

Studies on epitopes on low-density lipoprotein modified by 4-hydroxynonenal

Biochemical characterization and determination

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Oxidation of human low-density lipoprotein (LDL) was found to be accompanied by the generation of various reactive aldehydes. One of them, 4-hydroxynonenal (HNE), was shown to modify LDL to a form which represents a good model of oxidized LDL (ox-LDL). In order to investigate the epitopes newly formed on HNE-modified LDL, a polyvalent antiserum to HNE-LDL [anti-(HNE-LDL)] was raised in rabbits and the non-specific components were removed with native LDL coupled to CNBr-Sepharose 4B. Competitive fluorescence immunoassay analysis showed that anti-(HNE-LDL) recognized HNE-LDL, copper-oxidized LDL, HNE-albumin and to a lower extent HNE-modified high-density lipoprotein 3 (HNE-HDL₃) and ox-HDL₃ but not native LDL. A certain degree of cross-reactivity of the antibody with LDLs modified by either hexanal or 2,4-heptadienal was found. No reaction was obtained with LDL labelled with malondialdehyde. From the abilities of HNE-modified poly(L-amino acids) to compete with HNE-LDL for binding to anti-(HNE-LDL), it is postulated that lysine, tyrosine, arginine and histidine are involved in the formation of HNE-derived epitopes on apolipoprotein B (apo B). Using a double-sandwich fluorescence immunoassay [capture antibody: anti-(apo B); detection antibody: anti-(HNE-LDL)] we found that the HNE-derived epitopes were expressed at a far higher degree in ox-LDL and HNE-LDL than in native LDL.

INTRODUCTION

It is well known that elevated plasma levels of low-density lipoprotein (LDL) are associated with accelerated atherosclerosis. The mechanism for the action of LDL in atherosclerosis *in vivo* is not clear but several lines of evidence indicate that oxidatively modified LDL (ox-LDL) might play a significant role in atherogenesis [1–4]. Ox-LDL is able to cause accumulation of large amounts of cholesteryl esters in macrophages which is characteristic for early atherosclerotic lesions. Ox-LDL is highly cytotoxic, facilitates the recruitment and the retention of monocyte macrophages *in vitro* and in this way it might contribute to the progression of early fatty-streak lesion to the more complicated lesion *in vivo*. Antioxidants such as probucol, which have the ability to protect LDL from oxidation, were shown to prevent the progression of atherosclerosis in Watanabe-heritable hyperlipidaemic rabbits [5,6].

LDL oxidation is a complex process which is initiated by a free-radical lipid-peroxidation mechanism [2]. Experiments *in vitro* show that oxidation can be promoted by endothelial cells [7], smooth-muscle cells [8], monocytes and macrophages [9], as well as bivalent transition-metal ions such as copper or iron [10]. During the oxidative degradation of polyunsaturated fatty acids in LDL, a variety of reactive aldehydic products are generated which are capable of attaching covalently to apolipoprotein B (apo B). 4-Hydroxynonenal (HNE) is one of these major products. Compared with malondialdehyde (MDA), another major aldehyde product formed in LDL during oxidation, the concentration of HNE within the LDL particle is higher, even though its total production during the process of LDL oxidation is lower [11]. Studying the modification of LDL with certain

aldehydes derived from lipid peroxidation of polyunsaturated fatty acids, Jürgens *et al.* [12] showed that HNE was incorporated into LDL more rapidly than MDA *in vitro*. Thus HNE-modified LDL (HNE-LDL) became a subject of closer investigation.

