator and aminoguanidine, an 'anti-glycation' agent, upon oxidation.

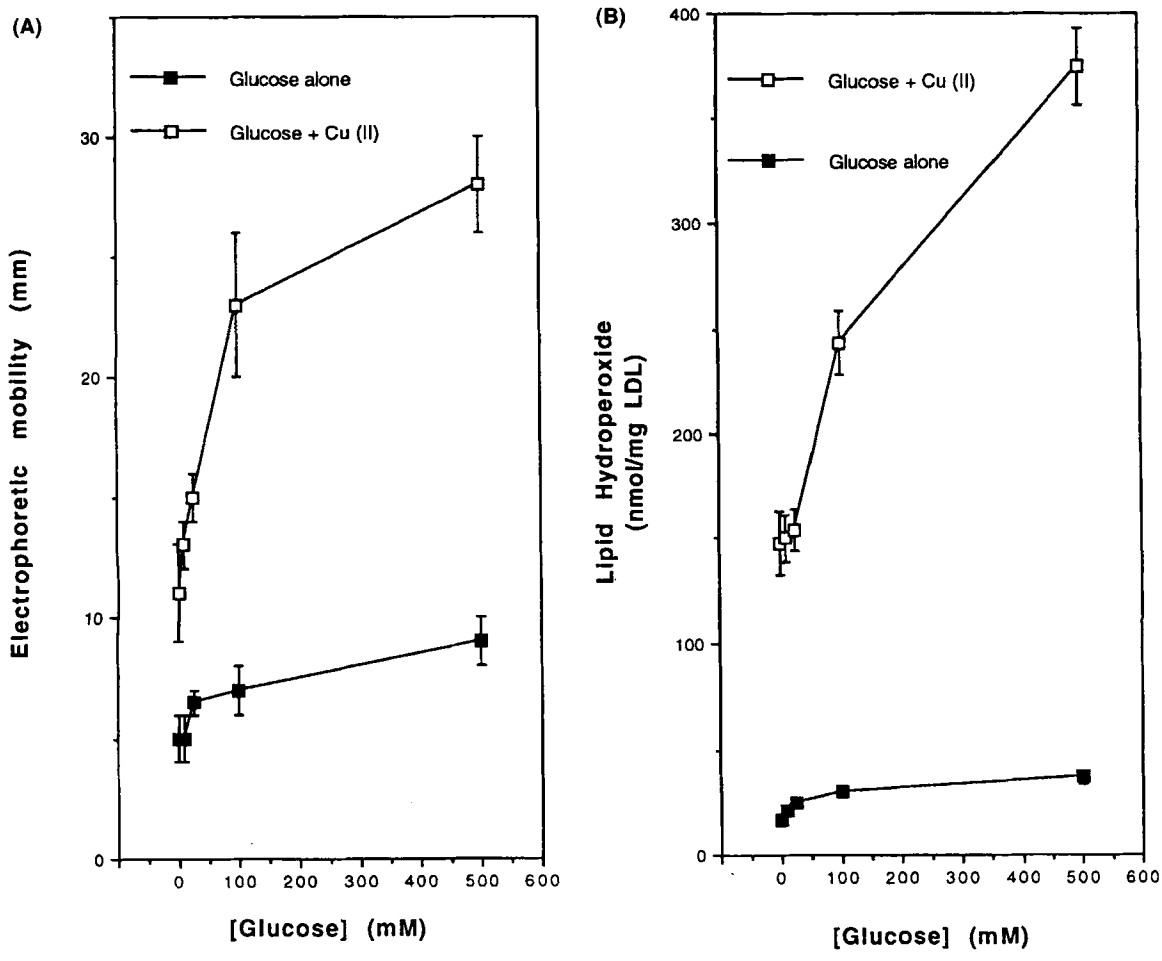

FIGURE 1 The effect of glucose concentration on the oxidation of human LDL. LDL (1 mg/ml) was incubated with glucose (0–500 mM) for 2 weeks with or without copper sulphate (0.5 μM). Changes in the electrophoretic mobility (A) and lipid hydroperoxide content (B) of the modified LDL and were determined as described in Methods and Materials. Unless otherwise indicated in this and all other tables/figures the results are mean ± standard deviation from least three experiments, in which assays were performed in triplicate.

