Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions

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Lipid peroxidation within human arterial lesions is thought to play an important role in the development of atherosclerosis. Peroxidation can be accelerated by the presence of 'catalytic' iron or copper ions. Gruel samples from advanced atherosclerotic lesions in the abdominal aortae of human cadavers were tested for pro-oxidant properties. All samples contained bleomycin-detectable iron and phenanthroline-detectable copper. Almost all gruel samples stimulated peroxidation of rat liver microsomes, and this was usually inhibited by the iron-ion chelator desferrioxamine. Some samples stimulated formation of hydroxyl radicals from H_2O_2 in the presence of ascorbate, a reaction again inhibited by desferrioxamine. We conclude that the interior of human advanced atherosclerotic lesions is a highly pro-oxidant environment, and that the use of copper or iron ions to promote peroxidation of low-density lipoproteins *in vitro* may be a valid model for events in the arterial wall.

INTRODUCTION

Accumulation of lipid-laden foam cells in the subendothelial region of arterial walls is an early event in the development of atherosclerosis [1,2]. Many foam cells arise from macrophages, probably by the scavenger receptor-mediated uptake of low-density lipoproteins (LDLs) that have undergone modification [1]. One likely physiologically important modifying event is the free-radical chain reaction of lipid peroxidation [1,3]. It is now widely thought that peroxidation of LDL lipids and covalent modification of apoprotein B are important events in atherogenesis [1,3], especially as chain-breaking antioxidant inhibitors of lipid peroxidation appear to have an anti-atherogenic action *in vivo* [1,4–8].

However, the mechanism by which LDL peroxidation occurs in the arterial wall is uncertain. Monocyte/macrophages, smooth-muscle cells and endothelial cells have all been shown to cause LDL peroxidation *in vitro*, but there is some controversy over the mechanisms involved. Lipoxxygenase activity may be important [1,9], but peroxidation could also be related to production of superoxide anion (O_2^-) and H_2O_2 by these cells (discussed in [1,9–11]). However, neither O_2^- nor H_2O_2 cause lipid peroxidation unless ions of such transition metals as iron or copper are present, to catalyse their conversion into more-reactive radical species such as hydroxyl ($\text{OH}^\bullet$) (reviewed in [12]). Indeed, transition-metal ions are probably required for all these cell-dependent oxidations of LDL [13,14], and copper ions are frequently used *in vitro* to obtain peroxidized LDL recognizable by the macrophage scavenger receptors [3,15–19].

Iron and copper ions capable of catalysing free-radical reactions such as lipid peroxidation do not exist in plasma from healthy human subjects [20–23]: these metals are safely bound to such proteins as transferrin and ferritin (iron) or caeruloplasmin and albumin (copper) [12,23]. If the studies described above [13–19] are indeed relevant to the situation *in vivo*, then 'catalytic' iron and copper ions should exist within atherosclerotic lesions. We therefore attempted to demonstrate this directly.

MATERIALS AND METHODS

Extracts from advanced atherosclerotic lesions in the abdominal aortae of cadavers were obtained by washing in water to remove blood, then incising lesions with a scalpel and scraping out semi-solid yellow gruel with the blunt side of a scalpel blade. Pooled gruel from several lesions in the same individuals was divided into portions and frozen immediately at -70°C . Storage at -70°C did not exceed 1 month, a period which does not itself cause oxidative deterioration of lipids ([27]; K. Carpenter & M. J. Mitchinson, unpublished work). Some samples were added to tubes containing butylated hydroxytoluene (final concn. $100\ \mu\text{M}$) to inhibit peroxidation during handling and storage.

Reagents

All reagents were of the highest quality available from Sigma Chemical Corp., except for desferrioxamine (a gift from Ciba-Geigy). The bleomycin assay for catalytic iron ions was performed by a modification of the method as described in [20]. Assay mixtures contained, in a final volume of 1 ml, the following reagents at the final concentrations stated and were incubated at 37°C for 1 h: DNA (0.2 mg/ml), bleomycin sulphate (0.05 mg/ml), MgCl_2 (5 mM), $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer, pH 7.4 (30 mM), ascorbate ($200\ \mu\text{M}$) and gruel sample or FeCl_3 standards. Reagents and buffers were freed of iron contamination by Chelex treatment and the pH re-adjusted to 7.4. Non-caeruloplasmin copper was measured essentially by the method of Gutteridge [21], except that absorbance at 532 nm was used to quantify the chromogen.