HNE-LDL showed decreased binding affinity to LDL receptors on human fibroblasts [13] and led to an overload of lipids in macrophages [14]. HNE-derived epitopes were found to exist not only on ox-LDL since they were also expressed on oxidized very-low-density lipoprotein and lipoprotein (a) [15]. Recently, Palinski *et al.* [16] developed polyvalent and monoclonal antibodies to reduced (by cyanoborohydride) HNE-LDL. These antibodies reacted with HNE-lysine conjugates on apo B and a positive immunostaining was obtained in the atherosclerotic lesions of WHHL rabbits [17]. Rosenfeld *et al.* [18] demonstrated that macrophage-derived foam cells within sections of the atherosclerotic lesions from the ballooned rabbit exhibit positive immunoreactivity with HNE-lysine-specific antibodies. Since it is not clear if reducing conditions during a reaction of HNE with apo B will occur *in vivo* and if lysine is the only amino acid residue involved in the attaching of HNE to the apo B molecule, we used human HNE-LDL modified under non-reducing conditions to immunize rabbits for production of an antiserum. This paper describes the specificity of this antiserum. Secondly, the immunochemical properties of HNE-derived epitopes formed during the LDL modification are compared with the epitopes derived from other aldehydes. Thirdly, certain HNE-modified poly(L-amino acids) were tested with a competitive fluorescence immunoassay to estimate their potential roles in the HNE modification. Finally, we compared the amount of HNE-derived epitopes on native LDL with ox-LDL and HNE-LDL by a double-sandwich fluorescence immunoassay.

Abbreviations used: HNE, 4-hydroxynonenal; MDA, malondialdehyde; LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo B, apolipoprotein B; ox-LDL, oxidized LDL; anti-(HNE-LDL), antiserum to HNE-modified LDL (HNE-LDL); PBS, phosphate-buffered saline.

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Table 1. Characteristics of five HNE-modified poly(L-amino acids)

The concentration of HNE was 6 mM in each case. Relative electrophoretic mobility is shown relative to the mobility of the relevant unmodified poly(L-amino acid). The HNE-LDL IC₅₀ is the concentration of HNE-modified poly(L-amino acid) (mean ± S.D.) required for 50% inhibition of the binding of anti(HNE-LDL) to HNE-LDL.

	pH	Relative electrophoretic mobility	HNE-LDL IC ₅₀ (μg/ml)
HNE-poly(L-lysine)	8.0	1.86	8.24 ± 0.35
HNE-poly(L-tyrosine)	10.5	–	39.50 ± 1.12
HNE-poly(L-arginine)	6.0	5.50	93.72 ± 0.73
HNE-poly(L-histidine)	6.0	–	2506.0 ± 35.0
HNE-poly(L-proline)	6.5	–	0

MATERIALS AND METHODS

Lipoprotein isolation

Lipoproteins were isolated from plasma of young healthy fasting donors in which serum lipoprotein (a) levels were lower than 1 mg/dl. Chloramphenicol (50 mg/l; Serva, Heidelberg, Germany), kallikrein inactivator (Trasylol, 100000 units/l; Bayer, Leverkusen, Germany), butylated hydroxytoluene (20 μM; Sigma, St. Louis, MO, U.S.A.) and EDTA (1 g/l; Merck, Darmstadt, Germany) were present during all steps of lipoprotein preparation to prevent lipid peroxidation and apo B cleavage by contaminating bacteria or proteinases. LDL (1.020–1.050 g/ml) and high-density lipoprotein 3 (HDL₃, 1.125–1.21 g/ml) were obtained by sequential ultracentrifugation and the density was adjusted by adding solid KBr. HDL₃ was purified once more on a heparin-coupled Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) to remove apo E. All lipoprotein fractions were verified by agarose-gel electrophoresis (Lipidophor; Immuno AG, Vienna, Austria). They were sterile-filtered for further use and stored at 4 °C. Protein content was measured by the method of Lowry *et al.* [19] with BSA (Sigma) as the standard.

Modification of the lipoproteins

HNE was synthesized as described previously [20]. MDA was obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane [21] and its content was measured as described [22]. Hexanal and 2,4-heptadienal were supplied by Aldrich (Steinheim, Germany). After extensive dialysis against N₂-saturated 0.1 M-phosphate-buffered saline (PBS), pH 7.4, containing 1 g of EDTA/l as well as 50 mg of chloramphenicol/l, 0.5 mg of LDL was incubated with 20 mM-MDA, 3 mM-HNE, 5 mM-2,4-heptadienal or 6 mM-hexanal in the dark at 37 °C for 5 h. Free aldehyde was removed by dialysis against the buffer mentioned above. The procedures of modification of poly(L-amino acids) were the same as for the lipoprotein except that the buffer pH was changed according to individual poly(L-amino acid) requirements [23]. Table 1 lists the experimental conditions of poly(L-lysine) (*M_r* 10000; Serva), poly(L-tyrosine) (*M_r* 40000–100000; Serva), poly(L-arginine) (*M_r* 11600; Sigma), poly(L-histidine) (*M_r* 5000; Serva) and poly(L-proline) (*M_r* 10000; Sigma).