TABLE I Effect of high and low concentrations of LDL on glycation and oxidation

Treatment	Electrophoretic mobility (mm) 200 μg/ml LDL	Electrophoretic mobility (mm) 1 mg/ml LDL
LDL	6.0 ± 1.0**	4.0 ± 2.0**
LDL + glucose	7.0 ± 1.0**	5.0 ± 1.0**
LDL + Cu(II)	9.0 ± 1.0*,**	10 ± 2.0*,**
LDL + Cu(II) + glucose	25 ± 2.0*	22 ± 4.0*
LDL + Cu(II) + glucose + DETAPAC	7.0 ± 1.0**	6.0 ± 1.0**
LDL + Cu(II) + glucose + AMG	11 ± 1.0*,**	12 ± 3.0*,**

LDL, 200 μg/ml and 1 mg/ml, was incubated with 25 and 500 mM glucose, respectively. The effect of adding Cu(II) (0.1 and 0.5 μM, respectively), aminoguanidine (10 mM) and DETAPAC (1 mM) is shown. The effect of each treatment upon the electrophoretic mobility (EM) was determined as described in Methods and Materials. * $p < 0.05$ when compared to unmodified LDL. ** $p < 0.01$ when compared to LDL modified by glucose and Cu(II).

Effect of Diethylenetriamine Pentaacetic Acid and Aminoguanidine

To investigate the contribution of glycation and oxidation to the chemotactic activity of LDL, conditions of extensive glycation and oxidation of 1 mg/ml LDL were used i.e. 500 mM glucose in the presence of 0.5 μM Cu(II).

In terms of [^{14}C]-glucose binding and boronate affinity chromatography to measure Amadori product formation, at this concentration of glucose, early glycation product formation was maximal after 2 weeks (results not shown). For [U-^{14}C]-glucose attachment, DETAPAC had no significant effect but aminoguanidine inhibited attachment by *ca.* 50%. The apparent reduction in glucose attachment in the presence of Cu(II) alone is a consequence of protein fragmentation and loss of trichloroacetic acid-precipitable material, as previously described.[25] In otherwords, oxidative fragmentation of protein leads to a loss in trichloroacetic acid-insoluble material, central to the measurement of [U-^{14}C]-glucose attachment, and an apparent decrease in glucose attachment. DETAPAC prevents this phenomenon and can even increase apparent levels of attachment since protein scission occurs as a consequence of autolysis.[25–27] Fluorescence, indicative of the formation of glycation end products and the accumulation of lipid hydroperoxides, was also assessed (Table II). The effects of including DETAPAC and aminoguanidine are shown.

Changes in electrophoretic mobility, fluorescence and hydroperoxide content of LDL incubated with glucose and Cu(II) (Tables I and II) were inhibited by the addition of both DETAPAC and aminoguanidine. However, whereas DETAPAC almost abolished changes in these parameters aminoguanidine merely inhibited them by *ca.* 50%.

Effect of Glucose on the Chemotactic Property of LDL

The optimal concentration of modified LDL for chemotaxis was found to be 50 μg/ml and this concentration was used throughout.

Figure 2 shows the changes in chemotactic property of LDL exposed to increasing concentrations of glucose in the presence of Cu(II) as described in Figure 1. Briefly, chemotactic index increased in proportion to the level of glucose to which the LDL had previously been exposed. The highest concentration, 500 mM, resulted in the greatest chemotactic index. Thus, to investigate the contribution of glycation and oxidation and the effects of DETAPAC and aminoguanidine, systems composed of 1 mg/ml LDL and 500 mM glucose were used.

Both glycation and oxidation were independently capable of increasing the chemotactic activity of LDL as demonstrated by the effects of Cu(II) or glucose alone upon the chemotactic

TABLE II Effect of DETAPAC and AMG on the glycation and oxidation of LDL

Treatment	Lipid hydroperoxide nmol/mg protein	mol glucose bound/mol LDL	Fluorescence (Ex: 340 nm/Em: 425 nm) as a ratio of control
LDL (control)	17 ± 2.0**	NA	1.0**
LDL + glucose	37 ± 3.0**	20 ± 2.1*,**	1.62 ± 0.05*,**
LDL + Cu(II)	148 ± 15*,**	NA	1.4 ± 0.05*,**
LDL + Cu(II) + glucose	375 ± 18*	14.7 ± 1.05*	2.32 ± 0.1*
LDL + Cu(II) + glucose + DETAPAC	13 ± 2.0**	23 ± 1.65*,**	1.05 ± 0.05**
LDL + Cu(II) + glucose + AMG	208 ± 21*,**	13.3 ± 0.51*	1.63 ± 0.10*,**

