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DIFFERING EFFECTS OF PROBUCOL AND VITAMIN E ON THE OXIDATION OF LIPOPROTEINS, CEROID ACCUMULATION AND PROTEIN UPTAKE BY MACROPHAGES

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Studies using ^{125}I-low density lipoprotein (^{125}I-LDL) show that probucol (10 μM) and α-tocopherol (100 μM) inhibit protein degradation in LDL exposed to Cu (II) *in vitro*. The inhibitory effect of α-tocopherol on protein fragmentation exceeded that of probucol. On the other hand, probucol was more able to inhibit lipid peroxidation. The subsequent uptake of Cu (II)-oxidised ^{125}I-LDL by murine peritoneal macrophages (MPM) was virtually unaffected by the presence of probucol during LDL oxidation. The same was not true for α-tocopherol which led to lower levels of ^{125}I-LDL uptake by MPM. Thus, it appears that although the antioxidant activity of probucol exceeds that of α-tocopherol for lipid oxidation, the reverse is true for protein degradation and, perhaps more significantly, for subsequent macrophage uptake.

Further studies used artificial lipoproteins composed of cholesteryl linoleate or cholesteryl arachidonate complexed with bovine serum albumin. Culture of these artificial lipoproteins with MPM resulted in protein uptake, protein degradation, cholesterol oxidation to cholest-5-en-3β,7β-diol and the intracellular accumulation of ceroid in MPM. The presence of α-tocopherol (0–100 μM) inhibited all of these processes. Probucol (0–10 μM) inhibited ceroid accumulation and cholesterol oxidation to the same degree as α-tocopherol (0–100 μM) but had no effect upon protein degradation and protein uptake. Control studies of lipoproteins incubated without cells showed that protein degradation by cell-independent processes was also inhibited by α-tocopherol, but not by probucol.

These observations are discussed in the context of the role of lipoprotein oxidation in atherogenesis.

KEY WORDS: Oxidation; vitamin E; probucol; macrophages; ceroid; lipoprotein uptake.

INTRODUCTION

The main source of lipid within the macrophage foam cells of atherosclerotic lesions is believed to be modified low density lipoprotein (LDL), probably oxidatively modified LDL, since oxidised LDL has been detected, using monoclonal antibodies, in human lesions.[1] Indeed, naturally-occurring autoantibodies to ceroid, a pigment found in all atherosclerotic plaques, cross-react with artificially oxidised LDL, suggesting that ceroid is composed, at least partly, of oxidised LDL.[2] Thus, the obvious choice of substrate in the investigation of LDL-cell interactions would appear to be human LDL. However, LDL from different individuals contains

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variable proportions of lipids, natural antioxidants and other substances. Therefore, we have made use of a model system for studying processes relevant to lipoprotein oxidation and subsequent cellular processing, using murine peritoneal macrophages cultured with artificial lipoproteins. These artificial lipoproteins consist of a single lipid species, such as cholesteryl linoleate (CL) or cholesteryl arachidonate (CA), which are known to be decreased in amount within atherosclerotic lesions,[3] and bovine serum albumin (BSA), a well characterised protein. To judge from electron microscopy, such artificial lipoproteins, prepared by an established technique,[4,5] are avidly taken up by a process resembling phagocytosis.[5] The uptake involves scavenger receptors, since acetyl LDL partially competes with it.[6] This uptake results in lipid-laden macrophages which resemble, by cytology and ultrastructure,[5] the foam cells found in human atherosclerotic plaques. The artificial lipoproteins used in these studies may have a naturally occurring counterpart in cholesterol ester- and albumin-rich particles obtained from human atherosclerotic plaques[7] and may also be considered as a model of cholesterol esters in LDL itself.[4,5,6]

Probucol, 4,4′ (isopropylidenedithio) *bis* [2,6 di-tert butylphenol], has a structural similarity to butylated hydroxytoluene and is known to inhibit LDL oxidation *in vitro*[8] and LDL uptake and degradation in Watanabe rabbits.[9] A necessary prerequisite for the comparative study of the antioxidant action of compounds is the use of physiologically relevant concentrations. The antioxidant effects of probucol and α-tocopherol (α-T) have been compared, since both compounds have been proposed as agents of potential use in human atherosclerosis. Plasma concentrations which can be achieved by oral administration of these two compounds are ca. 130 μM for α-T[10] and ca. 5–20 μM for probucol.[11] The concentration ranges tested in our investigations (0–10 μM probucol and 0–100 μM α-T) reflect this difference in achievable plasma concentrations in man.

The use of artificial lipoproteins permits the study of the effects of antioxidants on the oxidation of specific lipids[12] and proteins in the presence or absence of macrophages. Therefore, we have now used this model system to compare the antioxidant activities of dl-alpha tocopherol (α-T), and the hypocholesterolaemic and antioxidant drug probucol.

