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METAL ION RELEASE FROM MECHANICALLY-DISRUPTED HUMAN ARTERIAL WALL. IMPLICATIONS FOR THE DEVELOPMENT OF ATHEROSCLEROSIS

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Oxidation of low density lipoproteins (LDL) in blood vessel walls plays a significant role in the development of atherosclerosis. LDL oxidation *in vitro* is greatly accelerated by the presence of "catalytic" iron or copper ions, which have already been shown to be present within advanced atherosclerotic lesions. We demonstrate here that mechanical damage to human arterial wall samples (both normal and early or intermediate atherosclerotic lesions) causes release of "catalytic" iron and copper ions, to an extent increasing with the damage. It may be that traumatic (e.g. during angioplasty) or other injury to the vessel wall contributes to the generation of metal ions that can facilitate LDL oxidation and other free radical reactions, so promoting atherosclerosis.

INTRODUCTION

Accumulation of lipid-laden foam cells in the sub-endothelial 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 lipid peroxidation and subsequent modification of apoprotein B.[2,3]

The mechanism by which LDL peroxidation is initiated in the arterial wall is uncertain. Monocyte/macrophages, lymphocytes, smooth-muscle cells and endothelial cells can all induce LDL peroxidation *in vitro*, but there is controversy over the mechanisms involved. Lipoxygenase activity may be important,[1,4] but peroxidation may also involve production of superoxide radical ($O_2^{\cdot-}$) and H_2O_2 by these cells and/or the release of thiol compounds.[1,3,5–7] However, neither $O_2^{\cdot-}$ nor thiols can cause lipid peroxidation unless ions of such transition metals as iron or copper are present, to catalyse formation of more-reactive radical species.[8] Indeed, transition metal ions are probably required for all these cell-dependent oxidations of LDL,[1,3,5–7] and copper or iron ions are frequently used *in vitro* to obtain peroxidized LDL recognizable by the macrophage scavenger receptors.[1,3,5–9]

Iron and copper ions capable of catalysing free-radical reactions such as lipid peroxidation cannot be measured in plasma from healthy human subjects[10–12]: these metals are bound to such proteins as transferrin and ferritin (iron) or caeruloplasmin and albumin (copper). These bound metal ions do not, in general, accelerate lipid

peroxidation.[13,14] However, Smith *et al*[15] found that the contents of advanced atherosclerotic lesions (sometimes called "gruel") showed measurable levels of iron and copper ions when added to appropriate reaction mixtures and allowed to disperse. Such lesion contents were also demonstrated to accelerate microsomal lipid peroxidation *in vitro*[15] and to promote LDL oxidation.[16,17] Thus metal ion-dependent LDL oxidation is feasible within advanced lesions.[15]

However, these data are open to criticism because they were not accompanied by "controls" with normal arterial wall. This is difficult because there is no equivalent of "gruel" that can be sampled from the normal arterial wall, i.e. we cannot assay a solid lump of tissue. We wondered, however, to what extent injury to normal arterial wall could cause metal ion release. In the present paper, we show that mechanical damage to normal arterial wall and to histologically-defined lesions causes metal ion release and we discuss the relevance of these observations to the development of atherosclerosis.

MATERIALS AND METHODS

Tissue samples

Arterial samples were taken from cadavers at Addenbrooke's Hospital, Cambridge, UK, at time intervals after death ranging from 10.8 to 138 h (this time did not affect the results). The selected piece of aortic wall containing a lesion was cut out as an oblong; by inserting a scalpel blade and then stripping with forceps, the adventitia and outer media were stripped away from the whole specimen in the same plane of cleavage. The remaining piece of intima and inner media was divided into two by cutting with a scalpel, the cut dividing the lesion in two. Half the piece was immediately fixed in 10% formol saline for histology. The other half, destined for chemical examination, was treated differently; all the adjacent normal intima was trimmed away with a scalpel to attempt to ensure that the specimen consisted almost entirely of lesion material. It was then stored as soon as possible (always within 2 hours) under N_2 at −20°C until analysis (within 3 months). Normal aortic specimens were treated identically. The fact that the stripping away of outer media and adventitia preceded the sub-division of lesions ensured that the part used for chemistry included the same amount of media as shown by histology.

The fixed halves of the specimens were decalcified (necessary in some advanced lesions), embedded in paraffin wax and 5 μm sections stained with haematoxylin and eosin. Lesions were categorized according to histological appearance, into fatty streaks (defined as focal lesions consisting only of foam cells); intermediate lesions (having in addition early fibrous cap formation and what appeared to be early foci of lack of cellularity, suggesting foam cell death); and advanced lesions (defined as containing large acellular basal areas with cholesterol clefts).

The contents of advanced atherosclerotic lesions ("gruel") were obtained by washing the lesions in water to remove blood, then incising with a scalpel and scraping out the semi-solid yellow matter with the blunt side of a scalpel blade. Pooled contents from several lesions in the same individuals were divided into portions and frozen immediately at −70°C under N_2 or argon. Some samples were added to tubes containing butylated hydroxytoluene (final concn 100 μM) to inhibit peroxidation during handling and storage but this was found not to affect the results obtained.

