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DEPLETION OF ALPHA-TOCOPHEROL IN HUMAN ATHEROSCLEROTIC LESIONS

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Estimations of α-tocopherol content were made on a series of human necropsy samples of normal arterial wall and of atherosclerotic lesions. The results were compared with stage of lesion, shown by histology, and with the amounts of cholesterol and hydroxycholesterols in the same lesions. The ratio of α-tocopherol to cholesterol levels varied widely in normal arterial wall but was consistently low in lesions, especially in lesions rich in macrophage foam cells. The results suggested that significant accumulation of hydroxycholesterols, found almost exclusively in lesions, only occurred when α-tocopherol levels were low in relation to the cholesterol content. This suggests that oxidative activity in the lesion may lead to significant oxidation of constituents of low-density lipoprotein only after α-tocopherol has been depleted.

KEY WORDS: Atherosclerosis (human), lesions, normal artery, α-tocopherol, hydroxycholesterols, macrophages.

Abbreviations: GC, gas chromatography; HPLC, high pressure liquid chromatography; 7β-OH-CHOL, 7β-hydroxycholesterol; 26-OH-CHOL, 26-hydroxycholesterol; LDL, low-density lipoprotein; SDS, sodium dodecyl sulphate.

INTRODUCTION

The theory that oxidation of low density lipoprotein (LDL) within the arterial wall is a factor in the development of atherosclerotic lesions continues to attract increasing support.[1,2] Much of this support comes from experiments on cell-mediated lipoprotein oxidation *in vitro*, but evidence from human lesions is often more convincing.

For example, there is considerable chemical evidence that oxidised lipids are present in human lesions, notably oxidation products of cholesterol.[3–5] Naturally-occurring auto-antibodies to oxidised LDL are found in many patients with advanced atherosclerosis[6]; and antibodies raised in the laboratory against various forms of oxidised LDL react with components of human plaques.[7]

To judge from laboratory findings, the lipids of LDL particles, when exposed to oxidants, become appreciably oxidised only after a time-lag during which the LDL is depleted of the antioxidants it carries, including tocopherols and carotenoids.[8] It might be supposed, therefore, that human atherosclerotic lesions containing lipid oxidation products might be depleted of antioxidants. It was indeed shown many years ago that

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the ratio of α-tocopherol to total lipid was lower in human atherosclerotic aorta than in normal aorta, but there was no histology and the methods used were somewhat crude.[9]

The levels of α-tocopherol were therefore estimated, in a series of individual human atherosclerotic lesions from necropsy subjects, in order to examine this question more critically. Half of each lesion was examined histologically and the lipid composition of a part of each lesion was analysed by gas chromatography (GC).

MATERIALS AND METHODS

Arterial Samples

68 arterial samples were taken from 37 necropsy subjects (age range 8 y–95 y: mean 56.1 y ± 22.7 y (SD); M 17: F 20) at Addenbrooke's Hospital, Cambridge. The number of samples taken from each subject varied (1–4), as did the sites of samples (common carotid artery (10), thoracic (50) or abdominal aorta (8)). In 23 of the 37 subjects, normal arterial wall was sampled in addition to lesions. In 4 of the 37 subjects lesions were sampled but no normal artery, and in 10 out of the 37 subjects normal artery was sampled but no lesions. The samples formed part of a larger study of the lipid chemistry of arterial lesions.[5]

The selected piece of artery 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 bisected with a scalpel. One half 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 of lesion material with a little inner media only. It was then divided into two equal parts (one for GC, one for α-tocopherol estimation) and stored as soon as possible (within 2 hours) under nitrogen at –20°C until analysis. The normal arterial specimens were divided in the same way. The fact that the stripping away of outer media and adventitia preceded the subdivision of lesions ensured that the parts used for lipid analysis and α-tocopherol estimation included the same amount of media as shown by histology.

