Prooxidant iron and copper, with ferroxidase and xanthine oxidase activities in human atherosclerotic material

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Abstract Low density lipoproteins are highly sensitive to oxidation by copper salts, and such peroxidation is accompanied by macrophage scavenger receptor recognition. This study shows that fresh human atherosclerotic material (aneurysms and endarterectomies) can contain detectable amounts of redox active iron and copper that is chelatable from tissue homogenates. Such material is often prooxidant towards lipid peroxidation and deoxyribose degradation. Aneurysms and endarterectomies contain ferroxidase 1 activities, whereas only in aneurysms could caeruloplasmin be immunologically detected. Ferroxidase 2 activity, characteristic of a copper-oxidised lipoprotein complex, could not, however, be detected in any of the atherosclerotic samples. A third ferroxidase activity, attributable to xanthine oxidase, was present in several aneurysms and endarterectomies.

Key words: Atherosclerosis; Lipid peroxidation; Copper salts; Ferroxidase; Xanthine oxidase; Prooxidants

1. Introduction

Peroxidation of polyunsaturated fatty acids associated with low density lipoprotein (LDL) particles is thought to occur in the artery wall, and lead to the formation of a modified LDL recognised by the macrophage scavenger receptor [1,2]. Under such circumstances modified LDL uptake is not down-regulated and lipid-laden foam cells are often associated with atherosclerotic lesions [3]. The prooxidants that trigger lipid peroxidation of LDL in vivo are the subject of considerable current interest.

Copper salts have been shown to be remarkably effective at stimulating LDL oxidation [4]. However, copper salts are tightly sequested in biological systems and are not normally available to catalyse lipid peroxidation. Recently, several independent lines of research suggest that prooxidant forms of copper may be present in the circulation and artery walls to stimulate lipid peroxidation [5,6]. Most, if not all of the copper present in human plasma is associated with the protein caeruloplasmin, which has 6 tightly held copper atoms and a seventh that is easily dissociated (reviewed in [7]). This tightly held copper can be released in the presence of peroxynitrite [8] and structural modification of the protein changes its copper-dependent prooxidant/antioxidant activity [6].

An important observation by Smith et al. [5] showed that chelatable forms of iron and copper are present in gruel samples removed from atherosclerotic lesion at post-mortem. This together with the proposal that native caeruloplasmin is prooxidant in the presence of copper salts [6], prompted the present study.

2. Materials and methods

2.1. Clinical material and preparation of samples

Atheromatous material removed in the theatre was transported on ice to the laboratory, where the fresh material was immediately analyzed for reactive metals Remaining aliquots were stored in liquid nitrogen. Contaminating blood was removed, and samples were washed in ice-cold 0.15 M NaCl. Lesions were incised with a scalpel and fatty semi-solid gruel removed and homogenised in 2 ml of ice-cold 0.15 M NaCl, prepared in metal ion-free distilled water. Aneurysms were obtained from abdominal aortic, carotid and femoral arteries, and endarterectomies from coronary, carotid and femoral arteries.

Approximately 10 mg samples of endarterectomies and 100 mg of aneurysms were present per ml of homogenate. The ratio of cholesterol-to-protein present in endarterectomies was 0.233 ± 0.065 and that in aneurysms 0.027 ± 0.010, confirming that endarterectomies had a greater proportion of cholesterol.

2.2. Chelatable redox active copper

Copper that can be chelated by 1,10-phenanthroline is complexed to DNA, and redox cycled with mercaptoethanol to release thiobarbituric acid (TBA)-reactive material from DNA [9]. TBA reactive material released is quantitatively related to copper content by the use of a pure standard of cupric chloride.

2.3. Chelatable redox active iron

Iron is chelated from biological material by bleomycin which binds to DNA. The bleomycin–iron complex is redox cycled with ascorbate to release TBA reactive material from DNA [10]. TBA reactive material released from DNA is quantitatively related to iron content by the use of ferric chloride standards.

2.4. Caeruloplasmin assay

Radial immunodiffusion plates (Behring-Hoechst, Hounslow, Middx.) for low concentrations of caeruloplasmin (LC-plates) were used as detailed by the manufacturers, with reference to pure standards of caeruloplasmin (Behring-Hoechst).