Rat liver microsomes were obtained by differential centrifugation [28], except that the final pellet was washed twice with 20 mM- $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer, pH 7.4, and the pellet resuspended in this buffer. Deoxyribose degradation to thiobarbituric acid (TBA)-reactive material was measured essentially as described in [29]. Reaction mixtures contained, in a final volume of 1 ml, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer, pH 7.4 (10 mM), H_2O_2 (2.8 mM),

ascorbate (100 μM), gruel or FeCl_3 standard, and were incubated at 37 °C for 1 h.

Atomic absorption

Known masses of gruel were placed into dry clean plastic tubes and 0.5 ml of Chelex-treated water was added to each. The samples were vortex-mixed vigorously to form a suspension and placed on ice until required. Each sample was diluted 1:10 with water before use. A Perkin-Elmer 2380 spectrometer with autosampling system AS-1 and HGA-500 programmer at 2700 °C was calibrated with iron standards of 0.05, 0.1, 0.3 and 0.5 $\mu\text{g}/\text{ml}$, and the samples were measured at 248.3 nm. Peak heights were measured and concentrations determined from standard curves. Repeated analysis of the same gruel samples gave results that varied by $\leq 5.5\%$, and iron added to gruel samples (as FeCl_3) was quantitatively recovered.

RESULTS

Gruel from atherosclerotic lesions in the abdominal aortae of cadavers was obtained as described in the Materials and methods section. Because of limitations in sample size (some samples obtained were only 0.1 ml), every assay could not be performed on each sample. The time between death and sample collection was noted in every case; it varied from 8 h to 125 h. Statistical analysis showed no correlation between the results obtained and the time of sampling, the age of the subject or the sex of the subject.

Table 1. Presence of 'catalytic' metal ions in gruel from human atherosclerotic lesions

Results are corrected to a gruel mass of 5 mg, and absorbance values of blank determinations were subtracted. Standard curves of FeCl_3 (bleomycin assay) or CuSO_4 (phenanthroline assay) were used to calculate concentrations in μM . Insufficient material was available for each assay to be carried out on every sample.

Sample code	Bleomycin-detectable iron		Total iron (μM)	Phenanthroline-detectable copper (μM)
	(μM)	(% of total iron)		
P90574	2.3	26	9.0	0.2
P90584	7.2	39	18.4	0.4
P91006	0.4	5	7.3	—
P90727	1.1	10	11.3	—
P91004	1.2	2	56.3	2.4
P91027	1.1	8	13.9	—
P91030	0.3	2	16.5	—
P91031	0.7	4	16.4	7.0
P91035	0.1	2	4.3	1.3
P91038	0.7	5	13.2	—
P91037	0.6	14	4.3	0.5
P91045	1.7	6	26.8	16.5
P91041	1.3	10	12.8	1.2
P91242	1.9	28	6.7	0.8
P91244	0.3	10	2.9	0.3
P91076	0.2	0.5	44.0	0.3
P91075	0.6	2	32.9	0.3
P91058	0.9	13	6.8	16.7
P91060	0.2	7	3.0	2.4
P91053	0.1	1	7.5	0.6
P91366	1.1	—	—	0.21
P91369	0.7	—	—	28.6
P91490	1.2	—	—	0.2
P91491	2.6	—	—	0.3
P91606	0.03	—	—	0.2

Presence of catalytic iron

The bleomycin assay [20] was used to measure iron in the gruel. Duplicate assays upon the same gruel sample gave results varying by $\leq 15\%$. The bleomycin assay does not measure 'non-catalytic' iron attached to ferritin, transferrin or other proteins [20,25,30,31], but does measure low-molecular-mass iron complexes. Hence it is thought to be an assay that measures pro-oxidant forms of iron, i.e. iron capable of stimulating free-radical reactions [discussed in 20, 30, 31]. Results were expressed as μM bleomycin-detectable iron present in the assay solution when a fixed mass of gruel was added, using a standard curve with freshly prepared FeCl_3 solution in each experiment. Washing the scalpel blades (used to incise and scrape lesions) with distilled water did not cause bleomycin-detectable iron to appear in the water, so that contamination from this source could be ruled out. A similar control was performed for the experiments aiming to demonstrate the presence of catalytic copper (see below).