MATERIALS AND METHODS

Radioactive isotopes were obtained from Amersham International and all other reagents were supplied by Sigma Chemical Co. Student's-T tests were performed using Microsoft Excel 5.0, Apple MacIntosh.

Protein Degradation

Protein degadation, probably due to a combination of oxidative protein scission and cell-mediated proteolytic activity, was assessed in the medium of cell cultures and in parallel incubations performed in the absence of cells. Using radiomethylated [^{14}C]-BSA (less that 1% of lysine residues modified) to construct lipoproteins (18,000 dpm/mg), degradation was determined by measuring radioactive peptides soluble in 5% trichloroacetic acid (TCA), as previously described.[13,14]

Cell Culture

Murine peritoneal macrophages (MPM) were isolated from male BALB/c mice aged 6–8 weeks, as previously described.[14,15] Cells were resuspended in RPMI 1640 medium containing 10% lipoprotein-deficient foetal calf serum and plated at the desired cell number. The medium also contained 100 IU/ml penicillin and 100 mg/ml streptomycin sulphate. Cell cultures (2×10^6 cells/culture) were performed in 3 ml culture medium in 4 cm diameter glass dishes (Anumbra, Payne, Slough, Berks, UK) throughout. The macrophages were allowed to adhere by incubating for 3 hours at 37°C in an atmosphere of 5% CO_2. Non-adherent cells were removed by washing with phosphate-buffered saline. Fresh medium was added, and cells incubated overnight at 37°C in an atmosphere of 5% CO_2 prior to replacement of medium and introduction of the artificial lipoprotein emulsions. The cultures were then incubated in the presence of the emulsion for 24 hours.

Cells were harvested by removing the medium, washing the culture with PBS and adding 3 mls 0.33% ethylenediamine tetraacetic acid (EDTA) in RPMI with 10% lipoprotein-deficient foetal calf serum. Cells were then removed and retained. Any remaining adherent cells were scrape-harvested in 3 ml Hanks balanced salt solution. A cell pellet was prepared by centrifugation as previously described.[4,15] Cells were resuspended in 0.25 ml FACS fixative (1.5% formaldehyde, 0.1% BSA in phosphate buffered saline) by vortexing. Samples fixed in this way were stored at 4°C until flow cytometry. Such fixed samples were stable for up to 2 weeks.

Preparation, Protein Fragmentation and Cellular Uptake of Low Density Lipoprotein

Human low density lipoprotein (LDL) was prepared from normal individuals as previously described.[16] Briefly, blood from healthy volunteers was centrifuged in the presence of 1 mg/ml EDTA to obtain plasma. Lipid fractions were obtained from pooled plasma by ultracentrifugation and flotation through potassium bromide gradients. Centrifugations were performed at 100,000 g for 18 hours at 16°C in the presence of EDTA. Low density lipoprotein (LDL) was taken as the fraction that floated at a relative density of 1.063. LDL was radioiodinated with ^{125}I to 500 cpm/ng, as previously described.[16,17] Prior to experimentation LDL was dialysed overnight at 4°C against phosphate buffered saline containing Chelex-resin (Sigma) to remove any EDTA.

Radioiodinated LDL (3.5 mg/ml) was incubated at 37°C for 24 hours with or without 5 μM Cu (II) in the presence or absence of 10 μM probucol or 100 μM α-tocopherol. The levels of protein fragmentation were determined by measuring the TCA-soluble radiolabelled fraction, as described above. Lipid peroxidation was assessed by reaction with thiobarbituric acid and by the xylenol orange assay for lipid peroxides.[16] Prior to culture with murine peritoneal macrophages, LDL was chromatographed on a PD10 column. The uptake of such pre-incubated LDL by MPM on culturing with 100 μg/ml LDL for 5 hours was determined as previously described.[17]

Artificial Lipoprotein Preparation

Lipoproteins were prepared as previously described.[4,15] The molar ratio of lipid to BSA was 60:1. BSA was used at 10 mg/ml (150 μM) in phosphate-buffered saline

(PBS). The cholesteryl linoleate or cholesteryl arachidonate (CL or CA; 9 mM) was first dissolved in acetone (BDH-Analar), added to the BSA whilst vortexing and then sonicated for 1 minute. Acetone was then evaporated by gassing with a stream of nitrogen. The resulting emulsion was added to the culture medium at 50 μl/ml medium. Artificial lipoproteins containing vitamin E [dl-α-tocopherol (α-T)] or probucol were prepared in a similar fashion, but with the inclusion of known concentrations of α-T or probucol in the acetone used to dissolve the cholesterol ester.