2.5. Ferroxidase 1 activity

Caeruloplasmin catalyses the oxidation of ferrous ions to the ferric state, at pH 6.5, which bind to apotransferrin to produce a pink complex (A460nm). The kinetics of this reaction form an assay for ferroxidase activity [11]. In our assay apo-ovotransferrin was substituted for apotransferrin. The specific contribution by caeruloplasmin (ferroxidase 1) was determined by inhibiting it in the presence of 1 mM azide.

2.6. Ferroxidase 2 activity

Total ferroxidase activity was measured as described above and repeated in the presence of 1 mM azide. Ferroxidase 2 is an oxidised lipid–copper–protein complex which has enzyme-like activity which is not inhibited by azide.

2.7. Ferroxidase 3 activity

Xanthine oxidase has a ferroxidase activity [12] (ferroxidase 3) which can be inhibited by allopurinol. Assays were performed as described above. All ferroxidase activities were expressed as International Units.

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One IU of ferroxidase transforms 1 μmol of ferrous ions to the ferric state under defined conditions.

2.8. Xanthine oxidase

Xanthine oxidase activity was determined at pH 7.4 by the formation of uric acid (A293m) from hypoxanthine. One unit of xanthine oxidase converts 1 μmol of xanthine to uric acid per minute.

2.9. Lipid peroxidation and deoxyribose degradation

Linolenic acid micelles [13], and deoxyribose [14] were used as substrates for organic and inorganic oxygen radical damage respectively. 50 μl of linolenic acid (10 mg/ml) was incubated in 10 mM sodium phosphate buffer, pH 7.4 (840 μl), with 100 μl ascorbic acid (1 mM). 10 μl of homogenate was added to assess stimulation, or inhibition, of the reaction. The mixture was incubated at 37°C for 20 min and the amount of TBA-reactive material released from linolenic acid measured as fluorescence at 532 nm. A similar procedure was adopted for deoxyribose, where 10 mM deoxyribose was used instead of linolenic acid.

2.10. Other techniques

Thiobarbituric acid-reactive substances in atherosclerotic material were determined by heating homogenised samples with 28% (w/v) trichloroacetic acid and 1% (w/v) thiobarbituric acid for 15 min at 100°C. The resulting chromogen was extracted into butan-l-ol and its fluorescence measured at 532 nm. Total proteins were determined by the Lowry method.

3. Results

Freshly taken human atherosclerotic material removed at surgery and analysed in the intensive care laboratory within the shortest possible time often showed the presence of pro-oxidant forms of iron and copper that could be chelated from solution. Bleomycin-chelatable iron was detected in 7 of 13 endarterectomies (ETMS) and in 5 of 10 aneurysms (ARMS) whereas phenanthroline-chelatable copper was present in 12 ETMS and 6 ARMS (Table 1). Homogenates of different atherosclerotic material could both stimulate and inhibit model systems generating peroxyl radicals (linolenic acid micelles), and hydroxyl radicals (deoxyribose degradation). ETMS stimulated lipid peroxidation by 7% whereas ARMS stimulated by 4%. Mean values in the deoxyribose assay were 41% inhibition for ETMS and 1% stimulation for ARMS. Appropriate controls for endogenous TBA-reactive material were included.

Ferroxidase activity inhibitable by azide, and therefore attributable to caeruloplasmin, was present in both ETMS and ARMS, however, only in ARMS could the protein be recognised by an antibody assay (Table 2). Although TBA-reactive material was detectable in both ETMS and ARMS, we found no evidence for oxidised lipid–copper–protein complexes characteristic of material with ferroxidase 2 activity. Since most atherosclerotic samples had a higher total ferroxidase activity than could be accounted for by ferroxidase 1, plus 2, we assayed samples for ferroxidase activity dependent upon xanthine oxidase; here referred to as ferroxidase 3. Both ETMS and ARMS samples showed the presence of low and variable ferroxidase 3 activities inhibitable by allopurinol. The presence of xanthine oxidase activity was confirmed by measuring uric acid formation in the presence of hypoxanthine (Table 2). Four ETMS samples out of 8 showed ferroxidase 3 activity whereas 6 out of 8 ARMS samples showed the same activity (Table 2).