Table 1 shows that all the gruel samples analysed contained bleomycin-detectable iron ranging in concentration from 0.03 to 7.2 μM . The total iron content of some gruel samples was measured by atomic absorption: bleomycin-detectable iron usually represented only a small percentage of the total iron present (Table 1). Six of the gruel samples were divided into two immediately on collection; one portion was collected into butylated hydroxytoluene, the other not. No difference in levels of bleomycin-detectable iron was observed. Addition of FeCl_3 to gruel samples, followed by re-analysis, showed detection of $94 \pm 2\%$ of the added iron.

Presence of 'catalytic' copper

The phenanthroline assay [21] was used to measure non-caeruloplasmin copper ions. Duplicate assays on the same gruel samples showed results reproducible within 10% or less. Again, phenanthroline-detectable copper was present in all the gruel samples examined, at concentrations ranging from 0.2 to 28.6 μM (Table 1). Because of small sample size, not all assays could be performed on each sample. Collection of samples into butylated hydroxytoluene did not affect the results obtained. Addition of CuSO_4 to gruel samples, followed by reanalysis, showed detection of $99 \pm 7\%$ of the added copper.

Stimulation of lipid peroxidation by gruel samples

If the bleomycin-detectable iron and phenanthroline-detectable copper gruel are indeed capable of stimulating free-radical reactions, then the gruel might itself be able to stimulate such reactions. In order to investigate this, rat liver microsomes were used. In the presence of ascorbic acid, microsomes undergo lipid peroxidation, measurable by the TBA test, if iron ions are added [32]. Butylated hydroxytoluene was added with the TBA reagents to block peroxidation during the test itself and eliminate any possible artifacts due to stimulating effect of gruel components on the TBA test. Controls were performed without microsomes to correct for any TBA-reactive material present in the gruel itself or generated during its incubation [33]. Of 21 gruel samples tested, all but one stimulated microsomal lipid peroxidation (Table 2). Linear-regression analysis of data from gruel samples for which several assay results were available showed no significant correlation of the stimulation of peroxidation with the concentrations of bleomycin-detectable iron ($r = -0.09$, $n = 17$), phenanthroline-detectable copper ($r = -0.20$, $n = 14$) or the sum of both metals ($r = -0.21$, $n = 15$).

However, desferrioxamine, a powerful inhibitor of iron-ion-dependent lipid peroxidation [32,34], exerted variable protective

Table 2. Stimulation of microsomal lipid peroxidation by gruel from human atherosclerotic lesions

Gruel (0.005 g) was added to rat liver microsomes in the presence of ascorbate at pH 7.4 and incubated at 37 °C for 20 min as described in the Materials and methods section. Results are corrected for the low 'blank' values ($\leq 10\%$ of usual peroxidation rates) of microsomal lipid peroxidation observed in the absence of gruel samples, and for controls without microsomes ($12 \pm 5\%$ of the peroxidation rates observed). Peroxidation is expressed as A_{532} .