Oxidation of the lipoproteins was performed using a ratio of lipoprotein to Cu²⁺ of 0.3 mg of protein/ml to 10 μM-CuCl₂. Before oxidation, the lipoproteins were carefully dialysed against 0.01 M-PBS, pH 7.4, which was degassed and then saturated with N₂ before use. After incubation with Cu²⁺ at 37 °C, LDL (24 h) and HDL₃ (6 h) respectively, the lipoproteins were dialysed against 0.01 M-PBS (pH 7.4) containing 1 g of EDTA/l. Reactive amino groups in protein were estimated by the trinitrobenzene-

sulphonic acid assay [24]. The electrophoretic mobility of aldehyde-modified or oxidized LDL was measured relative to the non-modified native fraction on Lipidophor.

Production and purification of the antiserum

Rabbits were immunized by intracutaneous injections of HNE-LDL (500 μg of protein) emulsified in complete Freund's adjuvant. Two booster injections at multiple sites were given at 4-week intervals. The components non-specific for HNE adducts of this antiserum were adsorbed by affinity chromatography. Briefly, 10 mg of native LDL was coupled to about 2 ml of swollen CNBr-activated Sepharose 4B as described in the instruction manual (Affinity Chromatography; Principles and Methods; Pharmacia LKB Biotechnology, S-75182 Uppsala, Sweden) except that 0.1 mg of EDTA/ml was added to the working buffer. Then 1 ml of antiserum was incubated with 0.3 ml of LDL-coupled CNBr-Sepharose 4B under N₂ at room temperature for 3 h and the supernatant was collected. The same procedure was repeated three times with the fresh antigen-coupled gels and the purification efficiency was monitored by a competitive fluorescence immunoassay.

Solid-phase fluorescence immunoassay

For the binding assay, poly(vinyl chloride) microtitration plates (A/S Nunc, Roskilde, Denmark) were coated with 200 μl of HNE-LDL (1 μg/ml) in 50 mM-sodium carbonate buffer (pH 9.6) containing 1 mg of EDTA/ml at 4 °C overnight. After they had been washed once with washing buffer (50 mM-Tris/HCl buffer, pH 7.7, containing 9 g of NaCl/l, 0.2 g of Tween 20/l and 0.5 g of NaN₃/l) using a microtitration plate washer (Wallac Oy, Turku, Finland), 250 μl of blocking buffer [50 mM-NaH₂PO₄ · H₂O, 5 g of BSA/l and 60 g of sorbitol/l (Sigma)] was added to the wells to block the remaining binding sites. The wells were then washed twice and 200 μl of serial dilutions of antiserum were plated and incubated for 2 h at room temperature. After three washes, the amount of rabbit IgG bound was detected by adding 50 ng of a goat anti-rabbit IgG (Sigma) labelled with europium (DELFLIA, Eu-labelling kit 1244-302, Pharmacia) per well according to the manufacturer's description. The labelling yield was 10 Eu³⁺/IgG (mol/mol). After incubation for 1 h at room temperature, the plates were washed six times. Then 200 μl of enhancement solution (Pharmacia)/well was added to measure the fluorescence counts in the well with a 1234 DELFLIA research fluorimeter (Wallac Oy). The titre of the antibody was defined as the reciprocal of the greatest dilution of the antiserum that gave a specific binding three times greater than the normal rabbit serum used as a blank.

Competitive solid-phase fluorescence immunoassays were performed similarly, except that 100 μl of fixed dilution of the primary antibody was added together with an equal volume of assay buffer [50 mM-Tris/HCl buffer, pH 7.7, containing 5 g of BSA/l, 0.5 g of bovine globulin/l (Sigma), 0.5 g of NaN₃/l, 8 mg of diethylenetriaminepenta-acetic acid (Sigma)/l, 9 g of NaCl/l and 100 μl of Tween 20/l] containing increasing amounts of potential competitors. The results were expressed as *B/B₀*, where *B* is the amount of antibody bound to the coated antigen in the presence of competitor and *B₀* that in the absence of competitor.

