The effects of ascorbate and dehydroascorbate on the oxidation of low-density lipoprotein

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Ascorbate at concentrations of 60–100 μ M inhibits the modification of freshly prepared low-density lipoprotein (LDL) by macrophages. With ‘moderately oxidized’ LDL (produced by prolonged storage in a refrigerator), however, ascorbate does not inhibit LDL modification by macrophages and actually modifies the LDL itself in the absence of macrophages [Stait and Leake (1994) FEBS Lett. 341, 263–267]. We have now shown that dehydroascorbate can modify both ‘fresh’ LDL and moderately oxidized LDL in a dose-dependent manner to increase its uptake by macrophages. The modification of moderately oxidized LDL by ascorbate and dehydroascorbate or of ‘fresh’ LDL by dehydroascorbate is dependent on the presence of iron or copper.

In ‘fresh’ LDL, ascorbate inhibited conjugated-diene formation by copper. In moderately oxidized LDL, the number of conjugated dienes present was decreased rapidly in the presence of copper and ascorbate. Dehydroascorbate decreased the lag phase and increased the rate of copper-induced conjugated-diene formation in ‘fresh’ LDL (although in some experiments it inhibited the formation of conjugated dienes). The ascorbate-modified moderately oxidized LDL was taken up by macrophages by their scavenger receptors, as the uptake was inhibited by polyinosinic acid or fucoidan. Ascorbate and dehydroascorbate therefore have the potential to increase LDL oxidation under certain conditions, but whether or not they do so *in vivo* is unknown.

INTRODUCTION

Low-density lipoprotein (LDL) is well known to play an important role in atherosclerosis, the underlying cause of coronary heart disease and strokes. Oxidatively modified LDL is taken up by macrophages via scavenger receptors at a much greater rate than native LDL [1,2]. This leads to the formation of cholesterol-laden foam cells, which are characteristic of many atherosclerotic lesions [3]. Antioxidants are of importance in the protection of LDL against peroxidation and hence may be of importance in protection against atherosclerosis.

Ascorbic acid (vitamin C) is a major water-soluble antioxidant in plasma. It is a scavenger of aqueous free radicals [4,5], can regenerate α -tocopherol from the α -tocopheryl radical [6,7] including that in LDL [8] and can also regenerate the antioxidant probucol [9]. Ascorbate is able to protect plasma and LDL lipids from peroxidative damage [5,10,11]. Physiological concentrations of ascorbate inhibit LDL oxidation by copper ions or human monocyte-derived macrophages and help to preserve the endogenous lipid-soluble antioxidants of LDL [12,13]. Ascorbate also inhibits cholesterol chlorohydrin formation in LDL by myeloperoxidase/ H_2O_2/Cl^- [14].

Two mechanisms have been suggested by Retsky et al. [15] to explain how ascorbate protects against LDL oxidation; ascorbic acid may scavenge free radicals in the aqueous phase or dehydroascorbic acid (the oxidation product of ascorbic acid), or its decomposition products, might modify LDL such that less copper can bind to the LDL particle, thus increasing its resistance to oxidation by copper. Very recently, Retsky and Frei [16] have shown that ascorbate and dehydroascorbate no longer protect against the oxidation by copper of LDL that has already been partially oxidized by copper.

Ascorbate can act as a pro-oxidant as well as an antioxidant [4,17]. We have recently shown [18] that ascorbate is able to increase the modification, both by macrophages and in a cell-free

system, of LDL ‘autooxidized’ during prolonged storage. We showed that this modification was oxidative, since it was inhibited by the addition of the antioxidant butylated hydroxytoluene (BHT). We have now investigated this effect in greater detail and have also shown that, in our hands, dehydroascorbate can increase the modification of both ‘fresh’ and moderately oxidized LDL.

MATERIALS AND METHODS

Materials

Ham’s F-10 medium was purchased from ICN Flow, High Wycombe, Bucks., U.K. Ham’s F-10 medium specially formulated to be deficient in copper, iron, zinc and cysteine, Dulbecco’s modified Eagle’s medium (DMEM), foetal-calf serum (FCS), PBS (without calcium or magnesium), penicillin/streptomycin (5000 i.u./5000 μ g/ml), amphotericin B (250 μ g/ml), gentamicin (10 mg/ml), and glutamine (200 mM) were from Gibco, Uxbridge, Middx., U.K. $FeSO_4$, Phenol Red solution (0.5%, w/v), L-ascorbic acid (sodium salt), tetramethoxypropane, polyinosinic acid and fucoidan were from Sigma Chemical Co., Poole, Dorset, U.K. Dehydroascorbic acid was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Falcon multiwell tissue-culture plates (18 mm and 22 mm diameter wells) were purchased from Fahrenheit, Bradwell Abbey, Milton Keynes, Bucks., U.K., and Paragon electrophoresis Lipo gels were from Beckman. Female Swiss T.O. mice were from Tuck and Son, Battlesbridge, Essex, U.K.

Isolation and radioiodination of LDL

LDL (1.019–1.063 g/ml) was isolated from the blood of healthy volunteers by sequential density ultracentrifugation in the presence of EDTA as previously described [19] and then radiolabelled

Abbreviations used: BHT, butylated hydroxytoluene; DHA, dehydroascorbate; DMEM, Dulbecco’s modified Eagle’s medium; FCS, foetal-calf serum; LDL, low-density lipoprotein; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; apo B-100, apolipoprotein B-100.