Sample code	Amount of peroxidation (A_{532})	Sample code	Amount of peroxidation (A_{532})
P90584	0.014	P90006	0.020
P90727	0.021	P91004	0.021
P91031	0.026	P91030	0.032
P91366	0.019	P91035	0.003
P91038	0.017	P91037	0.004
P91045	0.010	P91041	0.006
P91036	0.020	P91242	0.078
P91244	0.025	P91076	0.064
P91075	0.097	P91058	0.062
P91060	0.033	P91053	0.010
		P91369	0

Table 3. Effect of the iron-ion chelator desferrioxamine on microsomal lipid peroxidation stimulated by gruel samples

Sample code	Inhibition of observed peroxidation (as A_{532}) by desferrioxamine (%)	Sample code	Inhibition (%)
No gruel, 100 μ M-FeCl ₃ added	94 $\pm$ 5 (n = 20)	P91038	73
		P91037	82
		P91041	0
P90584	7	P91036	63
P91006	67	P91244	0
P90727	56	P91076	0
P91004	7	P91058	78
P96035	6	P91060	61
		P91366	100

effects (Table 3), suggesting that some or all of the stimulation was indeed due to iron ions. Perhaps the failure to inhibit in some cases means that peroxidation is due to other metal ions, such as copper. Insufficient sample was available to test the action of desferrioxamine in all cases.

Stimulation of hydroxyl-radical generation by gruel samples

A mixture of ascorbic acid, H₂O₂ and iron (or copper) ions generates OH[•], which can be detected by their ability to degrade the sugar deoxyribose into fragments that, on heating with TBA at low pH, generate a pink chromogen [29]. The deoxyribose assay was therefore used to examine the ability of gruel samples to catalyze OH[•] formation from ascorbate and H₂O₂ at pH 7.4. Table 4 summarizes the results obtained. Of 24 samples tested, 16 were positive. Linear-regression analysis showed a weak correlation of the extent of deoxyribose degradation with bleomycin-detectable iron in the 21 samples for which both sets of data were available ($r = 0.48$): this correlation was not improved by omitting from the analysis the eight samples in which no deoxyribose degradation was observed ($r = 0.50$, $n = 13$). There

Table 4. Catalysis of OH[•] generation by gruel from human atherosclerotic lesions

OH[•] was measured by the deoxyribose method [29] as described in the Materials and methods section. Results have been corrected for the low apparent rate of OH[•] generation observed in reaction mixtures containing ascorbate, H₂O₂, gruel and buffer alone at pH 7.4 ($< 15\%$ of the values given above). This control corrects for any TBA-reactive material in the sample already or generated during its incubation.

Sample code	Deoxyribose degradation (A_{532})	Sample code	Deoxyribose degradation (A_{532})
P90584	0.136	P91242	0.151
P91006	None	P91244	0.028
P90727	0.030	P91076	0.005
P91004	None	P91075	None
P91027	0.013	P91058	0.077
P91030	None	P91060	0.046
P91031	None	P91053	0.058
P91035	0.039	P91366	None
P91038	0.059	P91490	0.198
P91037	0.054	P91554	0.300
P91041	None	P91577	0.026
P91036	0.036	P91606	None

was no correlation between deoxyribose degradation and phenanthroline-detectable copper ($r \sim 0$, $n = 16$).

Formation of TBA-reactive material from deoxyribose was inhibited by including the OH[•] scavenger mannitol (final concn. 50 mM) in the reaction mixtures, confirming that OH[•] was involved [29]. Desferrioxamine also inhibited. Table 5 summarizes the data obtained. Omission of deoxyribose from the reaction mixtures inhibited chromogen development by 85–95%, i.e. the gruel samples (in the amounts used in the assay) did not themselves contain or generate significant amounts of TBA-reactive material during the deoxyribose assay. This is an essential control in the deoxyribose assay [35].

DISCUSSION

Our results show that gruel from advanced human atherosclerotic lesions contains iron and copper ions detectable in the bleomycin and phenanthroline assays. These assays are thought to measure iron and copper in 'catalytic' forms that can accelerate free-radical reactions [12,20–23,30,31]. Hence iron and copper

Table 5. Action of desferrioxamine and mannitol on deoxyribose degradation by gruel from human atherosclerotic lesions

Experiments were performed as described in the legend to Table 4. Where indicated, mannitol or desferrioxamine were added to give the final concentrations stated. Because of lack of sample, not all gruel samples could be tested for effects of mannitol and desferrioxamine.