The fixed halves of the specimens were embedded in paraffin wax and 5 μm sections stained with haematoxylin and eosin. Lesions were categorised, 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 foci of lack of cellularity, suggesting an early lipid core); and advanced lesions (defined as containing well-established fibrous caps and large lipid cores, with cholesterol clefts). In all specimens, it was noted whether the residual media included was thin or fairly thick. Intermediate and advanced lesions were subclassified according to whether they were rich in foam cells (termed intermediate macrophage lesions and advanced macrophage lesions) or relatively acellular and fibrous (termed intermediate fibrous lesions and advanced fibrous lesions).

Extraction and workup of lipids

Lipids were extracted from the samples and processed for GC analysis as described previously.[4,5] Essentially the procedure consisted of weighing each sample (wet weight),

addition of internal standards (n-heptadecanoic acid, 5α-cholestane, and coprostane) and the antioxidant butylated hydroxytoluene (BHT), Bligh and Dyer extraction (with sonication), sodium borohydride reduction, saponification, and derivatisation to methyl esters and trimethylsilyl ethers. Processed samples were stored at −20°C under nitrogen until GC analysis, typically within 1 week.

Chemicals were as described previously, as was the cleaning procedure for analytical glassware.[4,5] Care was taken throughout to minimise exposure of samples to air, and sodium borohydride reduction, saponification, derivatisation and storage of samples were all under nitrogen.

Gas Chromatography

After the above workup, GC analysis was performed for all the samples as described previously.[5] Quantitation was by peak areas, measured electronically using an integrator, relative to internal standards.

α-Tocopherol estimations

Arterial samples (lesions 1–50 mg wet weight; normal artery 10–80 mg wet weight) were homogenised with 2.3 ml glass-distilled water in glass centrifuge tubes, using an IKA Ultra Turrax macerator, for 2 × 15 s on ice. The macerator was then rinsed with 1.3 ml water and this was combined with the homogenate. α-Tocopherol was extracted from these homogenates by the method of Burton *et al.*,[10] using SDS (0.1 M)/ethanol (HPLC grade)/ heptane (HPLC grade) (0.4 ml/4 ml/2 ml) with thorough vortexing after each stage of the sequential addition of these solvents. Plasmas were extracted similarly except that maceration was omitted, as was the SDS. Centrifugation was then carried out and the heptane layer was collected and stored at −20°C prior to analysis by HPLC using fluorescence detection and with external standard calibration, as described previously.[11]

Statistical analysis of data

Statistical evaluation of data was carried out using StatWorks™ software. Students's t-tests were unpaired, and significance was taken as $p \leq 0.05$. Marginal significance was taken as $0.05 < p < 0.10$. Insignificance was taken as $p \geq 0.10$.

RESULTS

The lipid analyses of the specimens, in which α-tocopherol was also measured, form a subset of a larger series of arterial lesions and the results of the lipid analyses are similar to those of the larger study.[5] The most consistently found lipid oxidation products, in almost all lesions, were cholest-5-en-3β,7β-diol (7β-hydroxycholesterol) and cholest-5-en-3β,26-diol (26-hydroxycholesterol). Both these compounds were undetectable, or occasionally found in only minute amounts, in normal artery specimens. In normal plasma the same hydroxycholesterols were undetectable in all the samples measured, implying that their mean levels were even lower than in normal artery.

None of the components measured showed any correlation with the time-interval between death and necropsy, gender or age of subject, lesion site, thickness of residual media, or with duration of storage before analysis.

Table I shows the content (mean levels and standard deviations) in lesions and normal arterial wall of α-tocopherol (as nmol/g wet weight), α-tocopherol relative to cholesterol (as mmol/mol), and the two hydroxycholesterols relative to cholesterol (as mmol/mol); the number of specimens in each category is also indicated. Table I also includes, for comparison, corresponding data for normal plasma analysed by a similar protocol. The main features of the results were as follows.