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Figure 1 Lipid hydroperoxide content of ^{125}I -labelled LDL and non-labelled LDL stored for various times

^{125}I -labelled LDL (2 mg of protein/ml) or non-labelled LDL (4–8 mg of protein/ml) were stored at 3 °C in phosphate buffer containing 100 μM Na_2EDTA for various periods. They were then assayed for hydroperoxides. The times shown are the intervals between starting the LDL isolation and assaying for hydroperoxides. The number beside the point on the graph represents the batch number of the LDL to enable comparison of the oxidation of the same batch of labelled and non-labelled LDL. Means $\pm$ S.E.M. of triplicate observations are shown. ■, Labelled LDL; □, non-labelled LDL.

with Na^{125}I using iodine monochloride [20]. The radioiodinated LDL was mixed with non-labelled LDL to give an initial specific radioactivity of 20–40 c.p.m./ng and diluted to 2 mg of protein/ml with buffer containing 100 μM EDTA. When used at 100 μg of protein/ml, this gives a final EDTA concentration of 5 μM . This standardization was considered important as, depending on its concentration, EDTA can either promote or inhibit LDL oxidation by cells [21].

Formation of moderately oxidized LDL

Moderately oxidized LDL was formed by storage of ^{125}I -labelled LDL or non-labelled LDL in the refrigerator at 3 °C, in a buffer containing 154 mM NaCl, 16.7 mM NaH_2PO_4 , 21.1 mM Na_2HPO_4 and 100 μM Na_2EDTA , pH 7.4, for a period of 10–51 weeks.

Modification of LDL by cells

Resident peritoneal macrophages were isolated from female Swiss T.O. mice by peritoneal lavage [22]. The peritoneal cells were plated at 0.75×10^6 cells/18 mm well, then incubated at 37 °C. After 3–4 h, the cells were washed with DMEM to remove non-adherent lymphocytes before being used for the modification of LDL. ^{125}I -labelled LDL (100 μg of protein/ml) was incubated for 16 or 18 h with macrophages (macrophage-modified LDL) or in cell-free wells (control LDL) at 37 °C under 5% CO_2 in 0.4 ml of Ham's F-10 medium supplemented with Phenol Red (final concn. 12 $\mu\text{g}/\text{ml}$) and gentamicin (50 $\mu\text{g}/\text{ml}$), and with concentrations of ascorbate or dehydroascorbate as specified. For experiments which involved an assay of thiobarbituric acid-reactive substances (TBARS), cells were plated in 22 mm-diameter wells at 1.5×10^6 peritoneal cells/well with 1 ml of medium/well. After incubation, the modified medium was collected and centrifuged at 500 g for 5 min to remove any detached cells.

Modification of LDL in the absence of cells

^{125}I -labelled LDL (100 μg of protein/ml) was incubated for 18 h in cell-free wells at 37 °C under 5% CO_2 in 0.4 ml of Ham's F-10 medium supplemented with Phenol Red (12 $\mu\text{g}/\text{ml}$) and gentamicin (50 $\mu\text{g}/\text{ml}$) and with various concentrations of ascorbate or dehydroascorbate. Ascorbate or dehydroascorbate at 100 μM did not affect the pH of the Ham's F-10 medium. For the time-course experiments, Ham's F-10 medium specially formulated to be deficient in iron, copper, zinc and cysteine was used, supplemented with Phenol Red and gentamicin as above, with the zinc (0.1 μM) added back, and with FeSO_4 or CuSO_4 as specified. Where specified, LDL was also incubated at 37 °C with CuSO_4 (10 μM) and ascorbate in Dulbecco's PBS without calcium or magnesium (8 g NaCl/l, 20 mg KCl/l, 200 mg $\text{KH}_2\text{PO}_4/\text{l}$ and 1.15 g $\text{Na}_2\text{HPO}_4/\text{l}$). The medium was collected into EDTA (1 mM) and BHT (20 μM) after the appropriate time of incubation and stored in the refrigerator until it was assayed for oxidation products or incubated with the 'target' macrophages.

Uptake of modified LDL by cells

For measurement of uptake of modified LDL, mouse J774 macrophage-like cells (plated at 150000 or 180000 cells per 22 mm-diameter well and used the day after) or mouse peritoneal macrophages (1.25×10^6 peritoneal cells per 22 mm-diameter well) were used ('target' macrophages). Macrophage-modified or control LDL was diluted to 10 μg of protein/ml in DMEM containing 20% (v/v) heat-inactivated FCS, amphotericin B (1 $\mu\text{g}/\text{ml}$), L-glutamine (2 mM) and penicillin/streptomycin (10 i.u./10 $\mu\text{g}/\text{ml}$) for the J774 cells or in DMEM containing 10% (v/v) FCS, penicillin/streptomycin (100 i.u./100 $\mu\text{g}/\text{ml}$) and gentamicin (50 $\mu\text{g}/\text{ml}$) for the peritoneal cells. The LDL was then incubated with the 'target' macrophages or cell-free wells (1 ml/well) for 22 h. The target macrophages would not have