Sample code	Deoxyribose degradation		
	Control	Plus mannitol (50 mM)	Plus desferrioxamine (100 μ M)
P90727	0.030	–	0.018
P91490	0.198	0.071	0.021
P91554	0.300	0	0
P91577	0.026	0	0

are certainly more available in the gruel than they are in human plasma [22,23]. Consistent with the presence of 'catalytic' metal ions, gruel samples were shown to be capable of stimulating lipid peroxidation (Table 2) and, in some cases, OH[•] generation (Table 4). The OH[•] generation was always inhibited by the iron-chelator desferrioxamine, and the lipid peroxidation often was. However, whereas all but one of the gruel samples showed the ability to accelerate lipid peroxidation, the ability to catalyse OH[•] production was more restricted. In addition, acceleration of lipid peroxidation was not correlated with metal-ion concentrations, and the extent of deoxyribose degradation was weakly correlated with bleomycin-detectable iron only. This general lack of correlation may be because the gruel samples contain not only pro-oxidants, but also a variable range of antioxidants. The metal ions may not only contribute to formation of species capable of initiating lipid peroxidation, but they could also act by decomposing traces of peroxides in the microsomes or in the gruel samples to form chain-propagating peroxy and alkoxyl radicals [12]. It is also the case that several low-molecular-mass iron complexes reactive in the bleomycin assay are able to stimulate lipid peroxidation, but not OH[•] generation [12,30,31].

Our data may explain a previous report that extracts of atherosclerotic lesions can modify LDL in such a way that it is taken up at enhanced rates by macrophages [36]. In addition, our data validate the use of copper ions as a means of preparing oxidized LDL *in vitro* [3,15–19] by showing that copper-dependent reactions are feasible *in vivo*. Indeed, Salonen *et al.* [37] have shown a positive correlation between plasma copper concentration and development of atherosclerosis in Finnish men. Most serum copper is bound to the protein caeruloplasmin, which has antioxidant properties [38], but this protein can easily break down to yield 'catalytic' copper [27], a process that could conceivably happen in atherosclerotic lesions. Attack of reactive oxygen species upon haem proteins [25,26] and/or upon ferritin [24] could give rise to the bleomycin-detectable iron measured. In addition, cell death, which occurs in many advanced lesions, could release catalytic transition-metal ions from intracellular storage sites [12,39,40].

These are two obvious potential objections to our studies. One is the use of material from human cadaveric lesions. It could be argued that, after death, rapid cell dissolution takes place in the lesions, with the release of catalytic metal ions. We attempted to avoid this problem by taking material as quickly after death as possible (8 h in some cases). However, it has been shown that the histological appearance of human atherosclerotic lesions is similar whether the lesions examined are taken from fresh human material (e.g. organs removed during transplant surgery) or from cadavers several days old (M. J. Mitchinson, unpublished work). When lesions were removed from cadavers more than 24 h old, we obtained no increase in any of the parameters measured (results not shown). There was no correlation whatsoever between any of the parameters measured and the interval between death and necropsy. A second objection is that we have not included a 'control' to distinguish between atherosclerotic lesions and 'normal' arterial wall. In fact, this is not possible because there is no equivalent of 'gruel' that can be sampled from the normal arterial wall. Our data show that the atherosclerotic lesion is a potentially pro-oxidant environment, but we cannot prove that the normal arterial wall is not itself also such an environment, although this seems unlikely.

In conclusion our data strongly support the concept that the interior of advanced human atherosclerotic lesions is a pro-oxidant environment. Our data support the proposal that free-radical damage to lipids [1], and perhaps to other biomolecules such as proteins and DNA [41], is important in atherogenesis. Of course, much earlier lesions will need to be studied to examine

the contribution of oxidative damage to the initial stages of lesion formation.

M.J.M. and B.H. thank the British Heart Foundation for research support. B.H. also thanks the Ministry of Agriculture, Fisheries and Food, and Nestlé Ltd., for support. C.S. is grateful to the Science and Engineering Research Council for a Research Studentship.

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Received 11 November 1991/27 February 1992; accepted 25 March 1992