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Iron released from transferrin at acidic pH can catalyse the oxidation of low density lipoprotein

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Abstract Low density lipoprotein (LDL) oxidation within the arterial wall may contribute to the disease of atherosclerosis. We have investigated the conditions under which transferrin (the major iron-carrying protein in plasma) may release iron ions to catalyse the oxidation of LDL. Transferrin that had been incubated at pH 5.5 released approximately 10% of its bound iron in 24 h, as measured by ultrafiltration and atomic absorption spectroscopy. Furthermore, transferrin co-incubated with LDL and L-cysteine at pH 5.5 resulted in the oxidation of the LDL as measured by thiobarbituric acid-reactive substances and electrophoretic mobility. This effect was observed at transferrin concentrations as low as 40% of its average plasma concentration. The release of iron from transferrin in atherosclerotic lesions due to a localised acidic pH may help to explain why LDL oxidation occurs in these lesions.

Key words: Acidic pH; Atherosclerosis; Iron ion; Low density lipoprotein; Oxidation; Transferrin

1. Introduction

The oxidation of low density lipoprotein (LDL) within the arterial wall may result in its increased uptake by macrophages converting them into cholesterol-laden foam cells, the migration of monocytes into the arterial wall, cytotoxicity and many other effects that are involved in the disease of atherosclerosis [1].

There is increasing evidence that iron may play an important role in promoting tissue damage at sites of inflammation [2]. Under normal physiological conditions, however, free iron does not exist in plasma or other extracellular fluids. It has been suggested, however, that free iron may become available following release from transferrin [3,4] or ferritin [5] under certain conditions.

Transferrin is the major iron transport protein of mammalian plasma. It is a polypeptide of M_r 80,000 which, in the presence of bicarbonate, can bind two iron ions per molecule [6]. Transferrin in plasma is only around 33% saturated with iron [7].

It has been reported that an elevated serum ferritin level is a risk factor for acute myocardial infarction [8], but not all studies agree with this [9]. The total iron-binding capacity of serum (mainly due to transferrin) has been reported to be a negative risk factor for myocardial infarction [9], and transferrin saturation has been reported to be inversely associated with cardiovascular mortality [10]. The epidemiology of iron status and atherosclerosis is, therefore, unclear.

We have investigated the effect of acidic pH on transferrin to determine whether or not it can release bound iron ions which could catalyse the oxidation of LDL.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; MES, *N*-morpholinoethanesulphonic acid; TBARS, thiobarbituric acid-reactive substances.

2. Materials and methods

2.1. Isolation of LDL

LDL (1.019–1.063 g/ml) was isolated from pooled normal human blood by sequential ultracentrifugation in KBr solutions at 4°C as described elsewhere [11].

2.2. Determination of iron release from transferrin

All solutions were prepared fresh and treated with Chelex-100 prior to use. Transferrin 30% saturated with iron (Boehringer-Mannheim) was incubated in triplicate at 6 mg/ml in a 0.2 M MES buffer (pH 7.4–5.5) containing 10 mM sodium bicarbonate for 24 h at 37°C. It was then filtered using a 30,000 M_r cut-off microcentrifuge filter tube (Whatman) at 9500 × *g* for 25 min. The filtrate was then assayed for iron ions by atomic absorption spectroscopy.

2.3. Co-incubation of transferrin and LDL

LDL (100 μg protein/ml) was incubated in triplicate in the presence or absence of transferrin (2.4 mg/ml) in a 0.2 M MES buffer (pH 7.4–5.5) containing 10 mM sodium bicarbonate and 180 μM L-cysteine in 22 mm tissue culture wells (Corning) for 44 h at 37°X.

2.4. Thiobarbituric acid-reactive substances (TBARS) assay

Samples and appropriate standards (0.25 ml) were taken in triplicate and to each was added 3 ml 0.335% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid. They were then heated at 95°C for 15 min, centrifuged at 1500 × *g* for 5 min at 4°C, and the absorbance measured at 535 nm.

2.5. Determination of LDL electrophoretic mobility

Samples (5 μl) were loaded onto a Beckman Paragon agarose gel and the gel electrophoresed at 100 V for 30 min, fixed, dryed and stained with the lipid stain Sudan black. The distance of LDL migration was then determined.