LDL, 1 mg/ml, was incubated with 500 mM glucose. The effect of adding Cu(II) (0.5 μM), aminoguanidine (10 mM) and DETAPAC (1 mM) upon the accumulation of lipid hydroperoxides and the generation of fluorescence characteristic of glycation is shown. Fluorescence is expressed as a ratio of a control of LDL incubated in buffer alone. * $p < 0.05$ when compared to unmodified LDL. ** $p < 0.01$ when compared to LDL modified by glucose and Cu(II). NA = not applicable.

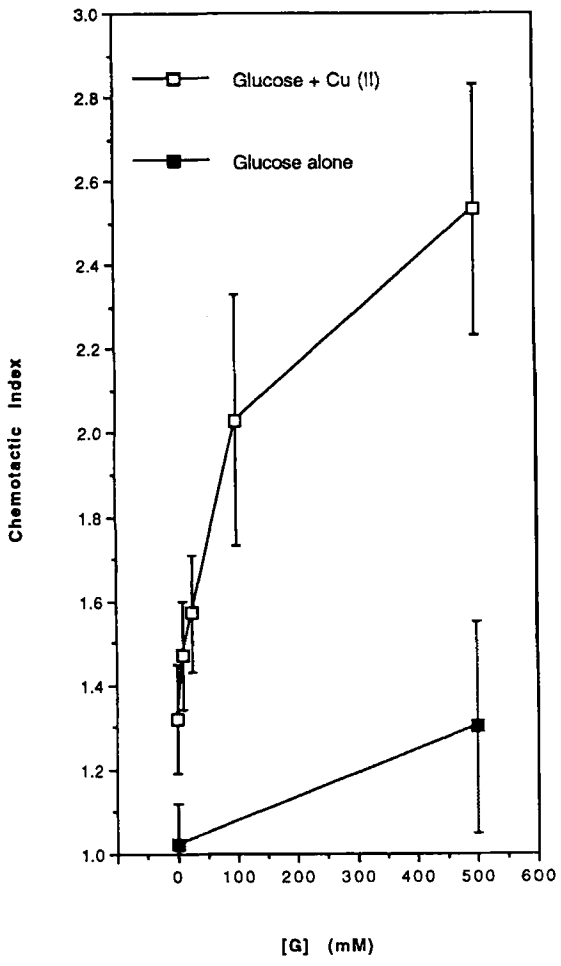

FIGURE 2 The effect of glucose concentration on the generation of chemotactic human LDL. LDL (1 mg/ml) was incubated with glucose (0–500 mM) for 2 weeks with copper sulphate (0.5 μM). Changes in the chemotactic nature of LDL for human monocytes were determined as described in Methods and Materials. Unless otherwise indicated in this and all other tables/figures the results are mean ± standard deviation from least three experiments, in which assays were performed in triplicate.

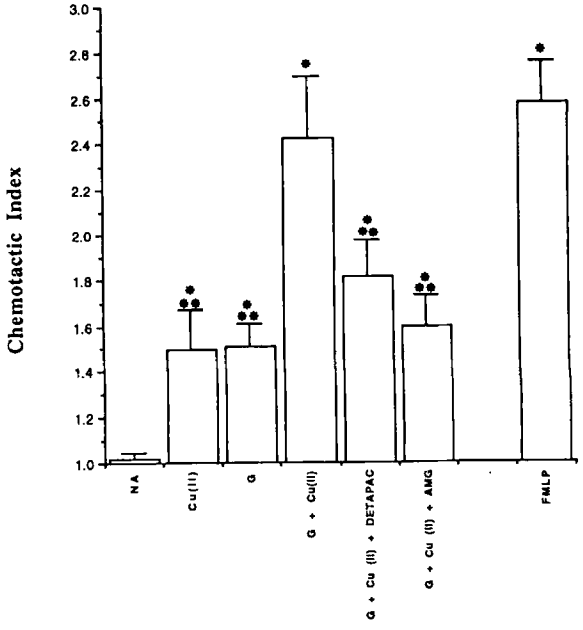

FIGURE 3 Effect of oxidation and glycation upon the chemotactic activity of LDL. LDL (1 mg/ml) was incubated with glucose (500 mM) and Cu(II) (0.5 μM), under the indicated conditions, in the presence or absence of DETAPAC (D) (1 mM) or aminoguanidine (A) (10 mM), as described in Methods and Materials. Chemotaxis of human monocytes towards each modified LDL (50 μg/ml) or FMLP (10^{-8} M) was determined as described in Methods and Materials. $^{*}p < 0.05$ when compared to unmodified LDL. $^{**}p < 0.05$ when compared to LDL modified by glucose and Cu(II).