Estimation of Cellular Uptake of Protein

Bovine serum albumin (BSA) was labelled at lysine residues by fluorescein isothiocyanate (FITC), as previously described.[18] Labelling by FITC resulted in less than 4% lysine modification. Labelling of 0.5–7% of the lysine residues does not affect BSA uptake. Cellular uptake of BSA (FITC-labelled) within artificial lipoproteins was monitored by flow cytometry[18] as described above, but with the laser tuned to emit 300 mW of light at 488 nm and emission monitored using a 530 nm band pass filter.[18]

Gas Chromatography

Products of CL or CA oxidation in culture medium were measured using gas chromatography (GC). Sample preparation and GC were performed as previously described,[12] using a Carlo Erba Mega 5360 gas chromatograph equipped with a 25 m 0V-1 Crossbond™ fused silica capillary column of diameter of 0.32 mm and film thickness of 0.1–0.15 μm (Mega, Milan, Italy). Hydrogen was used as the carrier gas. Amounts of compounds were measured relative to internal standards, as previously described.[12]

Ceroid Fluorescence Estimation by Flow Cytometry

Fluorescence in cells was analysed using a FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) flow cytometer equipped with a Spectra Physics 2025 argon ion laser, as previously described.[15] The laser was tuned to emit 300 mW of light in the UV wavelengths 351–363.8 nm. Forward light scatter and side scatter were also recorded.

Intact macrophages were identified by forward light scatter. Selection of intact populations of macrophages was verified on selected live, unfixed samples, using propidium iodide to stain dead cells as previously described.[18] Fluorescence was monitored using a 490 nm long pass filter in front of the detector. Mean (geometric) fluorescence intensities of the intact macrophage population were calculated using the Lysis II programme (Becton-Dickinson) for each sample. Mean fluorescence intensities were expressed relative to controls in which cells were exposed to medium alone, since this instrument, in common with all fluorimeters, measures light intensities in arbitrary units.[18]

RESULTS

The Effect of α-Tocopherol and Probucol upon LDL-Protein Fragmentation and Macrophage Uptake

The exposure of radioiodinated ^{125}I-LDL to 5 μM Cu (II) resulted in a time-dependent increase in protein fragmentation, as assessed by the formation of a radioactive trichloroacetic acid-soluble fraction (data not shown). Whereas α-T inhibited this fragmentation far more than probucol, suggesting that α-T protected apo-B from oxidative insult better than did probucol (Figure 1: Filled Bars), the reverse was true for the inhibition of lipid oxidation (Figure 1: hatched bars). Peroxide measurements shown in Figure 1 (narrow-hatched bars) also show that LDL

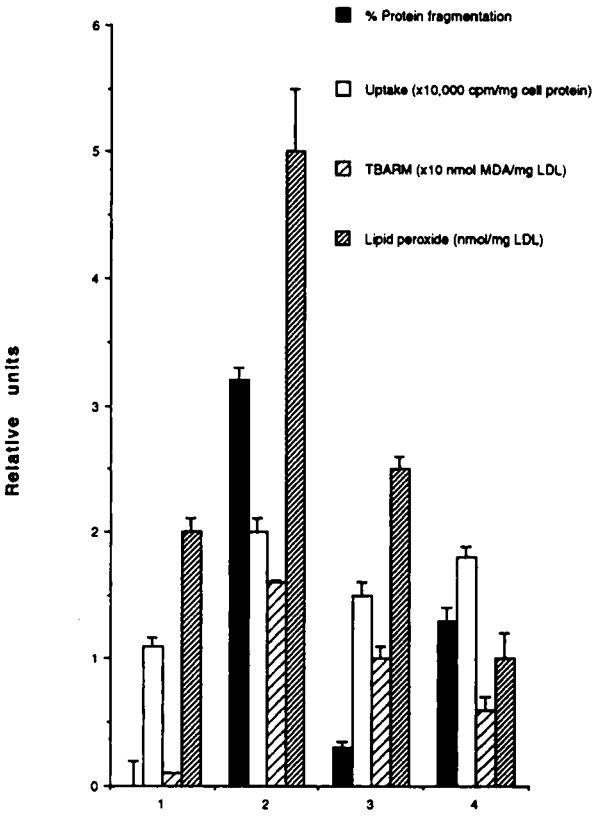

FIGURE 1 ^{125}I-LDL (3.5 mg/ml) was incubated alone [1] or with 5 μM Cu (II) for 24 hours at 37°C [2] in the presence of 100 μM vitamin E [3] and 10 μM probucol [4]. The level of protein fragmentation was monitored in terms of the production of a trichloroacetic acid-soluble radiolabelled fraction. Also shown is the effect upon the level of lipid peroxidation, determined by the xylenol orange assay of peroxides or measured as thiobarbituric acid-reactive material (TBARM). The effect of LDL oxidation and these antioxidants upon subsequent uptake of ^{125}I-LDL by mouse peritoneal macrophages (2×10^6/dish, cultured with 100 μg/ml ^{125}I-LDL over 5 hours) is also shown. The uptake of pre-oxidised LDL was reduced in the presence of vitamin E ($p < 0.05$), whereas there was no significant difference when probucol was included during LDL preoxidation. On the other hand, Apo-B fragmentation and lipid peroxidation was significantly reduced by both antioxidants ($P < 0.05$), with vitamin E differing from probucol ($p < 0.05$).