3. Results

Transferrin was incubated in the presence of bicarbonate at various pHs, subjected to ultrafiltration (M_r cut-off 30,000) to separate protein from free iron and the released iron determined by atomic absorption. The concentration of the 30% iron-saturated transferrin was 6 mg/ml and, therefore, contained around 45 μM iron. A higher concentration of transferrin was used for the atomic absorption work than was used for

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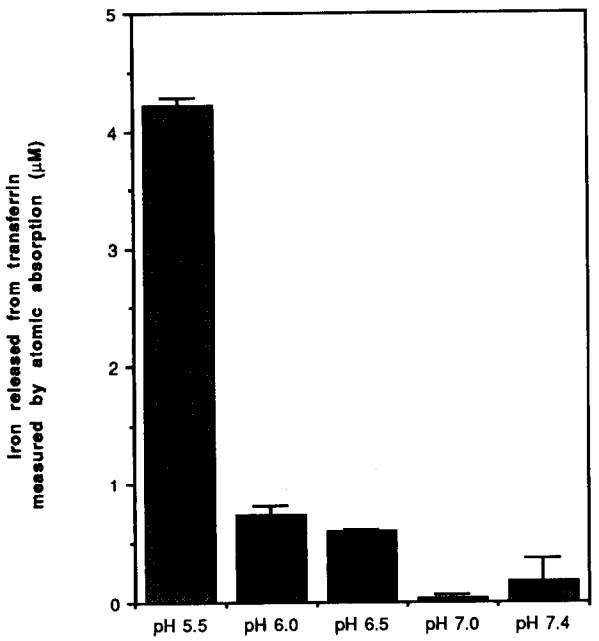

Fig. 1. The effect of pH on the release of iron from transferrin. Transferrin (6 mg/ml) was incubated for 24 h at 37°C in 0.2 M MES buffer of various pH values containing 10 mM $NaHCO_3$. It was then filtered through a 30,000 M_r cut-off filter and the filtrate assayed for free iron by atomic absorption spectroscopy. The histobars represent the mean ± S.E.M. of triplicates. These results were confirmed by an independent experiment.

oxidising LDL because of the lower sensitivity of the atomic absorption technique.

Transferrin incubated at pHs 7.4 and 7.0 released a negligible quantity of iron over 24 h (Fig. 1). At pHs 6.5 and 6.0 around 0.75 μM iron ions were released. At pH 5.5, however, around 4 μM iron ions were released from the transferrin, which represents around 10% of the total iron bound to transferrin.

When LDL was co-incubated with transferrin at pHs 7.4 and 7.0 over 44 h, the LDL was not oxidised, as evidenced by the lack of TBARS generated (Fig. 2). Very low levels (<3 nmol MDA equivalents/mg LDL protein) of TBARS were generated by LDL co-incubated with transferrin at pHs 6.0 and 6.5, and even less by LDL incubated in the absence of transferrin at these pHs. When LDL was co-incubated with transferrin at pH 5.5, however, the LDL was oxidised significantly. While the level of TBARS was modest, around 23 nmol MDA equivalents/mg LDL protein, the levels of TBARS generated by LDL incubated in the absence of transferrin at pH 5.5 was negligible.

The levels of TBARS were mirrored by the electrophoretic mobilities of the LDLs in agarose gels (Table 1). LDL that had been co-incubated with transferrin at pH 5.5 migrated about twice as far as native LDL or LDL incubated in the absence of transferrin at pH 5.5. LDL incubated in the presence or absence of transferrin at pHs 6.0–7.4 did not migrate significantly further than native LDL.

When LDL was co-incubated with transferrin at pH 5.5 at 100% of the average plasma concentration of transferrin (taken as 2.4 mg/ml [7]), around 22 nmol MDA equivalents/mg LDL protein were generated over 44 h (Fig. 3). As the concentration of transferrin was decreased, the levels of TBARS decreased in a dose-dependent manner, so that at 40% of the plasma concentration (0.96 mg transferrin/ml) around 10 nmol MDA equivalents/mg LDL protein were generated. At 20% of the plasma concentration (0.48 mg transferrin/ml) or in the absence of transferrin only, low levels of TBARS (<3 nmol MDA equivalents/mg LDL protein) were generated.