activity (Figure 3). Co-incubation with Cu(II) and glucose enhanced the chemotactic activity of LDL to a level which was, at the least, equal to the additive effect of incubation with Cu(II) or glucose alone.

DETAPAC and aminoguanidine differed in there respective abilities to inhibit changes in electrophoretic mobility, generation of novel fluorescence, accumulation of lipid hydroperoxides and the level of glucose attachment to protein (Tables I and II). However, DETAPAC and aminoguanidine were equally effective at inhibiting (ca. 50%) chemotaxis in response to LDL which had been glycated in the presence of Cu(II) (Figure 3). Thus, whereas the inhibitory effect of DETAPAC on the generation of chemotactic LDL is explicable on the basis of transition metal chelation and prevention of oxidative reactions the effect of aminoguanidine is not.

Checkerboard analysis confirmed that the effects of oxidation and glycation on cell movement due to LDL were due to chemotaxis rather than chemokinesis (Table III).

TABLE III Checkerboard analysis of oxidised and glycated LDL

Below membrane	Above membrane				
	DMEM	LDL	LDL + Cu(II) + glucose	LDL + Cu(II) + glucose + DETAPAC	LDL + Cu(II) + glucose + AMG
DMEM	1.00 ± 0.16	1.12 ± 0.10	0.95 ± 0.10	1.11 ± 0.15	0.99 ± 0.05
LDL	1.20 ± 0.20	1.04 ± 0.28	—	—	—
LDL + Cu(II) + glucose	2.42 ± 0.18	—	0.99 ± 0.13	—	—
LDL + Cu(II) + glocose + DETAPAC	1.75 ± 0.20	—	—	1.00 ± 0.14	—
LDL + Cu(II) + glucose + AMG	1.65 ± 0.16	—	—	—	0.90 ± 0.16

The indicated modifications of LDL were prepared as described (Figure 2). Chemotaxis of monocytes was determined, as described in Methods and Materials, when each modification was present either below or above the membrane or present in both compartments at the same time. Results are expressed as a chemotactic index (CI) and are mean ± SD for triplicate determinations. Results are representative of those from three separate experiments.

DISCUSSION

These studies confirm earlier findings that glucose can potentiate the oxidation of LDL in the presence of transition metals.[22,28,29] The oxidation of LDL was shown by changes in the lipid peroxide content and electrophoretic mobility, indicating oxidative damage to both the lipid and apolipoprotein B moieties of LDL.

Throughout our studies high levels of glucose have been used to facilitate maximal glycation in conjunction with oxidation using 1 mg/ml LDL. However, the level of LDL oxidation, under these conditions, was of the same magnitude as that achieved with pathophysiologically relevant concentrations of glucose in diabetes[30] and lower concentrations of LDL (Table I).

One possible mechanism for glucose-mediated oxidation of LDL is by the generation of reactive oxygen species during glucose autoxidation, the term used to describe the generation of oxidants by glucose in solution.[15] The mechanism is known to be transition metal-dependent, explaining the importance of Cu(II) in our system. The dependence of glucose-mediated oxidation on transition metals may have important implications for the development of atherosclerosis. Transition metals are known to be present in advanced atherosclerotic lesions[31] and this may be particularly important in diabetes where altered handling of transition metals is apparent.[32] Low levels of protein-bound metal ions have been implicated in the oxidative damage of tissues during ageing[33] and products of metal-catalysed reactions of protein-bound glucose have been shown to accumulate in proteins of diabetic patients.[34] It is therefore conceivable that LDL with altered chemotactic activity could be produced in diabetes and could contribute to accelerated arterial disease.