Figure 2 Time course of the effect of ascorbate on the oxidation of moderately oxidized LDL by iron or copper

Moderately oxidized ^{125}I -labelled LDL (41 weeks old, $100 \mu\text{g}$ of protein/ml) was incubated for up to 24 h in triplicate cell-free wells in Ham's F-10 medium specially formulated to be deficient in transition metal ions and to which either iron ($3 \mu\text{M}$) or copper ($8 \mu\text{M}$) was added. Freshly dissolved ascorbate ($100 \mu\text{M}$) was present in some of the wells. The LDL was collected into EDTA (1 mM) and BHT ($20 \mu\text{M}$) after various times and stored in the refrigerator until the end of the incubation. The LDL was then assayed for TBARS (**B**) or diluted to $10 \mu\text{g}$ of protein/ml in serum-containing DMEM and incubated with 'target' J774 macrophages or in cell-free wells for 22 h. The rate of uptake of the LDL was then measured (**A**). results are means $\pm$ S.E.M. of triplicates. The uptake of control LDL incubated for 24 h in the absence of iron, copper or ascorbate was $0.58 \pm 0.06 \mu\text{g}$ of LDL protein/mg of cell protein in 22 h. These results were confirmed by another experiment. ○, Copper; ●, copper and ascorbate; △, iron; ▲, iron and ascorbate.

further oxidized the LDL, because serum strongly inhibits LDL oxidation [23].

To assess the degradation of modified LDL, the radioactive non-iodide trichloroacetic acid-soluble degradation products in the medium were measured [19]. The degradation products in the cell-free wells were subtracted from the values for the wells containing macrophages. The cells were washed, lysed with 0.2 M NaOH , and the protein and radioactivity associated with the cells was determined [20]. The low levels of radioactivity that remained in the cell-free wells were subtracted from the cell-associated values. Since a lot of radioactivity remains in the cells

with oxidized ^{125}I -labelled LDL, the cell-associated values were added to the degradation values to obtain total LDL uptake [24].

TBARS assay

A method modified from that described by Bernheim et al. [25] was used to assay TBARS. BHT ($20 \mu\text{M}$) and EDTA (1 mM) were added to $250 \mu\text{l}$ samples of modified or control LDL ($100 \mu\text{g}$ of protein/ml) to prevent further oxidation. A 2 ml portion of 0.335% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid was added to each sample and incubated at

95 °C for 15 min. Standards were prepared using tetramethoxypropane to form malondialdehyde (MDA). The absorbance was read at 535 nm. The assay was not affected by ascorbate.

Lipid hydroperoxide assay

The method of El-Saadani et al. [26], modified as described in Wilkins and Leake [19], was used. Briefly, 1 ml of cholesterol colour reagent was added to 250 μ l samples of macrophage-modified or control LDL, and incubated in the dark for 30 min at room temperature. Standards were prepared using H₂O₂. The absorbance was read at 365 nm. Ascorbate was found to interfere with this assay, and thus the effect of ascorbate on the formation of lipid hydroperoxides could not be measured.

Conjugated-diene assay

Oxidation of LDL was measured by monitoring the formation of conjugated dienes, as described by Esterbauer et al. [27]. Briefly, 'fresh' or moderately oxidized LDL (50 μ g of protein/ml) was incubated in Dulbecco's PBS, without Ca²⁺ or Mg²⁺, containing CuSO₄ (5 μ M) in the presence or absence of ascorbic acid (100 μ M). The absorbance was read at 234 nm at 5 min intervals, at 37 °C, against reference cuvettes containing PBS, CuSO₄ and ascorbate as appropriate.

Agarose-gel electrophoresis

Moderately oxidized or 'fresh' ¹²⁵I-labelled LDL (100 μ g/ml) was incubated at 37 °C in Ham's F-10 medium under the conditions specified for the individual experiment. Samples (5 μ l) were then run on Beckman Paragon electrophoresis Lipo gels of 0.5% agarose at 100 V for 30 min and then fixed and stained with Sudan Black B according to the manufacturer's instructions. Native (non-incubated) LDL (100 μ g of protein/ml) was also applied to the gels.

RESULTS

Ascorbate increases the modification of moderately oxidized LDL by iron or copper

The moderately oxidized (or 'aged') LDL was formed by incubating LDL at 3 °C in the presence of 100 μ M EDTA for 10–51 weeks. There was a progressive increase in the levels of hydroperoxides in the LDL with storage until about 40 to 50 weeks, when they approached the maximum levels of lipid hydroperoxides that can be achieved by incubating LDL with CuSO₄ or cells [22] (Figure 1). LDL that had been labelled with ¹²⁵I and then stored at 2 mg of protein/ml at 3 °C contained more lipid hydroperoxides than the same batch of non-labelled LDL stored for the same time at a concentration of 4–8 mg/ml, although the initial hydroperoxide levels were not greatly altered by iodination.

The effect of ascorbate on the oxidation of moderately oxidized LDL was studied by incubating this LDL with 8 μ M CuSO₄ or 3 μ M FeSO₄ in Ham's F-10 medium specially formulated to be deficient in iron and copper. In the absence of ascorbate, there was no modification of the moderately oxidized LDL by iron, as detected by macrophage uptake, but the LDL was oxidized rapidly by copper after a lag period of about 4 h (Figure 2). In the presence of ascorbate, however, the LDL was oxidized rapidly by iron or copper, as measured by macrophage uptake or TBARS, and the lag period was abolished or at least was less than 1 h (Figure 2).