4. Discussion

The mechanism by which LDL is oxidised within the arterial wall is still unclear but it seems likely that it is catalysed by either copper or iron ions. There is some suggestion that iron status is associated with coronary heart disease, but this is highly controversial. The human normal aortic intima has been shown to contain 97 mg transferrin/100 cc of tissue [12] (approximately 8 μmol iron/l whole tissue) and 310 mg transferrin/kg dry tissue [13] (equivalent to 2.6 μmol iron/kg dry tissue). In addition, a human atherosclerotic plaque can contain 1.22 g transferrin/kg dry tissue (equivalent to 10 μmol iron/kg dry tissue) [13]. Gruel taken from human advanced atherosclerotic lesions has been shown to contain up to 56 μM catalytically active iron ions [14].

We have shown here that transferrin releases around 10% of its iron ions at acidic pH (Fig. 1), and that these ions are capable of oxidising LDL to increase its electrophoretic mobility (Table 1) and generate TBARS (Fig. 2). Welch [15] has shown that the pH required to remove the initial 10% of iron ions from transferrin is 5.4, whilist a pH of 4.3 is required to remove the final 10%: this supports the findings reported here. Transferrin incubated at pH 7.0 or over did not release its iron ions and could not catalyse the oxidation of LDL, suggesting that these ions are tightly bound within transferrin and are not able to redox-cycle in a way that can catalyse the oxidation of LDL.

We have shown previously that acidic pH can increase the oxidation of LDL by iron ions as evidenced by conjugated diene formation and macrophage degradation [16]. In this system where LDL and transferrin are co-incubated at acidic pH, the increase in LDL oxidation observed at pH 5.5 will be due not only to the release of iron ions from transferrin but also to the low pH. There was no LDL oxidation at pH 7.0 and above. This was not simply due to iron not being very efficient at oxidising LDL at these pH values, because ultrafiltration and atomic absorption spectroscopy showed that the iron was still bound to the transferrin at these pH values. A MES buffer was used because it does not precipitate iron ions and allows them to catalyse LDL oxidation more efficiently than in a phosphate

Table 1
The effect of pH on the oxidation of LDL coincubated with transferrin as measured by electrophoretic mobility

pH	Distance migrated (mm)	
	+ transferrin	− transferrin
5.5	20.5	10.0
6.0	10.5	10.0
6.5	10.0	10.0
7.0	10.0	10.0
7.4	10.0	10.0

LDL (100 μg protein/ml) was incubated for 44 h at 37°C in the presence or absence of transferrin (2.4 mg/ml) in 0.2 M MES buffer of various pHs containing 10 mM $NaHCO_3$ and 180 μM L-cysteine. The samples were then subjected to electrophoresis at 100 V for 30 min. The figures are the distances the LDL migrated in mm. Native LDL migrated 9.5 mm.

buffer (data not shown). Kuzuya et al. [17] have demonstrated that phosphate buffers inhibit LDL oxidation by iron ions, possibly by binding to the iron.

Transferrin is normally about 33% saturated with iron and has a mean plasma concentration of about 2.4 mg/ml [7]. The concentration of transferrin used in these experiments to oxidise LDL was also 2.4 mg/ml and was 30% saturated with iron, and would therefore contain around 18 μM iron ions. Around 10% of the total iron bound to transferrin was released at pH 5.5 (see Fig. 1). LDL oxidation was seen with concentrations of just 40% of the average plasma concentration of transferrin, when LDL oxidation was measured in terms of TBARS (Fig. 3) or electrophoretic mobility (data not shown). Since the normal arterial wall can contain around 55% of the plasma concentration of transferrin [12], if the pH were to fall low enough then sufficient iron ions may be released to catalyse the oxidation of LDL.

Transferrin at physiological concentrations or below can release catalytically active iron ions at acidic pH to catalyse the oxidation of LDL. But are atherosclerotic lesions acidic? The media of large arteries are one of the poorest perfused tissues in the human body and therefore have a low oxygen tension [18]. Atherosclerotic lesions in the intima re also poorly perfused and this may similarly result in ischaemia, and this may lead to local lactic acidosis and therefore a low pH. Macrophages in atherosclerotic lesions exhibit some of the characteristics of activated macrophages [19], and the pH measured near an activated macrophage can be as low as 3.6 [20]. In addition, atherosclerosis is now widely recognised as being in many ways a type of chronic inflammatory disease, and, by analogy to other inflammatory sites, the pH may be expected to fall below 7.4 [21]. It is thus possible that atherosclerotic lesions may experience an extracellular pH below 7.4, especially within a cluster of macrophages, although there is, as yet, no direct evidence to support this.