HNE-modified apo B and apo B were measured by a double-sandwich fluorescence immunoassay. A goat anti-(human apo B) (Sigma) was used as capture antibody (1 μg/ml and 200 μl/well) for both measurements. After blocking and washing as mentioned above, 100 μl of the fixed dilution of anti-(HNE-LDL) was added together with 100 μl of the sample for measuring HNE-derived epitopes on apo B. Meanwhile 100 μl of the fixed dilution of rabbit anti-(human apo B) (Behring, Marburg, Germany) and 100 μl of the same sample were taken to measure the content of

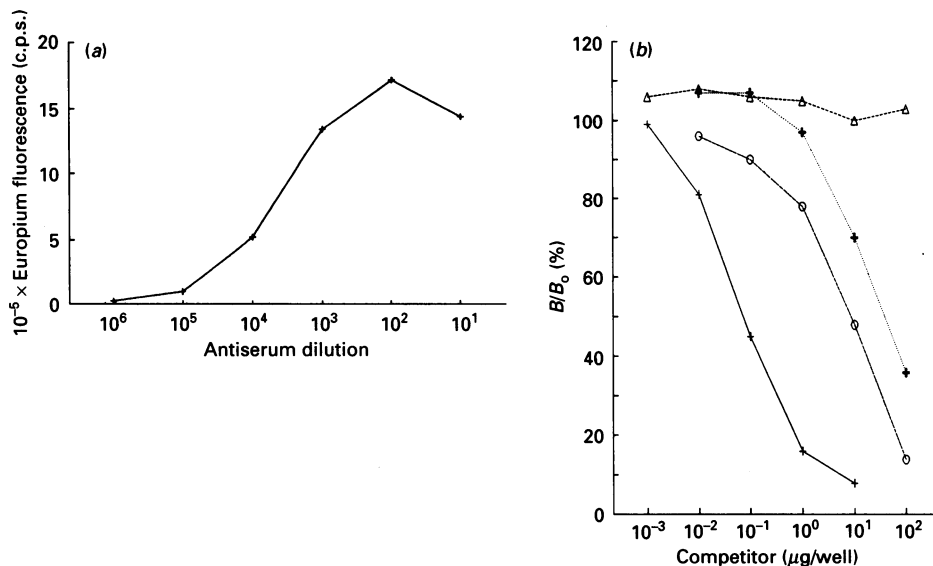


Fig. 1. (a) Binding of rabbit antiserum against HNE-LDL to immobilized human HNE-LDL (coated at 1 μg/ml) in a solid-phase fluorescence immunoassay and (b) solid-phase competitive fluorescence immunoassay of rabbit antiserum against HNE-LDL with human LDL (Δ), HNE-LDL (+—+) and ox-LDL (6 h, + ··· +; 24 h, ○)

(a) The amount of antibody bound was detected by using europium-labelled goat anti-rabbit IgG. (b) The wells were coated with human HNE-LDL (1 μg/ml), and a 1:10000 dilution of the antiserum was added in the absence or presence of increasing amounts of competitors. To each well, 50 ng of europium-labelled goat anti-rabbit IgG was added as the detection antibody. The results are expressed as described in the Materials and methods section. Each point represents the mean of triplicate determinations.

apo B. HNE-LDL, originating from the interaction of 0.5 mg of LDL/ml with 3 mM-HNE (26% of its active amino groups were modified) was used as the standard to evaluate HNE-derived epitopes. A standard curve was plotted in each assay. For apo B measurement, a lipid control serum (Precinorm L; Behring) was used as the standard. The values of HNE-derived epitopes and apo B were calculated from the relevant standard curve. The final result for HNE-derived epitopes on apo B was expressed as the value of HNE-derived epitopes divided by the value of apo B.