Reagents

Reagents were of the highest quality available from Sigma Chemical Co.

Assay of "catalytic" metal ions

The bleomycin assay for catalytic iron ions was performed by a modification[18] of the method of Gutteridge *et al.*[10] Reagents and buffers were freed (as far as possible) of metal ion contamination by Chelex treatment and the pH readjusted to 7.4. Non-caeruloplasmin copper was measured by the method of Gutteridge[11] as described in.[18] Results are corrected to a fixed mass of arterial wall or gruel (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.

Just before assay, samples of approximately 10 mg of normal wall and lesions were weighed, placed into robust plastic tubes and homogenised in 1 ml of ice-cold deionized double distilled water for either 10 or 30 sec as indicated using a Polytron homogeniser, speed mark 6. The resultant homogenates were kept on ice and analysed for their copper and iron contents as described above. Controls consisting of 1 ml of water only were taken through the same process to check for contamination with these metals due to the procedures used. The scissors used for mincing, or the scalpels, did not release metal ions when incubated in distilled water. The contents of advanced lesions could often be assayed directly because they dispersed in the reaction mixture during incubation.

RESULTS

In agreement with previous results, the contents of advanced lesions from the abdominal aortae of cadavers contained significant quantities of iron and copper ions, catalytic for free radical reactions, as measured by the bleomycin[10,18] and phenanthroline[11,12,18] assays. Concentrations of bleomycin-detectable iron ranged from 0.1 to 2.6 μM and those of phenanthroline-detectable copper from 0.2 to 28.6 μM.

When normal arterial walls were finely minced with scissors and assayed in the reaction mixtures, bleomycin-detectable iron and phenanthroline-detectable copper could be measured (data not shown). Inclusion of proteinase/peptidase inhibitors (5 μg/ml aprotinin, 35 μM pepstatin, 5 μM leupeptin) in the assay mixtures did not decrease the amounts of metal ion detected. When normal arterial walls were more vigorously disrupted by homogenization using a Polytron homogenizer, considerable quantities of catalytic iron and copper ions were detected in the homogenate (Table 1). In the case of iron, concentrations were comparable to those obtained when gruel samples (normally assayed directly in the reaction mixture) were similarly homogenized (Table 1). In the case of copper ions, homogenization of gruel produced levels that tended to be higher than those observed when gruel was simply allowed to dissolve in the reaction mixture. Table 1 also shows that homogenization of vessel wall samples containing fatty streaks, intermediate fibrous lesions and fibrous lesions led to release of similar amounts of "catalytic" metal ions.

TABLE 1
Bleomycin-detectable iron and phenanthroline-detectable copper in human arterial wall samples after homogenization.

Sample homogenized	Bleomycin-detectable iron (μM)	Phenanthroline-detectable copper (μM)
Normal wall	3.04 ± 2.44 (26)	4.86 ± 2.80 (10)
Gruel	5.43 ± 3.95 (6)	68.8 ± 41.6 (6)
Fatty streaks	3.69 ± 1.61 (7)	3.26 ± 6.40 (6)
Intermediate fibrous lesions	4.13 ± 2.88 (5)	–
Advanced fibrous lesions	2.65 ± 1.20 (9)	5.26 ± 4.35 (5)

Known masses of arterial wall or gruel were vigorously homogenized in a Polytron for 30 sec and the bleomycin and phenanthroline assays carried out as described in the Materials and Methods section. Results are corrected for control absorbances obtained in the absence of sample or bleomycin and are corrected to 5 mg tissue wet weight. Results are mean ± SD (numbers of samples are given in parentheses).

DISCUSSION

Our previous data showed that the contents of advanced human atherosclerotic lesions contain 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 (reviewed in[18]). The present paper reports an extension of this data, showing that mechanical injury to normal arterial walls and earlier lesions causes comparable levels of catalytic metal ions to be liberated. The significant amount of copper ion liberated is especially worthy of note since copper ions are powerful catalysts of LDL oxidation.[3,9]

It is widely thought that injury to the vessel wall *in vivo*, e.g. mechanically (haemodynamically, or during angioplasty[19]), by ischaemia/reperfusion, by viral infection or by blood-borne toxins, is responsible for the development of atherosclerosis.[1,20] Plaque fissure also plays an important role.[20] Our data suggest that rupture of lesions, and even injury to the normal vessel wall, might cause release of metal ions that could facilitate LDL oxidation in the sub-endothelial space. Release of catalytic metal ions into the plasma, creating a localized pro-oxidant environment, might also occur. We recognize, of course, that the severity of the disruption techniques used is very great when compared to the subtle endothelial injuries that can initiate atherosclerosis,[20] but the events we describe may be relevant to events occurring during plaque fissure and in the accelerated development of atherosclerosis subsequent to angioplasty.[19] Many cell types in the atherosclerotic lesion release $O_2^{\cdot-}$, H_2O_2 and thiols. In the presence of transition metal ions, damaging free radical reactions will be enormously accelerated.[5-9,21]

Our data also illustrate the difficulties in performing "control" experiments alongside studies on advanced lesions. The use of homogenates of normal vessel wall (e.g. in[22]) may not be a valid procedure because of the release of metal ions during homogenization.

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