There was a slow increase in the electrophoretic mobility of

Table 1 Time course of the increase in electrophoretic mobility of moderately oxidized LDL incubated with ascorbate

Moderately oxidized ¹²⁵I-labelled LDL (19 weeks old, 100 μ g/ml) was incubated at 37 °C in complete Ham's F-10 medium with or without ascorbate (100 μ M) for various intervals up to 24 h. Samples were then electrophoresed on agarose gels. The distance of the band from the origin was then measured. Native (non-incubated) moderately oxidized LDL migrated 8.5 mm from the origin. These results were confirmed by another experiment.

Period of incubation (h)	Distance from origin (mm)	
	Control	100 μ M Ascorbate
0	9	9.5
1	9	12
2	9	13
4	9	15
6	9.5	17
8	10	19
24	15	22

moderately oxidized LDL over 24 h in complete Ham's F-10 medium, presumably due to its further oxidation by the iron in this medium (but this may not have been enough to cause a substantial increase in macrophage uptake, as this occurs later than increased electrophoretic mobility [28]) (Table 1). The increase in electrophoretic mobility was much more rapid in the presence of ascorbate, with increased mobility being seen within the first hour of incubation.

Ascorbate (100 μ M) completely prevented the formation of conjugated dienes by copper in the 'fresh' LDL (results not shown). Moderately oxidized LDL had a much higher initial absorbance at 234 nm than the 'fresh' LDL, presumably due to the presence of conjugated-diene lipid hydroperoxides or hydroxides or other lipid-peroxidation products that had formed in it during prolonged storage. The absorbance was increased by copper, but only modestly, presumably because only relatively small amounts of non-oxidized lipids were left in the LDL. The addition of ascorbate and copper to the moderately oxidized LDL caused a very rapid decrease in the absorbance, after which the absorbance remained essentially unchanged (results not shown). A solution of 100 μ M ascorbate in PBS oxidized gradually with time, as determined by the decrease in its absorbance at 265 nm, presumably due to the presence of contaminating iron or copper in the buffer (Figure 3). The rate of oxidation was increased greatly by 5 μ M CuSO₄, such that all of the ascorbate was oxidized by 20 min.

Ascorbate-modified LDL is taken up by macrophage scavenger receptors

The increased uptake by mouse peritoneal macrophages of ascorbate-modified moderately oxidized LDL or of copper-oxidized LDL was almost completely inhibited by polyinosinic acid and was partially inhibited by fucoidan (Table 2). Much of the radioactivity in moderately oxidized ¹²⁵I-labelled LDL modified by ascorbate accumulated inside macrophages. This would be consistent with oxidized LDL being resistant to lysosomal proteases [24,29,30]

Dehydroascorbate increases the modification of both moderately oxidized and 'fresh' LDL

Dehydroascorbate (100 μ M), the initial oxidation product of ascorbate, caused a large increase in the modification of both

Figure 3 Oxidation of ascorbate by copper

Ascorbate (100 μ M in PBS) was incubated with or without CuSO_4 (5 μ M) and the absorbance at 265 nm was measured at 5 min intervals. The spectrophotometer was blanked with PBS. $\circ$, Ascorbate alone; $\bullet$, ascorbate with copper. These results were confirmed by another experiment.

Table 2 Uptake of ascorbate-modified moderately oxidized LDL by macrophage scavenger receptors

Moderately oxidized ^{125}I -labelled LDL (29 weeks old, 100 μ g of protein/ml) was incubated for 18 h in complete Ham's F-10 medium with or without ascorbate (100 μ M). 'Fresh' ^{125}I -labelled LDL (4 weeks old, 100 μ g of protein/ml) was incubated in PBS with copper (25 μ M). The LDLs were then diluted to 10 μ g of protein/ml in serum-containing DMEM to which polyinosinic acid [poly(I)] (25 μ g/ml) or fucoidan (50 μ g/ml) was added. The LDLs were then incubated with mouse peritoneal macrophages or in cell-free wells for 22 h. The rates of degradation and cell association were then measured. The sum of these represents the total uptake of the LDL. The uptake of native (non-incubated) LDL was also measured. means $\pm$ SEM for triplicate wells are shown. These results were confirmed by another experiment.

LDL	Scavenger-receptors competitor	Uptake of ^{125}I -labelled LDL by mouse peritoneal macrophages (μ g of LDL protein/mg of cell protein in 22 h)		
		Degraded	Cell-associated	Total uptake
Native 'fresh'	No addition	0.81	0.09	0.90 $\pm$ 0.08
Native moderately oxidized	No addition	1.11	0.58	1.69 $\pm$ 0.22
Control moderately oxidized	No addition	3.27	1.90	5.17 $\pm$ 0.52
	Poly(I)	0.26	0.64	0.90 $\pm$ 0.14
	Fucoidan	2.44	1.07	3.51 $\pm$ 0.28
Ascorbate-modified moderately oxidized	No addition	19.5	10.7	30.2 $\pm$ 2.2
	Poly(I)	1.42	2.76	4.18 $\pm$ 0.10
	Fucoidan	6.01	3.29	9.30 $\pm$ 0.17
Copper-oxidized	No addition	27.9	22.2	50.1 $\pm$ 2.8
	Poly(I)	0.18	3.57	3.75 $\pm$ 0.05
	Fucoidan	5.04	5.34	10.4 $\pm$ 0.36

'fresh' and moderately oxidized LDL in Ham's F-10 medium, whereas 100 μ M ascorbate increased the modification of moderately oxidized LDL but not 'fresh' LDL (Table 3). The radioactivity of dehydroascorbate-modified LDL also accumulated in the cells.