RESULTS

Characteristics of the antiserum to human HNE-LDL

When human HNE-LDL was used to immunize rabbits, the antiserum produced contained components non-specific for HNE adducts since human LDL is heterologous to rabbit. In order to get rid of these non-specific components, we applied the rabbit antiserum to a CNBr-Sepharose 4B column to which native human LDL was coupled. During the process of antiserum purification the ability of native human LDL to compete with HNE-LDL for binding to the antiserum decreased gradually along with the titres of antibody when monitored by the fluorescence immunoassay (results not shown). Fig. 1(a) shows the binding properties of anti-(HNE-LDL) to HNE-modified human LDL after purification of the antibody by affinity chromatography. Fig. 1(b) depicts the binding characteristics of anti-(HNE-LDL) to native, HNE-modified and oxidized LDL. Although a 10-fold concentration of native LDL was used in this test, no sign of competition with HNE-LDL was observed. However, when two different oxidatively modified LDLs were used to compete with HNE-LDL for binding to anti-(HNE-LDL), strong competition was observed. The extent of competition of ox-LDL depended on the degree of oxidative modification of LDL.

To test whether anti-(HNE-LDL) recognizes HNE adducts attaching only to apo B, we used HNE to modify other plasma proteins such as the apolipoproteins of HDL₃ and albumin. Fig.

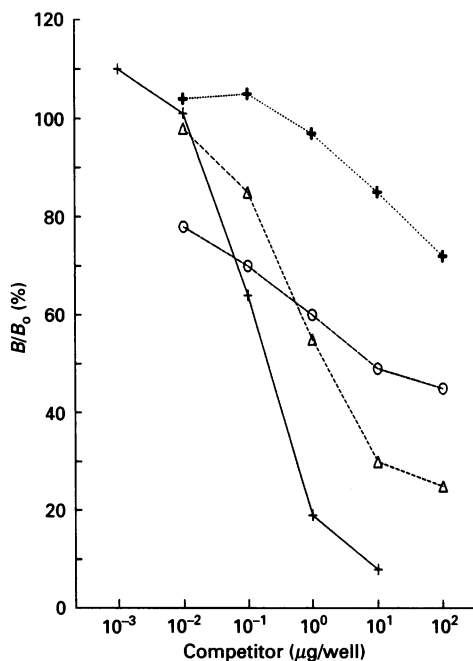


Fig. 2. Solid-phase competitive fluorescence immunoassay of rabbit antiserum against HNE-LDL with HNE-LDL (+—+), HNE-BSA (Δ), HNE-HDL₃ (○) and ox-HDL₃ (+ ··· +)

2 shows that both of these two HNE-modified proteins bind to anti-(HNE-LDL) to a certain degree (HNE-albumin > HNE-HDL₃). Furthermore, oxidized HDL₃ also shows binding ability, but this is lower than that of HNE-modified proteins.

Cross-reactivity of anti-(HNE-LDL) with LDLs modified by 2,4-heptadienal, hexanal and MDA

During the process of LDL oxidation, a number of highly

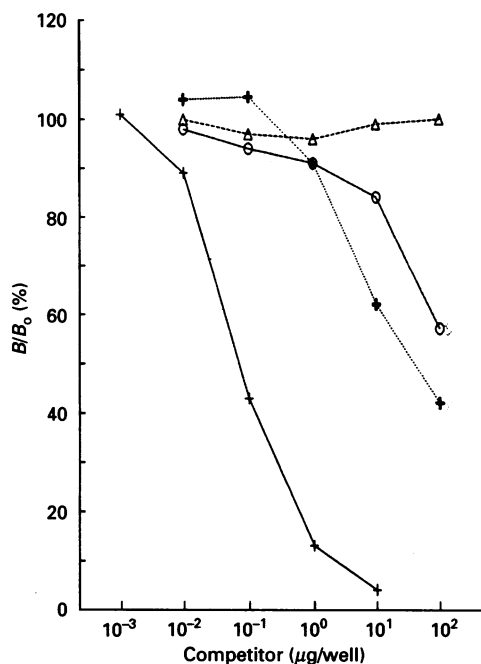


Fig. 3. Solid-phase competitive fluorescence immunoassay of rabbit anti-serum against HNE-LDL with human LDLs modified by HNE (+—+), MDA (△), 2,4-heptadienal (○) and hexanal (+.....+)