exposed to buffer alone over 24 hours accumulates a detectable amount of peroxide, as previously shown.[19] The subsequent uptake of LDL by macrophages was reduced if pre-exposed to Cu (II) and 100 μM α-T, more so than by pre-exposure to Cu (II) and 10 μM probucol (Figure 1; Open Bars).

Studies using artificial lipoproteins were performed to further elucidate the different effects of probucol and α-tocopherol upon lipoprotein modification and cellular handling. The use of artificial lipoproteins, instead of LDL, avoids the experimental variation common to LDL studies, primarily due to variations in lipid and antioxidant content.

The Effect of α-Tocopherol and Probucol upon Artificial Lipoprotein Degradation and Uptake by Macrophages

The culture of murine peritoneal macrophages (MPM) with CL/^{14}C-BSA or CA/^{14}C-BSA resulted in the production of trichloroacetic acid (TCA)-soluble radiolabelled peptides (Figure 2A/2B). Whereas α-T substantially inhibited protein degradation, probucol had no significant inhibitory effect.

Control studies in which CL/^{14}C-BSA or CA/^{14}C-BSA was incubated under identical conditions to macrophage cultures, but without cells, resulted in a much decreased level of BSA degradation (Figure 3), reduced further by α-T but not by probucol. Control incubations of BSA with solvent alone (not shown) did not differ from that of BSA alone.

Prior to experimentation approximately 0.7% of BSA is TCA-soluble. This increases to 0.9% in 24 hours and is unaffected by either probucol or α-T (Figure 3). The presence of TCA-soluble radioactivity (in the absence of cells or lipids) is probably due to autolytic hydrolysis or the release of a small amount of non-covalently bound ^{14}C-formaldehyde[13,14] and is unaffected by either probucol, α-T or by the addition of acetone, used during the preparation of artificial lipoproteins.

The culture of MPM with CL/FITC-BSA or CA/FITC-BSA resulted in the intracellular accumulation of FITC, monitored by flow cytometry (Figure 4A/4B). The inhibitory effect of α-T upon protein uptake resembled its effect on protein degradation (Figure 4A). Probucol caused little or no change in protein uptake (Figure 4B). The similar trend in BSA degradation and BSA uptake by macrophages shows that protein degradation, at least partially due to oxidative mechanisms (since both degradation and uptake is inhibited by α-T), is associated with lipoprotein uptake by macrophages.

The Effect of α-Tocopherol and Probucol upon Lipid Peroxidation in Artificial Lipoproteins

The culture of MPM with CL/BSA or CA/BSA resulted in the depletion of linoleate (18:2) or arachidonate (20:4) and the formation of the major oxidation product of the cholesterol esters, cholest-5-en-3β,7β-diol,[12] in the cell medium, measured by GC (Table 1).

Control studies during which CL/BSA or CA/BSA were incubated without cells ("No cells" control) under identical conditions also led to the depletion of linoleate (18:2) or arachidonate (20:4), respectively, although to a lesser degree. This was also inhibited by both α-T and probucol (Table 1).

Depletion of fatty acid (18:2 or 20:4, present in CL and CA, respectively) was greater for CA, presumably due to the greater oxidisability of this fatty acid.