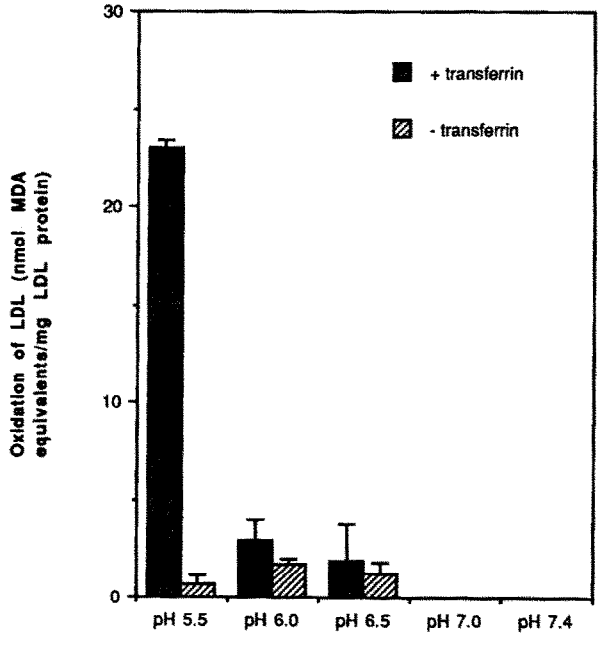

Fig. 2. The effect of pH on the oxidation of LDL co-incubated with transferrin as measured by TBARS. LDL (100 μg protein/ml) was incubated for 44 h at 37°C in the presence or absence of transferrin (2.4 mg/ml) in 0.2 M MES buffer of various pH values containing 10 mM $NaHCO_3$ and 180 μM L-cysteine. Samples were then assayed for TBARS. The mean ± S.E.M. of triplicate incubations are shown. These results were confirmed by four independent experiments.

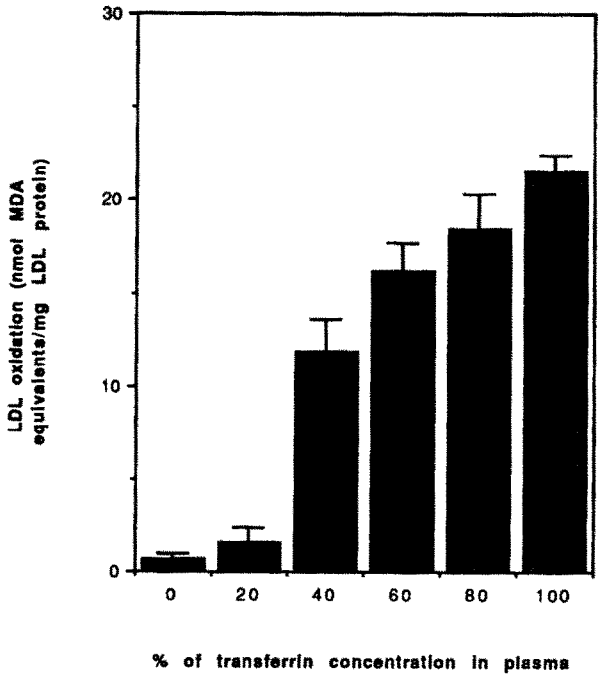

Fig. 3. The oxidation of LDL co-incubated with various concentrations of transferrin at acidic pH. LDL (100 μg protein/ml) was incubated for 44 h at 37°C in the presence of various concentrations of transferrin in 0.2 M MES buffer, pH 5.5, containing 10 mM $NaHCO_3$ and 180 μM L-cysteine. The mean transferrin concentration in plasma was taken as 2.4 mg/ml. Samples were then assayed for TBARS. The mean ± S.E.M. of triplicate incubations are shown. These results were confirmed by four independent experiments.

The oxidation of LDL in the arterial wall, resulting in an atherogenic form, is probably catalysed by transition metal ions. It is possible that low local pH values within the arterial wall may release iron from transferrin (or copper from caeruloplasmin [22]) which may catalyse this oxidation of LDL.

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