In contrast with ascorbate [18], dehydroascorbate was not protective against the modification of 'fresh' LDL by macrophages and in fact increased their modification of LDL to a form taken up rapidly by macrophages (Table 4). There appeared to be some modification of LDL by the iron in the Ham's F-10

medium during the incubation in the absence of macrophages, as the uptake of the control LDL by the 'target' macrophages was greater than that of the native (non-incubated) LDL. In the presence of macrophages, the modification of the LDL was increased further. Dehydroascorbate caused a dose-dependent increase in the modification of LDL in both the presence and absence of macrophages. Dehydroascorbate also modified moderately oxidized LDL with a similar potency to its ability to modify 'fresh' LDL (Table 5). Dehydroascorbate also modified moderately oxidized LDL and 'fresh' LDL in terms of increased

Table 3 Effects of ascorbate and dehydroascorbate on moderately oxidized and 'fresh' LDL

Moderately oxidized ^{125}I -labelled LDL (30 weeks old, 100 μg of protein/ml) or 'fresh' ^{125}I -labelled LDL (5 weeks old, 100 μg of protein/ml) was incubated for 18 h in triplicate cell-free wells in Ham's F-10 medium with freshly dissolved ascorbate (100 μM) or dehydroascorbate (100 μM). The LDL was then diluted to 10 μg of protein/ml in serum-containing DMEM and incubated with J774 macrophages or in cell-free wells for 22 h. The rates of degradation and cell association were then measured. The sum of these represents the total uptake of the LDL. Means $\pm$ S.E.M. for triplicates are shown. These results have been confirmed by other experiments.

LDL	Addition	Uptake of ^{125}I -labelled LDL by macrophages (μg of LDL protein/mg of cell protein in 22 h)		
		Degraded	Cell-associated	Total uptake
'Fresh'	No addition	0.19	0.16	0.36 ± 0.06
	Ascorbate	0.22	0.06	0.28 ± 0.01
	Dehydroascorbate	1.49	0.85	2.34 ± 0.11
Moderately oxidized	No addition	0.68	0.40	1.07 ± 0.01
	Ascorbate	2.80	1.81	4.61 ± 0.13
	Dehydroascorbate	2.53	1.79	4.32 ± 0.21

Table 4 Effect of dehydroascorbate on 'fresh' LDL

'Fresh' ^{125}I -labelled LDL (5 weeks old, 100 μg of protein/ml) was incubated for 18 h in triplicate wells with mouse peritoneal macrophages (MØ-modified LDL) or in cell-free wells (control LDL) in Ham's F-10 medium and the indicated concentration of dehydroascorbate. The LDL was then diluted to 10 μg of protein/ml in serum-containing DMEM and incubated with 'target' J774 macrophages or in cell-free wells for 22 h. The rates of degradation and cell association were then measured. The sum of these represents the total uptake of the LDL. The uptake of native (non-incubated) LDL is also shown. Means $\pm$ S.E.M. of triplicates are shown. These results were confirmed by another experiment.

[Dehydroascorbate] (μM)	LDL	Uptake of ^{125}I -labelled LDL by macrophages (μg of LDL protein/mg of cell protein in 22 h)		
		Degraded	Cell-associated	Total uptake
0	Native	0.19	0.05	0.23 ± 0.09
0	MØ-modified	1.08	0.86	1.94 ± 0.02
	Control	0.44	0.41	0.86 ± 0.04
1	MØ-modified	1.12	0.92	2.04 ± 0.06
	Control	0.45	0.41	0.86 ± 0.05
3	MØ-modified	1.17	0.97	2.14 ± 0.03
	Control	0.58	0.47	1.06 ± 0.04
10	MØ-modified	1.17	0.99	2.16 ± 0.07
	Control	0.56	0.60	1.16 ± 0.07
30	MØ-modified	1.49	1.22	2.70 ± 0.08
	Control	1.26	1.11	2.37 ± 0.02
100	MØ-modified	1.85	1.46	3.31 ± 0.06
	Control	2.03	1.88	3.92 ± 0.14

electrophoretic mobility, whereas ascorbate only increased the mobility of moderately oxidized LDL (Table 6).

In the absence of iron, there was no modification of the LDL by either ascorbate or dehydroascorbate, as measured by macrophage uptake (results not shown). In the absence of copper, there was no modification of 'fresh' (Figure 4, legend) or moderately oxidized LDL (results not shown) by dehydroascorbate as measured by conjugated-diene formation, and dehydroascorbate added in the absence of iron or copper did not oxidize 'fresh' LDL as measured by TBARS or electrophoretic mobility (results not shown).