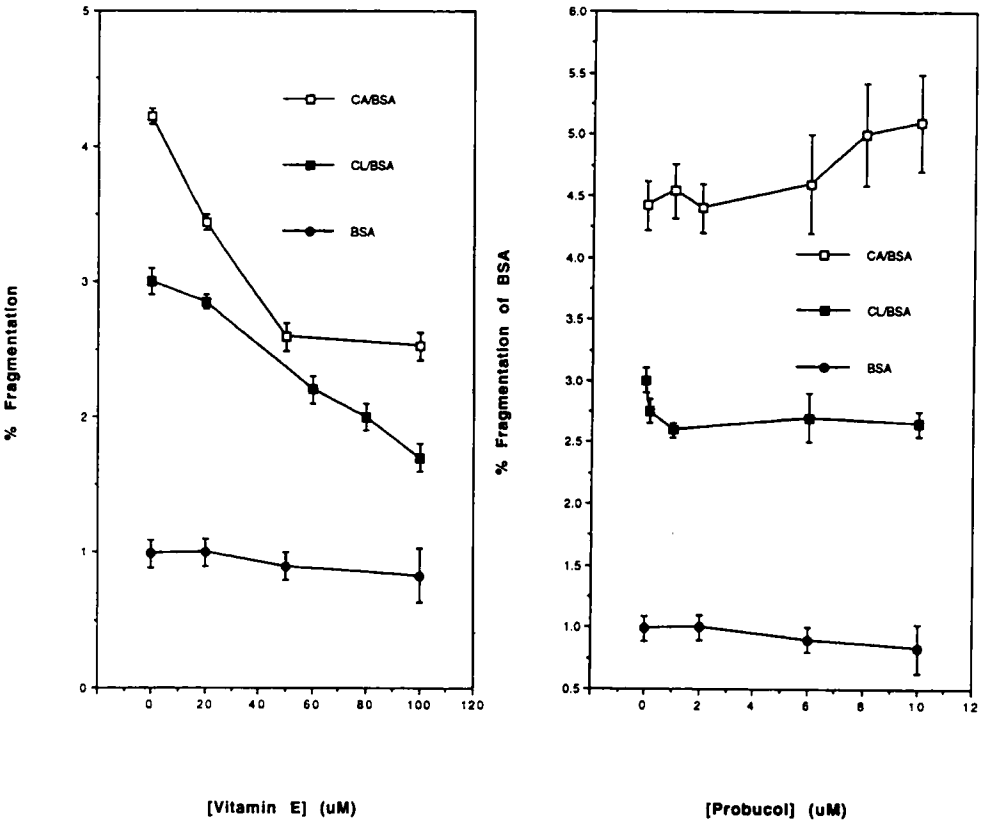

FIGURE 2 Mouse peritoneal macrophages (2×10^6/dish) were cultured in medium containing ^{14}C-BSA, CA/^{14}C-BSA or CL/^{14}C-BSA for 24 hours. BSA degradation was assessed by measuring the radioactivity in a trichloroacetic acid-soluble fraction, as described. The effect of including increasing concentrations of (α-T (0–100 μM) or probucol (0–10 μM) is shown. Values in this and all other figures are the mean ± SD of a minimum of 3 experiments.

However, despite the greater rate of CA oxidation, negligible amounts of cholest-5-en-3β,7β-diol were produced, when compared to CL.

Probucol (0–10 μM) and α-T (0–100 μM) both inhibited this lipid oxidation within artificial lipoproteins. However, probucol appeared to be the more effective inhibitor of lipid oxidation on a molar basis (Table 1).

The Effect of α-Tocopherol and Probucol Upon Ceroid Accumulation in Macrophages

The culture of murine peritoneal macrophages with CL/BSA or CA/BSA resulted in the intracellular accumulation of ceroid, an autofluorescent pigment which can be monitored by flow cytometry.[15] Probucol (0–10 μM) and α-T (0–100 μM) inhibited ceroid fluorescence accumulation to a similar extent (Figure 5A/5B).

Ceroid accumulation in macrophages is a marker of lipoprotein oxidation, which is taken up and processed by macrophages to form intracellular ceroid deposits.

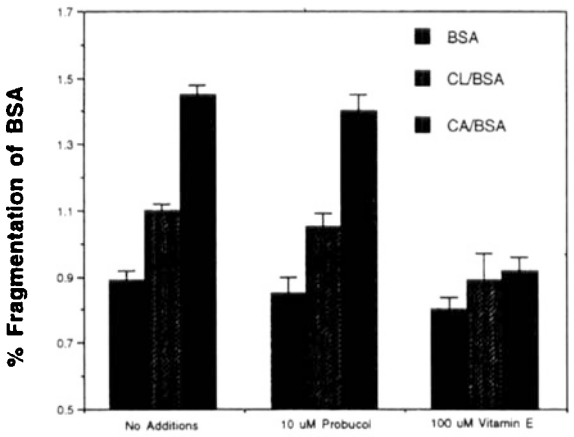

FIGURE 3 ^{14}C-BSA, CA/^{14}C-BSA or CL/^{14}C-BSA were incubated for 24 hours under identical conditions as described for cell culture but in the absence of cells. BSA degradation was assessed by measuring the radioactivity in a trichloroacetic acid-soluble fraction, as described. The effect of including 100 μM α-T or 10 μM probucol is shown. BSA degradation within CL-BSA was significantly lower ($P < 0.05$) in the presence of α-T than in the absence of added antioxidant, but probucol had no effect.