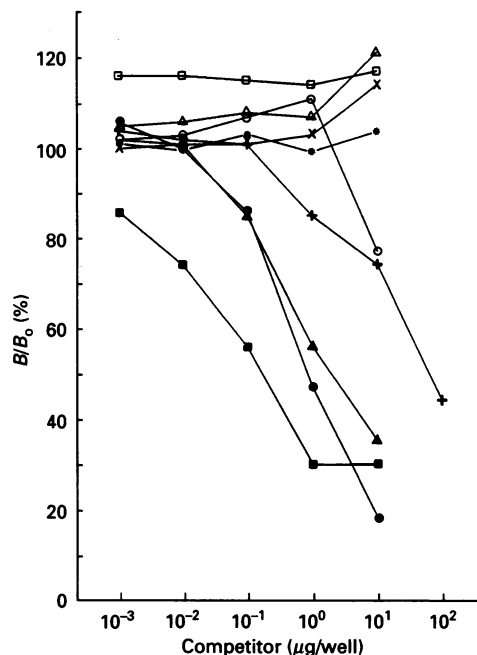


Fig. 4. Solid-phase competitive fluorescence immunoassay of rabbit anti-serum against HNE-LDL with various poly(L-amino acids)

□, Lys; ■, HNE-Lys; ○, Tyr; ●, HNE-Tyr; △, Arg; ▲, HNE-Arg; ×, His; +, HNE-His; *, HNE-Pro. Each value represents the mean of triplicate determinations.

reactive short-chain aldehydes were generated and some of them could covalently attach to apo B [11]. The immunochemical relationship between LDL modified by HNE and LDLs modified by the three other important aldehydes was investigated (Fig. 3). MDA was selected since it was generated during LDL oxidation in quantities comparable with HNE and our earlier experimental data demonstrated that 93% of MDA is distributed in the aqueous phase. In contrast, 98% of 2,4-heptadienal remained within the lipid phase of LDL. The aldehyde generated at the highest concentration during LDL oxidation was hexanal [11]. As shown in Fig. 3, only MDA-LDL does not compete with HNE-LDL for binding to the anti-(HNE-LDL) antibody, whereas the LDLs modified with 2,4-heptadienal and hexanal in part do.

Since the inability of MDA-LDL to compete with HNE-LDL for the antibody could be the result of not enough modification, we compared the relative electrophoretic mobilities, amounts of reactive amino groups and native apo B epitopes on LDLs

modified by the different aldehydes (Table 2). Of the four modified LDLs tested, the MDA-modified LDL showed the highest degree of modification as expressed by its relative electrophoretic mobility. MDA-LDL also lost the most reactive amino groups. However, the amounts of native apo B epitopes left on MDA-LDL are similar to those on HNE-LDL.

Evaluation of the amino acid residues involved in the formation of HNE-derived epitopes on apo B

As an approach to exploring which amino acid residues are involved in the modification of apo B by HNE, five poly(L-amino acids) were incubated with HNE under different pH conditions (Table 1). The increased relative electrophoretic mobilities of HNE-poly(L-lysine) and HNE-poly(L-arginine) were measured by agarose-gel electrophoresis but those of the others could not be estimated because they were not present in the system used (Lipidophor). Both untreated and HNE-treated poly(L-amino

Table 2. Modification indexes of LDL modified by HNE, MDA, 2,4-heptadienal and hexanal

The reactive amino groups (mol of NH_2 /mol of apo B) were calculated assuming an M_r of 513000 for apo B [25]. For measurement of expression of native apo B epitopes, 200 μl of lipoprotein (1 μg of apo B/ml) in coating buffer was added to each well of microwell plates at 4 °C overnight. After the plates had been washed and blocked as described in the Materials and methods section 200 μl of rabbit anti-(human apo B) serum (diluted 1:20000) was added to bind to coated apo B, and 200 μl of europium-labelled anti-rabbit IgG was used as detection antibody.