α-Tocopherol

α-Tocopherol concentrations (Table I) appeared not significantly different in normal artery and plasma, if, for the sake of comparison, the assumption is made that 1 g wet weight of artery is equivalent to 1 ml of plasma. Fatty streak α-tocopherol concentration was not significantly different to normal artery, whereas advanced macrophage lesions ($p = 0.028$) and advanced fibrous lesions ($p < 0.001$) had significantly higher α-tocopherol concentrations than normal artery. When all lesions were considered together as a single category, the mean level in lesions was more than three times that of normal artery, and this was significant ($p < 0.001$). When intermediate fibrous and macrophage lesions were considered together, and advanced fibrous and macrophage lesions combined likewise, advanced lesions emerged as significantly higher than intermediate lesions ($p = 0.022$).

When α-tocopherol was expressed relative to cholesterol (Table I), concentrations in normal artery were significantly higher than plasma ($p = 0.027$) the mean value for normal artery being just over twice that for plasma. However, lesions (all combined as a single category) were significantly lower than normal artery ($p < 0.001$), the mean value for lesions being just under a fifth of that for normal artery. Lesions were also significantly lower than plasma ($p < 0.001$). Of the individual categories of lesions, advanced fibrous lesions appeared significantly lower than normal artery ($p = 0.030$). When intermediate fibrous and macrophage lesions were considered together, and advanced fibrous and macrophage lesions likewise, concentrations in intermediate lesions and advanced lesions both emerged as significantly lower than those in normal artery ($p = 0.050$ and $p = 0.006$ respectively).

Cholesterol

Cholesterol concentrations (Table I) were not significantly different in plasma and normal artery, assuming 1 g wet weight is equivalent to 1 ml. Cholesterol levels were significantly higher in each category of lesion than in normal artery ($p \leq 0.006$). The mean cholesterol concentration for lesions (all categories combined) was 11.4 times that of normal artery, and this was significant ($p < 0.001$). Concentrations in advanced lesions were significantly higher than intermediate lesions ($p = 0.030$), and were also significantly higher than fatty streaks ($p = 0.040$). Fatty streaks were not significantly different to intermediate lesions.

7β-Hydroxycholesterol

7β-Hydroxycholesterol concentrations (expressed relative to cholesterol) were below the detection limit of the analytical protocol in plasma (Table I), whereas in normal artery it was detectable at very low levels in some of the samples, and undetectable in others. 7β-Hydroxycholesterol levels were significantly higher, by a factor of 7.3 (for the mean values), in all lesions combined than in normal artery ($p = 0.038$) and also

higher in each category of lesion than in normal artery ($p \leq 0.003$) with the exceptions of intermediate fibrous lesions ($p = 0.61$; insignificant) and advanced fibrous lesions ($p = 0.08$; marginally significant). Fatty streaks were higher than advanced lesions, with marginal significance ($p = 0.058$), but were not significantly different to intermediate lesions.

26-Hydroxycholesterol

Levels of 26-hydroxycholesterol (expressed relative to cholesterol) in plasma were below the detection limit of the analytical protocol (Table I), whereas in normal artery it was detected at very low levels in some of the samples but was undetectable in others, as was also the case for 7β-hydroxycholesterol (see above). It was significantly higher in all categories of lesions, combined and separate, than in normal artery ($p \leq 0.006$). The mean level for lesions was 32.8 times higher than that for normal artery ($p < 0.001$). Intercomparisons of lesion categories revealed 26-hydroxycholesterol as significantly higher in advanced lesions than in intermediate lesions ($p = 0.004$) or in fatty streaks ($p = 0.031$). Fatty streaks and intermediate lesions were not significantly different.

DISCUSSION

Variation between subjects

There was a high degree of variation between individual specimens in the results of our analyses. These presumably arise because of individual differences between subjects, and probably also because of the variation, even within a single lesion, over time. Each sample represents a single time-point, the time of death, or more correctly the time of sampling. Presumably during life the chemical composition of lesions changes continuously due to such variations as lipid content of the diet and oxidant-antioxidant balance. In addition it is unlikely that any individual lesion is histologically and chemically homogeneous throughout. In spite of these considerations, some definite patterns emerge from our data.