In the experiment shown in Figure 4, dehydroascorbate added at the start of the experiment greatly shortened the lag phase before oxidation and increased the rate of conjugated diene formation in 'fresh' LDL incubated with copper. (In some experiments, however, dehydroascorbate inhibited the oxidation

Table 5 Effect of dehydroascorbate on moderately oxidized LDL

Moderately oxidized ^{125}I -labelled LDL (31 weeks old, 100 μg of protein/ml) was incubated for 18 h in triplicate in cell-free wells in Ham's F-10 medium and the indicated amount of dehydroascorbate. The LDL was then diluted to 10 μg of protein/ml in serum-containing DMEM and incubated with 'target' J774 macrophages or in cell-free wells for 22 h. The rates of degradation and cell association were then measured. The sum of these represents the total uptake of the LDL. The uptake of native (non-incubated) LDL is also shown. Means $\pm$ S.E.M. of triplicates are shown. These results were confirmed by another experiment.

[Dehydroascorbate] (μM)	Uptake of ^{125}I -labelled LDL by macrophages (μg of LDL protein/mg of cell protein in 22 h)		
	Degraded	Cell-associated	Total uptake
Native LDL	0.29	0.28	0.57 ± 0.04
0	0.51	0.39	0.90 ± 0.04
1	0.49	0.46	0.95 ± 0.07
3	0.52	0.51	1.03 ± 0.04
5	0.64	0.55	1.19 ± 0.05
30	1.11	1.01	2.12 ± 0.04
100	1.80	1.60	3.40 ± 0.20

Table 6 Electrophoretic mobility of moderately oxidized and 'fresh' LDL incubated with ascorbate or dehydroascorbate

Moderately oxidized ^{125}I -labelled LDL (14 weeks old, 100 μg of protein/ml) and 'fresh' LDL (1 week old, 100 μg of protein/ml) were incubated for 18 h in the presence of ascorbate (100 μM) or dehydroascorbate (100 μM) in complete Ham's F-10 medium. Samples were then subjected to agarose-gel electrophoresis. The distance of each band from the origin was measured. These results were confirmed by another experiment.

Type of LDL	Distance from origin (mm)		
	Control	100 μM Ascorbate	100 μM Dehydroascorbate
'Fresh'	7.5	7.5	10
Moderately oxidized	12.5	20	19.5

of 'fresh' LDL when added at the start, but increased it greatly when added shortly afterwards). When the addition of dehydroascorbate was delayed until the most rapid part of the propagation phase, it immediately increased the rate of formation of conjugated dienes. The addition of ascorbate during the propa-

Figure 4 Effect of ascorbate and dehydroascorbate added during the propagation phase of LDL oxidation by copper

'Fresh' ^{125}I -labelled LDL (3 weeks old) ($50 \mu\text{g}$ of protein/ml) was incubated at 37°C in a dual-beam spectrophotometer in silica cuvettes in the presence or absence of CuSO_4 ($10 \mu\text{M}$ in excess of that bound to the EDTA present). Ascorbate ($100 \mu\text{M}$) or dehydroascorbate ($100 \mu\text{M}$) were added either at the start of the experiment or during the most rapid phase of conjugated-diene formation (in this case at 62 min, as indicated by the arrow, although the time varied with different LDL preparations). The reference cuvettes contained copper and ascorbate or dehydroascorbate as appropriate, but no LDL. The absorbance at 234 nm was measured at 2 min intervals and the absorbances at zero time were subtracted. There was no change in absorbance in the absence of copper with LDL alone, with LDL plus ascorbate or with LDL plus dehydroascorbate. The results are representative of four experiments. —, LDL + copper; ---, LDL + Cu + ascorbate after 62 min; ···, LDL + Cu + dehydroascorbate after 62 min; - · - ·, LDL + dehydroascorbate at zero time.

gation phase resulted in an immediate fall of the absorbance at 234 nm before it reached a plateau at a level lower than it was before the addition (Figure 4).

The effects of ascorbate and dehydroascorbate on LDL were not due to any contaminating iron or copper, as the levels of iron and copper in $100 \mu\text{M}$ ascorbate were less than 1 nM each, and the levels in $100 \mu\text{M}$ dehydroascorbate were undetectable, as determined by atomic-absorption spectrometry (in a 100 mM solution of ascorbate and a 10 mM solution of dehydroascorbate).

DISCUSSION

The study described here demonstrates that ascorbate can promote the oxidation of moderately oxidized LDL by either iron or copper in terms of TBARS, electrophoretic mobility and macrophage uptake. Moderately oxidized LDL was produced by prolonged storage in a refrigerator and, although it was stored in the presence of EDTA, it contained a high level of lipid hydroperoxides, migrated somewhat faster in agarose gels and was sometimes taken up somewhat faster by macrophages than 'fresh' LDL. We still refer to this LDL as being moderately oxidized because it was taken up by macrophages far more slowly than was highly oxidized LDL.