Thus, probucol again appears to be the more effective antioxidant on a molar basis. Although all data presented on ceroid are based on flow cytometric analysis, a limited number of studies using cell staining by oil-red O, with or without alcohol-xylene extraction of neutral lipids[4] exhibited similar trends for the effect of both of these antioxidants. The use of cell staining techniques, however, provides only a crude index of intracellular ceroid accumulation.[15]

DISCUSSION

Notwithstanding any effects upon cell growth of antioxidants such as α-T and probucol,[20,21] antioxidants (such as α-T) are able to inhibit the oxidation of both lipid and protein, as well as inhibit lipoprotein uptake and ceroid accumulation in macrophages, whereas probucol appears to significantly alter lipid oxidation and ceroid accumulation alone (Table 1 and Figures 1 and 2). Studies with human LDL (Figure 1) show that copper-mediated oxidation of LDL leads to the accumulation of lipid oxidation products and protein fragmentation. Probucol inhibited lipid peroxidation more than vitamin E at the concentrations used, whereas the reverse was true for both protein fragmentation and subsequent uptake by macrophages.

The exposure of macrophages *in vitro* to artificial lipoproteins containing cholesteryl linoleate or arachidonate has previously been used to circumvent experimental variation typically associated with LDL studies. Studies have shown that culture of artificial lipoproteins with macrophages results in lipid oxidation,[12] ceroid accumulation[4,15] and cell damage.[22] To judge from all previous experiments, these three events appear to be closely correlated *in vitro*, and all are inhibited by α-T.

α-T (0–100 μM) and probucol (0–10 μM) inhibited ceroid accumulation resulting from exposure to either CL/BSA or CA/BSA, as well as inhibiting the oxidation of

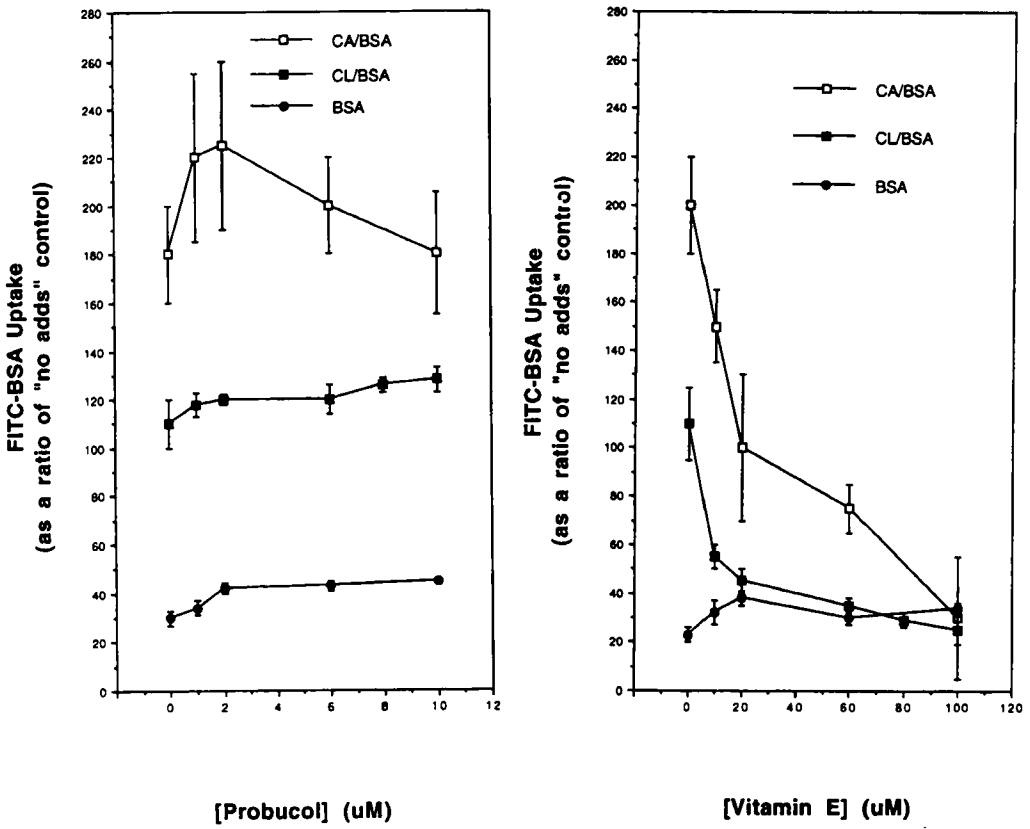

FIGURE 4 Mouse peritoneal macrophages (2×10^6/dish) were cultured in media containing FITC-BSA, CA/FITC-BSA or CL/FITC-BSA for 24 hours. FITC-BSA uptake by cells was assessed by flow cytometry, as described. The effect of including increasing concentrations of probucol (0–10 μM) or α-T (0–100μM) is shown.

both cholesterol esters to a similar extent (Table 1 and Figure 4). On a molar basis probucol would therefore appear to be the more potent antioxidant. In these studies probucol did not appear to have an inhibitory effect upon lipoprotein uptake by macrophages *in vitro*. Indeed, in our studies probucol and α-T, at physiologically relevant concentrations, differed markedly with respect to inhibiting protein uptake and degradation. α-T inhibited both these parameters in cultures of MPMs and artificial lipoproteins whereas probucol did not (Figures 1, 2 and 3).