	Relative electrophoretic mobility	Reactive amino groups		Expression of native apo B epitopes	
		(mol of NH_2 /mol of apo B)	(%)	($10^{-3} \times \text{c.p.s.}$)	(%)
Native LDL	1.0	630.5 ± 4.9	100	1166.0 ± 7.1	100
HNE-LDL	1.6	464.0 ± 11.3	74	985.5 ± 34.6	84
MDA-LDL	2.5	219.5 ± 3.5	35	979.5 ± 34.6	84
2,4-Heptadienal-LDL	1.4	434.5 ± 16.3	69	1181.0 ± 35.4	101
Hexanal-LDL	1.3	412.0 ± 52.3	65	1074.5 ± 3.5	92

Table 3. Levels of HNE-derived epitopes on isolated LDL

Goat anti-(human apo B) (200 ng) was used for coating the wells. After the unspecific binding sites had been blocked (see the Materials and methods section), 100 μ l of rabbit anti-(HNE-LDL) serum (diluted 1000-fold with the assay buffer) and 100 μ l of each sample were incubated in the wells to determine HNE-derived epitopes on apo B. In parallel, 100 μ l of rabbit anti-(apo B) serum (diluted 10000-fold) and 100 μ l of each sample were incubated in the relevant wells to measure the levels of apo B. Europium-labelled goat anti-rabbit IgG (50 ng) was used as the detection antibody. Results are means \pm s.d. ($n = 3$).

	HNE-apo B/native apo B
LDL	0.0056 \pm 0.0025
HNE-LDL	0.65 \pm 0.078
Ox-LDL	0.81 \pm 0.97

acids) were tested in a competitive fluorescence assay. As depicted in Fig. 4, all untreated poly(L-amino acids) were unable to compete with HNE-LDL for binding to anti-(HNE-LDL) except poly(L-tyrosine) (the maximum competitive inhibition in the experiment was 24%). If the concentration of the competitor is fixed, the ranking of the tested HNE-poly(L-amino acids) is as follows: HNE-poly(L-lysine) > HNE-poly(L-tyrosine) > HNE-poly(L-arginine) > HNE-poly(L-histidine) (Table 1), although the maximum competitive inhibition of HNE-poly(L-lysine) (70%) is lower than that of HNE-poly(L-tyrosine) (83%). HNE-poly(L-proline) does not compete with HNE-LDL for binding to anti-(HNE-LDL).

Measurement of HNE-derived epitopes on apo B

A double-antibody sandwich fluorescence immunoassay was established to measure the HNE-derived epitopes on apo B. In order to focus on the modification of the aldehyde on apo B, we used a goat anti-(human apo B) antibody as the capture antibody. Since the level of apo B in the tested samples could interfere with the results, the apo B concentration was determined as well as the concentrations of HNE-derived epitopes in the same sample at the same time. The final result was expressed as the ratio of HNE-derived epitope/apo B. Table 3 gives data for isolated LDL. The ratio of HNE-derived epitope/apo B from HNE-LDL was 100-fold higher than that of native LDL. Ox-LDL (copper-induced for 24 h) had the highest ratio.

DISCUSSION

It is well known that during oxidation of LDL a lot of reactive aldehydic products are generated and many of them can attach covalently to apo B [11]. The use of antibodies against these oxidation-specific aldehyde epitopes has accelerated the progress of studies on the atherogenicity of ox-LDL [15,17,18,25–27]. The antibody used in this paper could recognize HNE-LDL and ox-LDL but not native LDL, as demonstrated by competitive fluorescence immunoassay (Fig. 1b). This is consistent with our earlier results obtained using an agarose double-immunodiffusion test [15]. Further tests showed that not only HNE-LDL but also other HNE-modified proteins (e.g. HNE-HDL₃, HNE-albumin) could be recognized by the antibody, suggesting that this antibody is specific for HNE-derived epitopes regardless of the origin of the protein. This result is similar to our earlier report [15] and the data reported by Palinski *et al.* [16] on a monoclonal antibody to 4-HNE-reduced LDL which recognized 4-HNE-reduced LDL as well as 4-HNE-reduced albumin.

In order to characterize the specificity of anti-(HNE-LDL) in

more detail, we compared the binding abilities of the LDLs modified by four kinds of aldehyde to anti-(HNE-LDL). A slight cross-reactivity of anti-(HNE-LDL) with 4-hydroxyoctenal had been found earlier [15]. However, unexpectedly, LDLs modified by 2,4-heptadienal and hexanal could also compete with HNE-LDL for binding to the antibody whereas MDA-LDL could not, which is in agreement with previous results [15] (Fig. 3). This might be due to the fact that MDA, as a bivalent aldehyde, might react in a completely different manner from univalent aldehydes, such as HNE, hexanal and 2,4-heptadienal, with certain residues on apo B.