Ascorbate has been shown to lengthen the lag phase, in terms of the formation of conjugated dienes, before LDL is oxidized by copper ions [27]. This is in agreement with our results with 'fresh' LDL in which ascorbate totally prevented conjugated-diene formation by copper. The initial levels of conjugated dienes were much higher in moderately oxidized LDL than in 'fresh' LDL and were decreased very rapidly by a combination of copper and ascorbate. This may be surprising at first sight, as ascorbate resulted in increased TBARS, electrophoretic mobility

and macrophage uptake of moderately oxidized LDL. Ascorbate added during the propagation phase of 'fresh' LDL oxidation by copper also very rapidly decreased the absorbance due to conjugated dienes at 234 nm. We suggest that the mechanism to explain these observations involves the reduction by ascorbate (asc) of Cu^{2+} to Cu^+ with the formation of the semidehydroascorbate radical.

Cu^+ ions are believed to react rapidly with lipid hydroperoxides (LOOH) to form lipid alkoxyl radicals ($\text{LO}^\cdot$):

Alkoxyl radicals have a number of fates, but one of them is to be converted into aldehydes by β -scission, which results in the fragmentation of the carbon backbone of the fatty acid. The aldehydes would then react with lysyl and maybe other amino acid residues in the apolipoprotein B-100 (apo B-100) of LDL and increase the net negative charge and macrophage uptake of the LDL. The reaction of Cu^{2+} with lipid hydroperoxides is thought to be slower (if it occurs at all):

In addition, the lipid peroxy radicals formed ($\text{LOO}^\cdot$) may not fragment into aldehydes as rapidly as do the lipid alkoxyl radicals. The same considerations may also apply to iron, as ascorbate would reduce Fe^{3+} to Fe^{2+} , and Fe^{2+} would react faster than Fe^{3+} with lipid hydroperoxides:

The rapid fragmentation of the carbon backbone of the conjugated dienes by β -scission would lead to a rapid fall in the

absorbance at 234 nm if the aldehydes or hydrocarbons that form were to absorb less at this wavelength.

After the very rapid changes induced in moderately oxidized LDL by ascorbate plus copper, no more changes were observed in terms of the absorbance at 234 nm, whereas with copper alone they were. The ascorbate would have been converted into semidehydroascorbate and then to dehydroascorbate and this, or one of its degradation products, may possibly have modified apolipoprotein B-100 to prevent copper binding and LDL oxidation, as suggested by Retsky et al. [15]. The modification of the apolipoprotein B-100 by aldehydes, which leads to the increased electrophoretic mobility and macrophage uptake of moderately oxidized LDL in the presence of ascorbate plus copper or iron, may not be instantaneous and may proceed slowly over a period of hours, even after lipid oxidation itself has stopped.

Kanner and Mendel [31] studied the effects of ascorbic acid and metal ions in a β -carotene/linoleate solution and found that ascorbic acid was a pro-oxidant at concentrations up to 1 mM and that the pro-oxidant effects were enhanced when linoleic acid hydroperoxides were added.

Ascorbate has been shown to react with lysyl residues under autoxidizing conditions to form N^{ϵ} -(carboxymethyl)lysine, due to the formation of oxidation products of ascorbate that may attack the ϵ -amino group of lysine [32]. It is conceivable that the same thing may happen when ascorbate (or dehydroascorbate) is incubated with moderately oxidized LDL in the presence of iron or copper ions and that this may contribute to the increased electrophoretic mobility or macrophage uptake.

Ascorbate-modified LDL appeared to be taken up rapidly by mouse peritoneal macrophages by their scavenger receptors, since the macrophage competitors for scavenger receptors, polyinosinic acid and fucoidan, competed effectively for its uptake. This may mean that ascorbate-modified moderately oxidized LDL is taken up by the 'classical' scavenger receptor cloned by Kodama et al. [33], but a number of other scavenger receptors that bind oxidized LDL have now been identified (see, e.g. [2,34]), and some of these may possibly be involved, although not all of them bind polyinosinic acid and fucoidan.

Dehydroascorbate, in contrast with ascorbate, could increase the modification of both 'fresh' and moderately oxidized LDL. The effect of dehydroascorbate on both types of LDL was dose-dependent, and large effects were seen at 30 and 100 μ M. The reported concentrations of dehydroascorbate in normal human plasma differ greatly with values of 3 μ M [35], 2–5 μ M [36], 6 μ M [37], 12 μ M [38] and 29 μ M [39] being reported. Lunec and Blake reported that the mean dehydroascorbate level in the synovial fluid of patients with rheumatoid arthritis was 33 μ M [38]. It may therefore be of interest that foam cells derived from macrophages are present in the synovial membranes in rheumatoid arthritis and that they stain with antibodies against oxidized LDL [40]. The immediate hydrolysis product of dehydroascorbic acid is 2,3-diketogulonic acid, whose concentration in normal human plasma is reported to be about 14 μ M [35], but its effects on LDL oxidation are unknown.

Dehydroascorbate greatly decreased the lag phase and increased the rate of conjugated-diene formation when 'fresh' LDL was incubated with copper (although sometimes it inhibited the oxidation when added at the start of the experiment, but increased it when added shortly afterwards). It also increased the rate of conjugated-diene formation when added during the propagation phase. Dehydroascorbate decomposes to a wide variety of over 50 degradation products [41], and Takagi et al. have shown that dehydroascorbate and 2,3-diketogulonic acid produce free radicals when they degrade and greatly increase the

oxidation of linoleic acid, much better than ascorbate does [42]. These free radicals may possibly have been responsible for increasing the modification of the 'fresh' or moderately oxidized LDL in our experiments. In addition, Jore et al. [43] have demonstrated that dehydroascorbic acid can accept an electron from the α -tocopheroxyl radical in ethanolic solution, converting it into an α -tocopherol cation. This may prevent the α -tocopheroxyl radical from reacting with another lipid peroxyl or alkoxyl radical and therefore reduce its antioxidant activity, and this effect may possibly contribute to the pro-oxidant effect of dehydroascorbate on 'fresh' LDL.