During these studies over relatively long periods one's concern might be the role of hydroperoxide decomposition to aldehydes and subsequent protein modification, leading to increases in electrophoretic mobility. However, the reader should note the mild nature of oxidation in these systems of glucose-mediated changes in LDL. This is suggested by the relatively small increases in lipid hydroperoxide levels and electrophoretic mobility, the greatest movement being less than half that typically generated by Cu(II)-mediated oxidation of LDL.[19] Also, electrophoretic mobility and lipid hydroperoxide content increased with glucose concentration, shown in Figure 1, and do not suggest the exhaustion of oxidisable lipid.

This is the first demonstration that glucose-mediated oxidation of LDL results in chemotaxis for human monocytes. The chemotactic activity of Cu(II)-oxidised LDL[35,36] has been attributed to lipid oxidation products of LDL such as lysophosphatidylcholine.[36] In our own studies, addition of higher concentrations of Cu(II) up to 4 μM (which is still lower than the concentrations used in most studies) increased the extent of oxidation of LDL but did not lead to a further enhancement of chemotactic activity (results not shown). This confirms that only minimal oxidation is required for chemotaxis.[36]

Incubation of LDL with glucose, in the absence of Cu(II), did not lead to any significant oxidation but was just as effective at increasing the chemotactic activity of LDL as incubation with Cu(II) alone i.e. oxidative modification. The chemotactic activity of glycated protein has been previously reported and it has been suggested that the interaction between soluble glycated protein and specific-receptors for advanced glycation end products on monocytes is responsible for increased monocyte chemotaxis,[37] a mechanism which could play role in attracting monocytes into the vessel wall.

Glycation of LDL under oxidising conditions increased the chemotactic activity of LDL above which glycation or oxidation alone could achieve. Furthermore, DETAPAC, which almost entirely inhibited the oxidation of LDL exposed to Cu(II) and glucose, attenuated, but did not completely inhibit, the chemotactic activity of the modified LDL. This confirms that oxidation is not a necessary prerequisite for increased chemotactic activity of glycated LDL.

Other groups have reported a direct relationship between the formation of fluorescent products and the chemotactic activity of glycated protein.[38] Fluorescent products increased with increasing concentrations of glucose and with 500 mM glucose, increased to a maximum of 1.4-fold compared with control. However, as previously reported,[39] the increase in fluorescent products due to oxidation was inhibited by DETAPAC. DETAPAC did not affect the attachment of glucose to LDL. It is therefore probable that DETAPAC inhibits the chemotactic activity of LDL by an antioxidant effect as opposed to any anti-glycation effect. Although early reports suggest DETAPAC inhibits the binding of glucose to protein, subsequent and more recent reports suggest that this observation was a consequence of experimental sensitivity and the loss of acid-soluble peptide.[25–27] That DETAPAC failed to inhibit glucose binding confirms recent reports from this laboratory.[22]

The effect of aminoguanidine on the glucose-mediated oxidation of LDL and subsequent chemotactic activity is less clear. The way in which aminoguanidine behaves as an anti-glycation agent is not entirely understood. Previous studies have shown that aminoguanidine inhibits the production of advanced glycation end products.[40] Aminoguanidine may also react with Amadori products and prevent further rearrangement processes during later stages of glycation chemistry.[41] However, aminoguanidine has also been shown to have both pro-[27,42] and anti-oxidant effects[43,44] which may also contribute to the inhibitory effect of aminoguanidine on the chemotactic activity of modified LDL. Thus, the partial inhibitory effect of aminoguanidine on the chemotactic activity of modified LDL may be due to a combination of pro-oxidant, antioxidant and/or anti-glycation effects.

These results suggest that glycation and oxidation may act together or independently as mechanisms to increase monocyte chemotaxis, most probably via the production of different chemoattractant substances. The potentiation of LDL oxidation by glucose was not exclusively responsible for the increase in chemotactic activity of the modified LDL. Conversely, because glycation of LDL may coexist with oxidation, glycating LDL in conditions where oxidation is limited (i.e. in the absence of transition metals or in the presence of transition-metal chelators), may not reveal the full extent of the chemotactic activity of glycated LDL *in vivo*.

The relationship between, glycation, oxidation and chemotaxis could be important in atherosclerosis. Human lesions have been shown to contain transition metals,[29] glycated protein[45,46] and oxidised LDL-derived lipid.[47] Indeed our recent studies in human atheroma suggest that the chemistry of glycation might contribute to lipid oxidation.[46] Certainly in diabetes, where hyperglycaemia and altered handling of transition metals may coexist, the relationship between glycation, oxidation and chemotaxis could play a significant role in the enhanced vascular damage observed.

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