α-Tocopherol

The results demonstrate clearly that α-tocopherol concentrations (in nmol/g wet weight) are elevated in lesions compared with normal artery and plasma. As expected, cholesterol is also much higher in lesions than in normal artery and plasma. However, when α-tocopherol is expressed relative to cholesterol, normal artery levels are higher than those of plasma, and lesion levels emerge as lower than those of either normal artery or plasma. Thus lesions appear to be poor in α-tocopherol, not in absolute terms but when considered in relation to the cholesterol content. In plasma there is one α-tocopherol molecule for approximately every 200 cholesterol molecules, whilst in normal artery there is one α-tocopherol molecule for roughly every 100 cholesterol molecules. In lesions, however, there is one α-tocopherol molecule for roughly every 500 cholesterol molecules.

The reason for this apparent relative depletion of α-tocopherol is unknown, but explanations can be suggested, and a combination of effects may be responsible for the findings. It is unlikely that decomposition of α-tocopherol on storage of the lesions might be responsible for the observed depletion, as α-tocopherol content (nmol/g wet

TABLE I
Alpha-tocopherol, cholesterol and hydroxycholesterols in plasma, normal artery and lesions of different stages.

Category	n	α-Tocopherol (nmol/g wet weight)		Cholesterol (μmol/g wet weight)		α-Tocopherol/cholesterol (mmol/mol)		7β-OH-CHOL/cholesterol (mmol/mol)		26-OH-CHOL/cholesterol (mmol/mol)	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Plasma	28	26.58*	8.01	5.33*	1.04	5.03	1.26	< 0.06	–	< 0.06	–
Normal artery	34	33.44	39.97	6.63	6.99	10.72	13.20	0.30	0.99	0.13	0.57
Fatty streak	5	48.93	22.99	32.56	26.60	2.06	1.30	7.00	13.09	1.61	1.20
Intermediate fibrous lesion	3	69.06	62.53	22.93	25.75	3.59	1.18	0.60	0.56	1.75	3.03
Intermediate macrophage lesion	7	59.11	26.96	58.00	41.82	1.62	1.36	1.99	1.38	4.93	3.18
Advanced fibrous lesion	13	193.8	124.5	101.5	67.03	2.45	1.77	0.88	1.00	4.61	3.65
Advanced macrophage lesion	6	73.23	34.88	102.8	78.67	1.10	0.83	2.10	1.35	6.20	2.19
All lesions[+]	34	112.5	102.6	75.67	63.33	2.08	1.53	2.20	5.12	4.26	3.26
Intermediate lesions**	10	62.1	37.1	47.4	40.0	2.21	1.56	1.57	1.34	3.97	3.34
Advanced lesions[++]	19	156	118	102	68.7	2.02	1.64	1.27	1.23	5.11	3.28

Abbreviations: 7β-OH-CHOL, 7β-hydroxycholesterol; 26-OH-CHOL, 26-hydroxycholesterol.
Footnotes: *Assuming 1 g is equivalent to 1 ml.
[+] Combined data for all the lesions (fatty streaks, intermediate fibrous and macrophage lesions, advanced fibrous and macrophage lesions).
** Combined data for intermediate fibrous and macrophage lesions.
[++] Combined data for advanced fibrous and macrophage lesions.

weight or mmol/mol cholesterol) showed no correlation with storage time between necropsy and extraction, or with interval between death and necropsy (data not shown).

One possibility is that, in the lesions, the reason that the cholesterol accumulation outstrips that of α-tocopherol is because the α-tocopherol levels are somewhat diminished by oxidation occurring within the lesions. The hydroxycholesterols, oxidation products of cholesterol, are indeed significantly higher in lesions than in normal artery or in plasma, and this would appear to support this hypothesis. In the present study the ratio of α-tocopherol to polyunsaturated fatty acid (linoleate plus arachidonate) was found to be significantly lower ($p = 0.013$) in lesions (mean level of α-tocopherol 14.59 ± 19.40 mmol/mol polyunsaturate) than in normal artery (mean level of α-tocopherol 43.87 ± 64.09 mmol/mol polyunsaturate). Thus α-tocopherol is depleted in lesions not only in relation to the cholesterol content but also in relation to the polyunsaturated fatty acid content, possibly due to the lowering of α-tocopherol by oxidation in the lesions. Further evidence for lipid oxidation within the lesions has been described previously.[5]