We have confirmed the results of Retsky et al. [15] that, in the absence of LDL, ascorbate is completely oxidized by copper within 20 min. In the presence of LDL, ascorbate appears to be more stable to oxidation by copper and does not become exhausted for about 2 h [15], which may be due to the LDL binding some of the copper ions such that they are not able to oxidize the ascorbate [15]. In the experiments shown here, ascorbate would thus presumably be present during the first 2 h or so of the incubations. By contrast, dehydroascorbate would be expected to be present for only 20–30 min [15], after which time it would have degraded to other products, which may possibly be responsible for modifying the LDL. As ascorbate would oxidize steadily to dehydroascorbate, which would itself decompose into its degradation products producing free radicals, the question arises as to why ascorbate does not itself increase the rate of formation of conjugated dienes in LDL. The reason may be that the rate of production of free radicals during ascorbate decomposition would be much less than that produced when a bolus of dehydroascorbate itself is added, being limited by the rate of conversion of ascorbate into dehydroascorbate. In addition, some ascorbate may still be present when the dehydroascorbate is decomposing into its degradation products and this ascorbate may scavenge some of the free radicals generated.

Retsky et al. [15] found that ascorbic acid was only protective after its oxidation to dehydroascorbic acid. Preincubation of LDL with dehydroascorbate protected it against subsequent oxidation by Cu^{2+} [15]. They suggested that dehydroascorbate or its degradation products may covalently modify apo B-100 so that copper cannot bind to it and catalyse LDL oxidation. The reason for the apparent difference between our results and those of Retsky et al. is unknown, but may relate to the levels of lipid hydroperoxides in the LDL at the start of the experiments, especially as in some of our experiments dehydroascorbate inhibited the oxidation of 'fresh' LDL, but greatly increased it if added shortly after the incubation with copper was started.

Retsky et al. also showed that the oxidation of ascorbic acid is dependent on metal ions, but that the decomposition of dehydroascorbate is not. We have shown that LDL modification by dehydroascorbate is dependent on the presence of iron or copper ions. This therefore suggests that a breakdown product of dehydroascorbate, whose decomposition is dependent on copper or iron, is responsible for the oxidation of the LDL rather than dehydroascorbate itself, or that copper or iron are needed to propagate the lipid peroxidation or lipid hydroperoxide decomposition in LDL.

Whilst this manuscript was in preparation, Retsky and Frei published a paper [16] showing that ascorbate and dehydroascorbate were no longer protective against LDL oxidation if the LDL had been preincubated with copper to induce its partial oxidation. Indeed, ascorbate stimulated the oxidation of LDL pre-oxidized by copper for more than 3 h. When ascorbate was added to the LDL during the propagation phase of its oxidation by copper, there was an immediate decrease in lipid hydroperoxides, although these were not transformed into lipid

hydroxides as they expected. As discussed above, we suggest that these lipid hydroperoxides may break down in the presence of ascorbate into aldehydes. Retsky and Frei [16] found no decrease in lipid hydroperoxides when dehydroascorbate was added during the propagation phase. In fact, their data appear to show an increase in the rate of lipid hydroperoxide formation after the addition of dehydroascorbate, which agrees with our results showing that the rate of conjugated-diene formation was increased upon addition of dehydroascorbate during the propagation phase.

LDL accumulates in regions of arteries prone to lesion development and is retained in these regions for longer than in lesion-resistant areas [44]. Cholesterol feeding increases the residence time of LDL in lesion-prone areas [44]. If a small amount of oxidation of LDL were to occur during this time, as occurs during storage in a refrigerator over a period of months [45], then ascorbate could possibly promote further modification of LDL in the artery wall.

Epidemiological studies have shown an inverse relationship between the plasma levels of ascorbate in different European populations and the occurrence of coronary heart disease [46,47] and stroke [46]. Death from cardiovascular disease has been reported to be inversely related to ascorbate intake, although more strongly in males than in females [48]. However, not all epidemiological studies suggest that ascorbate may be protective against cardiovascular disease. Two studies on antioxidant vitamin intake [49,50] showed that ascorbate had no apparent effect. It would be interesting to look at the epidemiology of plasma dehydroascorbate levels (if they could be measured accurately) and cardiovascular disease.

In summary, both ascorbate and dehydroascorbate can stimulate the oxidation of moderately oxidized LDL, and dehydroascorbate can also increase the oxidation of 'fresh' LDL. Taken together with other studies, our results suggest that both ascorbate and dehydroascorbate have the *potential* to increase or decrease the modification of LDL in atherosclerotic lesions, although we suspect that ascorbate supplementation may if anything protect against LDL oxidation in atherosclerotic lesions. Further studies will be needed to determine if this is true.

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