The increased degradation of artificial lipoproteins in the presence of macrophages is likely to result from cell proteolysis and/or free radical-mediated fragmentation, the latter caused either by peroxidising lipid or by oxidants produced during the macrophage respiratory burst. Control studies, performed in the absence of macrophages, confirmed that the peroxidation of cholesterol esters is associated with protein degradation (Figure 2). Protein degradation by lipid peroxide-derived free radicals generated by the oxidation of unsaturated lipids in the absence of cells has previously been shown during *in vitro* studies.[13,23] Whether such extracellular oxidation of unsaturated lipids, catalysed by trace metals such as copper or iron,

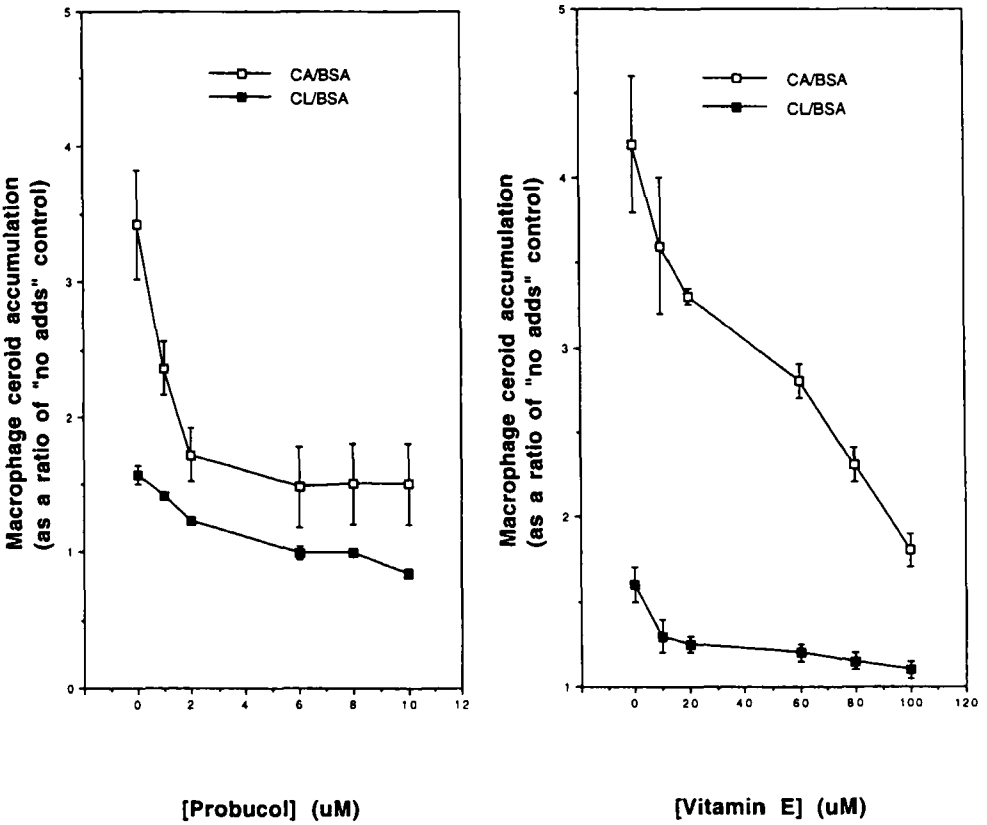

FIGURE 5 Mouse peritoneal macrophages (2×10^6/dish) were cultured in medium containing CA/BSA or CL/BSA for 24 hours. Ceroid accumulation was assessed by flow cytometry. The effect of including increasing concentrations of probucol (0–10 μM) or vitamin E (0–100 μM) is shown.

contributes to the lipoprotein uptake and ceroid formation is at present uncertain but seems likely, based on these and recent studies.[24] In the presence of macrophages protein degradation far exceeded that of controls performed in the absence of cells. It is therefore probable that the macrophages themselves play a significant part in protein degradation. The inhibitory effect of α-T upon protein degradation in the presence of macrophages is compatible with the suggestion that degradation may be due to oxidants produced during the macrophage respiratory burst[25,26] although evidence against the role of superoxide-mediated oxidation of lipoproteins generated by the macrophage respiratory burst has recently been reported.[27]

Whereas the inhibitory effect of probucol and α-T for the oxidation of cholesterol esters (Table 1) did not appear to differ, the same was not true for protein damage (Figures 1 and 2). Despite the inability to reduce protein degradation in artificial lipoproteins, probucol (0–10 μM) inhibited ceroid accumulation to a similar extent as α-T (0–100 μM). Thus, ceroid accumulation in macrophages appears to be dependent on lipid peroxidation. The inability of probucol to inhibit the degradation of protein and its subsequent uptake is perhaps not surprising. Probucol appears to act as an antioxidant solely for lipids and has previously been shown to inhibit the