Alternatively, oxidation of cholesterol within the lesions may occur as a consequence of the relative depletion of α-tocopherol. The artery is known to display a degree of selectivity in its retention of plasma components. For example, LDL is found in normal artery at approximately twice its concentration in plasma, whereas albumin only occurs at about a quarter of its plasma concentration.[12] Thus it is conceivable that the depletion of α-tocopherol (relative to cholesterol) in lesions might be due to some mechanism other than removal by oxidation. However this might seem less likely in view of the fact that in normal artery the ratio of α-tocopherol to cholesterol appears to be actually higher than in plasma or in the lesions, the lesions being lower than the plasma.

Another possible explanation is that the α-tocopherol to cholesterol ratio is lowered in lesions compared with normal artery because of permeation of plasma lipoprotein into the lesion, and that the α-tocopherol to cholesterol ratio is further diminished by oxidation within the lesion. The reason for the α-tocopherol to cholesterol ratio being higher in normal artery than plasma is unknown, but the ability of normal artery differentially to sequester plasma components has been alluded to above.

Relationship between α-tocopherol and hydroxycholesterols

The above discussion is based on consideration of the mean values of the relevant parameters. If we now consider the values of the same parameters in each of the individual arterial specimens, and plot α-tocopherol expressed in mmol/mol cholesterol vs. each of the hydroxycholesterols, also expressed in mmol/mol cholesterol (Fig. 1), it can be seen that the α-tocopherol to cholesterol ratio varied widely in normal artery but was always low in lesions. The lesions, containing significant amounts of both 7β-hydroxycholesterol and 26-hydroxycholesterol, always had α-tocopherol/cholesterol levels of less than 10 mmol/mol. The findings thus suggest that the hydroxycholesterols were only found in significant amounts when α-tocopherol was depleted below a threshold level (Fig. 1). This can be compared with the data for LDL oxidation *in vitro*, where it has been shown that endogenous antioxidants in the LDL particle, including α-tocopherol, are severely depleted before lipid peroxidation begins to increase rapidly.[8]

The ability of α-tocopherol to act as a radical scavenger and thus inhibit or delay lipid peroxidation is well known and can explain the findings for 7β-hydroxycholesterol, which is a product of free radical-mediated oxidation.[13] However,