Furthermore, we wanted to investigate more closely which amino acid residues apart from lysine are involved in the formation of HNE-derived epitopes on apo B. We approached this by modifying several poly(L-amino acids) with HNE and used these products as competitors for the binding of HNE-LDL to anti-(HNE-LDL) in a fluorescence immunoassay. We found that HNE-poly(L-lysine), HNE-poly(L-tyrosine), HNE-poly(L-arginine) and HNE-poly(L-histidine) (to a minor extent) were able to compete. The HNE-modified poly(L-amino acids) did not completely inhibit the binding of the antibody to HNE-LDL even when the concentration of the competitors was raised or different competitors were combined (results not shown). One possible explanation might be that modification of LDL by HNE leads to changes in the folding of apo B on the surface of LDL. Therefore our polyclonal antiserum to HNE-LDL might also contain antibodies directed against epitopes of apo B that were buried in the lipid shell before modification of LDL and might not contain HNE. Thus it seems to be impossible to block completely the binding of anti-(HNE-LDL) to HNE-LDL by HNE-derived epitopes formed on the polymers of amino acids.

Analysing the amino acid composition of HNE-LDL, Jürgens *et al.* [28] proved that, after modification of LDL by HNE, the contents of lysine, tyrosine, serine and histidine residues were decreased; Fong *et al.* [29] found that cooper-ion-induced oxidation of LDL resulted in a consistent decrease in histidine, lysine and proline residues. However, in our experiments HNE-poly(L-proline) had no ability to bind to anti-(HNE-LDL). Since the aliphatic side chain of proline has no functional groups, it probably cannot interact with HNE. As nucleophiles, however, arginine, lysine and histidine are good targets for attack by electrophilic compounds such as HNE. As to tyrosine, the hydroxy group on the phenolic ring possibly takes part in the reaction. The competitive ability of poly(L-serine) could not be tested since the polymer was not soluble in any buffers used [23]. To summarize, lysine, tyrosine, arginine and histidine residues are the likely candidates for interaction of apo B with HNE.

The susceptibility of LDL to oxidation might be an important factor in atherosclerosis, and several methods have been developed for assaying it [11]. The most commonly used assays are based on the determination of thiobarbituric acid-reactive substances or conjugated dienes [11,30]. However, the methodology of measuring the oxidation of LDL still needed to be improved because thiobarbituric acid-reactive substances represent only minor products in ox-LDL and there is only a poor correlation with the final products of lipid peroxidation [31]. Therefore a sandwich fluorescence immunoassay was established. In order to evaluate HNE-derived epitopes independently of the concentration of apo B, the latter was measured concomitantly with the determination of the HNE-derived epitopes. It is worth mentioning that the ratio of HNE-derived epitope/native apo B in the assay does not represent either the absolute amount of HNE-derived epitopes on apo B or the percentage of modified apo B in the native apo B because the concentration of standard HNE-LDL used in the assay was expressed as the protein concentration before modification. However, the changes assayed

would reflect the degree of oxidative modification with regard to the protein moiety. The data in Table 3 demonstrate that the number of HNE-derived epitopes on HNE-LDL, as recognized by our anti-(HNE-LDL), is more than 100-fold higher than that of native LDL. The highest amount of HNE-derived epitopes was found on ox-LDL.

The concept that oxidative modification of LDL takes place *in vivo* has been suggested by several authors, although most of them stressed this in relation to the cells and blood vascular walls [17,18,25–27]. However, Salmon *et al.* [32] described an e.l.i.s.a. technique able to recognize MDA-LDL in the serum of patients with cardiovascular diseases; Parums *et al.* [33] showed that antibodies to ox-LDL were detected in sera of 20 patients with clinical chronic periaortitis but not in healthy young adults. These findings are in agreement with a recently published article showing a strong correlation of autoantibodies against MDA-LDL with progression of carotid atherosclerosis in Finnish men [34]. Avogaro *et al.* [35,36] and Shimano *et al.* [37] isolated a small more-electronegative LDL in human plasma. The crucial point, however, is to develop an assay by which a possible modified LDL can be directly detected and measured in serum in order to avoid generation of artifacts during the preparation of LDL.

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