TABLE 1

Mouse peritoneal macrophages (2×10^6/dish) were cultured in medium (3 ml) containing CL/BSA for 24 hours. Medium was removed and then analysed by gas chromatography for products of CL oxidation. Control incubation performed under identical conditions of culture but without cells is also shown. Values are the average ± range obtained in a minimum of two represenatative experiments and are expressed as μg present in 3ml experimental medium. Cholesterol and unsaturated fatty acid (linoleate and arachidonate) present in CA/BSA and CL/BSA prior to experimentation were 230 ± 20 μg and 180 ± 10μg, respectively, measured by GC.[NA = not applicable, NEG = negligible, 20:4 = Arachidonic acid, 18:2 = Linoleic acid, (+) cells = with macrophages and (−)cells = without macrophages].

	20:4	18:2	Cholesterol	Cholest-5-en-3β,7β-diol
(+) cells				
CL/BSA:	NA	31 ± 5.23	185 ± 5	9.16 ± 0.99
+ 10 μM probucol	NA	100 ± 9.3	188 ± 13	0.1 ± 0.06
+ 10 μM α-tocopherol	NA	73 ± 10	156 ± 21	0.22 ± 0.05
CA/BSA	8.04 ± 0.25	NA	60 ± 2.36	NEG
+ 10 μM probucol	82 ±	NA	145 ± 7	NEG
+ 100 μM α-tocopherol	72 ● 12	NA	210 ± 15	NEG
(−) cells				
CL/BSA	NA	42 ± 9.7	233 ± 79	7.36 ± 0.5
+ 10 μM probucol	NA	112 ± 15	187 ● 29	0
+ 100 μM α-tocopherol	NA	162 ± 49	165 ± 20	0
CA/BSA	35 ± 6	NA	135 ± 10	NEG
+ 10 μM probucol	112 ± 1	NA	193 ± 5	NEG
+ 100 μM α-tocopherol	125 ± 1	NA	226 ± 18	NEG

oxidation of lipid within LDL without preserving antioxidants within the lipoprotein itself, unlike natural antioxidants.[28] In our studies it is possible that although the level of lipid oxidation was greatly reduced by probucol (with or without cells) there remained sufficient products of lipid oxidation able to damage protein. Lipid oxidation is associated with the production of hydrogen peroxide[16,19] (and presumably its free radical products of decomposition) able to damage protein which would remain unaffected by an extremely lipophilic antioxidant such as probucol. More significantly perhaps, the extremely lipophilic nature of probucol is likely to reduce its ability to become closely associated with BSA. A close association of antioxidant with protein is essential in the inhibition of protein oxidation, a process which is site-specific for both hydrogen peroxide and lipid peroxide.[13,14,23]

Table 1 also shows that the oxidation product, cholest-5-en-3β,7β-diol, is not generated by cholesterol arachidonate to a GC-detectable level, despite the enhanced level of oxidation by arachidonate. Although the reasons for this are unclear, it is possible that the rates of oxidation do not permit the accumulation of this product, which may oxidise further. Alternatively, the chemical manner in which oxidising linoleate may interact with cholesterol to generate this product[12] may differ from that of oxidising arachidonate.

Ceroid pigment is an autofluorescent polymer of oxidised lipid and protein.[29] Lipid-laden cells within early human lesions (fatty streaks) contain ceroid, which led to the suggestion that macrophage-mediated lipid oxidation might be involved in the development of atherosclerosis.[30] The importance of the ceroid found in human atherosclerosis is that it is probably at least partly composed of oxidised LDL. The evidence in favour of this includes the shared characteristic multilamellar ultrastructure,[31] the fact that naturally-occurring antibodies to ceroid cross-react with

oxidised LDL,[2] the depletion of linoleate and arachidonate in both ceroid and oxidised LDL[31] and the similarity of their fluorescence characteristics to each other and to lesion-derived LDL.[31] Since the effect of probucol appears to be primarily as an antioxidant for lipids, shown by studies using either LDL or artificial lipoproteins, then its beneficial effects *in vivo*[9] and in cell culture systems imply that lipid peroxidation is the primary cause of an elevated atherogenic nature of oxidised LDL, an observation in keeping with current consensus,[32] rather than the oxidation of apo-B, *per se*. However, the more potent effects of α-T in preventing protein oxidation (monitored by degradation) and subsequent uptake of LDL and artificial lipoproteins by macrophages shown within these experiments may suggest otherwise. These *in vitro* findings may also suggest that lipid peroxidation may not be the sole cause of apo-B modification relevant to the development of atherosclerosis.

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