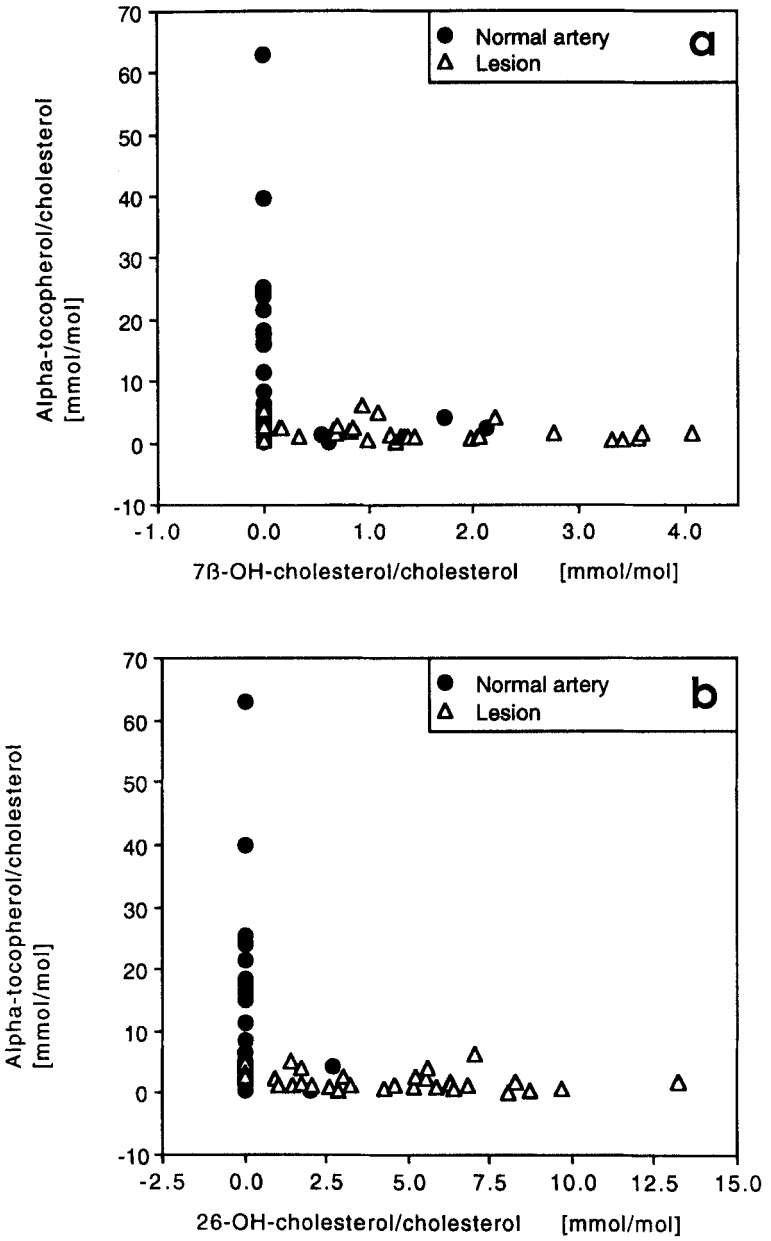

FIGURE 1 Graphs of α-tocopherol vs. (a) 7β-hydroxycholesterol and (b) 26-hydroxycholesterol, for normal artery and for atherosclerotic lesions. α-Tocopherol, 7β-hydroxycholesterol and 26-hydroxycholesterol are all expressed as mmol/mol cholesterol.

26-hydroxycholesterol is a product of the cytochrome P-450 sterol 26-hydroxylase enzyme (also termed sterol 27-hydroxylase) and is not produced by free radical oxidation.[13,14] The findings therefore raise the question of whether α-tocopherol is also able to inhibit this enzyme. Inhibition of another of the cytochrome P-450 enzymes by various antioxidant radical scavengers, including butylated hydroxytoluene (N.B. α-tocopherol was not tested), has been reported *in vitro*.[15] Both 7β-hydroxycholesterol and 26-hydroxycholesterol are toxic to a variety of cell types, including human monocyte-macrophages,[16] and may thus contribute to the cell death which is a feature of advanced lesions. Thus the putative inhibition of their formation *in vivo* by α-tocopherol suggests a possible mechanism by which α-tocopherol may exert a protective effect against atherosclerosis.

Role of macrophages

Comparison of results from different histological types of lesions is difficult because of the small numbers of specimens within each division, but there appears to be a trend for lower α-tocopherol/cholesterol ratios and higher hydroxycholesterol content in macrophage-rich rather than macrophage-poor (fibrous) lesions (Table I). This provides some circumstantial evidence to support previous observations that most lipoprotein oxidation occurs in collections of macrophage foam cells. These previous observations include: (i) Ceroid pigment, thought to arise at least partly from LDL oxidation, is found in macrophages rather than in any other cell types within lesions[17]; (ii) A similar, more significant preponderance of hydroxycholesterols in foam cell-rich lesions was found in the larger study of systemic artery lesions,[5] of which the specimens examined here formed a part; (iii) Lipid oxidation products, including hydroxycholesterols, are as abundant in pulmonary artery lesions as in systemic lesions (Carpenter *et al.*, submitted), which may reflect the known ability of macrophages to deploy their oxidative mechanisms even in low oxygen environments.

CONCLUSIONS

The finding that atherosclerotic lesions appear depleted in α-tocopherol compared to normal artery and plasma (standardised for cholesterol in each case), is consistent with the epidemiological findings that α-tocopherol is inversely associated with ischaemic heart disease[18–21] and lend further support to the idea that dietary antioxidants may protect against atherosclerosis.

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