Table 1
Chelatable iron and copper, and prooxidant/antioxidant activities of atherosclerotic material

	Endarterectomies (n = 13) ± S.E.M.	Aneurysms (n = 10) ± S.E.M.
Phenanthroline-chelatable copper (nmol/mg protein)	0.3 ± 0.1	0.3 ± 0.2
Bleomycin-chelatable iron (nmol/mg protein)	0.7 ± 0.3	0.4 ± 0.2
Effect on linolenic acid micelles (% stimulation [S] or inhibition [I])	42% I–109% S	34% I–113% S
Effect on deoxyribose degradation (I or S)	89% I–40% S	95% I–181% S

Table 2
Ferroxidase activities of atherosclerotic material

	Endarterectomies (n = 13) ± S.E.M.	Aneurysms (n = 10) ± S.E.M.
Ferroxidase 1 (caeruloplasmin, azide inhibitable) (IU/mg protein)	7.0 ± 2.8	6.3 ± 1.4
Caeruloplasmin (μmol/mg protein)	0	14.3 ± 3.4
Ferroxidase 2 (oxidised lipid/copper complex not inhibited by azide) (IU/mg protein)	0	0
TBA-reactivity (nmol MDA/mg protein)	0.4 ± 0.2	0.2 ± 0.1
Ferroxidase 3 (xanthine oxidase inhibited by allopurinol) (IU/mg protein)	2.4 ± 1.7 (n = 8)	0.5 ± 0.2 (n = 8)
Xanthine oxidase activity (U/mg protein)	0.6 ± 0.5 (n = 8)	0.3 ± 0.1 (n = 8)

4. Discussion

The current hypothesis that lipid peroxidation is a key event in the development of atherosclerosis [15] requires that prooxidant factors exceed antioxidant defenses in order to promote molecular damage. Studies with isolated LDL preparations have uniformly shown that copper salts are devastatingly effective at promoting LDL modification for recognition by the scavenger receptor of macrophages. However, simple copper salts do not exist in vivo because the metal is readily ligated to ubiquitous amino residues, and chelatable copper only appears in separated human plasma when it is stored or mishandled [16], due to proteolytic damage to caeruloplasmin. Caeruloplasmin is regarded as an important plasma antioxidant towards iron-driven free radical reactions [17,18] since it has a ferroxidase activity [19]. This activity can be inhibited by azide.

Copper fragments released from degraded caeruloplasmin are known to cause peroxidation of lipoproteins, and the resulting oxidised lipid–copper–protein complex has a ferroxidase-like activity similar to that of caeruloplasmin [20], but which is not inhibited by azide. Intact native caeruloplasmin has recently been ascribed a prooxidant property towards copper-stimulated LDL oxidation [6], which changes to an antioxidant activity when the protein is partly degraded [6]. The reported presence of chelatable redox active copper in gruel samples taken at post mortem [5] and its possible synergism with native caeruloplasmin in promoting LDL oxidation prompted us to

look for both components in fresh human atherosclerotic samples removed at surgery.

Endarterectomies and aneurysms contained similar amounts of chelatable copper and ferroxidase 1 activity. However, caeruloplasmin could only be detected by radial immunodiffusion in aneurysms. This in part may reflect the poor sensitivity of an assay system developed for use with plasma, as well as the difference in origin and structure of endarterectomies and aneurysms. Aneurysms will contain substantial amounts of artery wall structure, whereas endarterectomies are more comparable to the post-mortem samples of gruel previously reported [5]. Surprisingly, no ferroxidase 2 activity characteristic of copper-oxidised lipoproteins was detectable by our assay technique in either materials. Since the total ferroxidase activity often exceeded that which could be accounted for by ferroxidase 1 and 2 activities, we assumed other molecules were contributing ferroxidase activities. Xanthine oxidase has been reported to have a significant ferroxidase activity [12] and both ferroxidase and xanthine oxidase activities inhibitable by allopurinol were present in several atherosclerotic samples.

We conclude that chelatable forms of copper and iron are present in freshly taken atherosclerotic material, and that other pro-oxidants such as xanthine oxidase and possibly caeruloplasmin (when reactive copper is present) may also contribute to the development and amplification of oxidative processes in atherosclerotic lesions. There were, however, no statistically significant correlations between chelatable metals and the stimulation of lipid peroxidation, suggesting that other pro-oxidants are also involved.

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