Binding characteristics of scavenger receptors on liver endothelial and Kupffer cells for modified low-density lipoproteins

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Previous studies showed that both endothelial and Kupffer cells contain specific recognition sites of oxidized low-density lipoprotein (OxLDL), in addition to recognition sites which recognize OxLDL and acetylated LDL (AcLDL). We have determined the binding characteristics of the recognition sites for OxLDL on Kupffer cells and endothelial cells (OxLDL-specific binding-site) in comparison to the recognition site for AcLDL on endothelial cells, which recognizes both AcLDL and OxLDL (Ac/OxLDL binding site). The capacity of Kupffer cells to bind OxLDL ($B_{\max} = 779$ ng of ^{125}I -OxLDL/mg of cell protein; $K_d = 6$ $\mu\text{g/ml}$) was comparable to the binding-capacity of endothelial cells ($B_{\max} = 803$ ng of ^{125}I -OxLDL/mg of cell protein; $K_d = 5$ $\mu\text{g/ml}$). The effect of net charge of modified LDL on its affinity for the recognition sites on Kupffer and endothelial cells was evaluated using competition studies. The affinity of AcLDL for the Ac/OxLDL binding site was greatly increased from 460 $\mu\text{g/ml}$ to 4 $\mu\text{g/ml}$ with increasing extent of modification and thus net charge. The Ac/OxLDL binding-site on endothelial cells also

displayed an increased affinity towards LDL with an increasing degree of oxidation. The affinity of OxLDL for the Ac/OxLDL binding-site appeared to be about 4-fold higher than that of AcLDL with a similar extent of modification. At higher degrees of oxidation of LDL, the affinity for the OxLDL-specific site on endothelial and Kupffer cells was also strongly enhanced; the OxLDL-specific binding-site possesses a higher affinity for mildly oxidized LDL as compared with the Ac/OxLDL binding-site. It is concluded that recognition of OxLDL by both the OxLDL-specific binding-site and the Ac/OxLDL binding-site on liver endothelial and Kupffer cells depends on the net negative charge of modified LDL. The similarity in binding pattern of these binding sites makes it likely that the newly described 95 kD OxLDL binding protein on Kupffer cells [Y. B. De Rijke and Th. J. C. van Berkel, *J. Biol. Chem.* (1994), **269**, 824–827] contains a recognition site with similar structural elements as described earlier for scavenger receptors.

INTRODUCTION

Scavenger receptors are suggested to play a role in the development of atherosclerosis (for review see Steinberg et al. [1]). Various modifications of low-density lipoprotein (LDL), including acetylation [2], malondialdehyde treatment [3] and oxidative modification [4], result in effective recognition of LDL by scavenger receptors [5–7].

At present, oxidatively modified LDL (OxLDL) is considered to be a highly atherogenic particle as previously proposed by Steinberg et al. [1]. After being oxidized in the intima, OxLDL is probably taken up by scavenger receptors(s) on macrophages. As a result of the accumulation of OxLDL, these cells are converted into cholesterol-rich foam cells [8,9], which is a characteristic feature of early atherosclerotic lesions. Recently, scavenger receptors have been cloned from bovine liver and lung [10,11]. It appeared to be a membrane-bound 220 kDa protein, containing a positively charged C-terminal domain which is implicated in ligand binding [12,13].

Although vascular endothelial cells and macrophages possess scavenger receptors, the liver is quantitatively the most important organ in the scavenger receptor-mediated elimination of modified lipoproteins from the blood circulation [5,7]. Therefore, the hepatic scavenger receptors may constitute a protection system against the pathological effects of OxLDL. Evidence for this hypothesis was provided by previous studies on the fate of modified LDL *in vivo* demonstrating that, in rats, acetylated LDL (AcLDL) was mainly cleared via endothelial liver cells [5],

whereas OxLDL was mainly taken up by Kupffer cells [7]. Studies with isolated liver cells showed that OxLDL association with endothelial and Kupffer cells could be completely inhibited by OxLDL and only partly by AcLDL, while AcLDL association with endothelial and Kupffer cells was completely blocked by both AcLDL and OxLDL [7]. This suggests that, besides the Ac/OxLDL-recognizing scavenger receptor, the liver contains a second scavenger receptor, recognizing only OxLDL.

Recent ligand blotting studies from our laboratory confirmed this hypothesis [14]; it was demonstrated that both endothelial and Kupffer cell membranes express a 220 kDa protein recognizing both AcLDL and OxLDL, which probably corresponds with the scavenger receptor cloned by Kodama et al. [10] and Rohrer et al. [11]. In addition to this protein, a 95 kDa protein, specifically recognizing OxLDL, could be identified on Kupffer cell membranes. This OxLDL binding-protein forms the most likely candidate for the specific ‘oxidized lipoprotein receptor’ in the liver.

In this study, we have determined the binding parameters of the OxLDL-specific binding sites and the Ac/OxLDL binding sites on both liver endothelial and Kupffer cells. In addition we have compared the requirements for ligand recognition by the OxLDL-specific site with that of the Ac/OxLDL binding site.

MATERIALS AND METHODS

Materials

Collagenase (type IV), BSA (fraction V) and *t*-butylated 4-

Abbreviations used: LDL, low-density lipoprotein; Ac, acetylated; Ox, oxidized; REM, relative electrophoretic mobility; BHT, *t*-butylated 4-hydroxy-toluene; TNBS, trinitrobenzenesulphonic acid.

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hydroxytoluene (BHT) were obtained from Sigma. Na^{125}I in 0.1 M NaOH (13.5 mCi/ μg) was purchased from Amersham, Buckinghamshire, U.K.

Isolation of low-density lipoproteins and modification

Blood from healthy volunteers was collected into $\text{K}_3\text{-EDTA}$ - (1 mg/ml) containing tubes. Plasma was separated from the blood and BHT (40 μM) in Me_2SO ($\leq 0.1\%$ v/v) was added. LDL ($\rho = 1.019\text{--}1.063$ g/ml) was isolated by density gradient ultracentrifugation as described by Redgrave et al. [15]. LDL was acetylated as previously described by Basu et al. [16]. The extent of acetylation was varied as follows. Under gentle stirring in an ice/water-bath, 1 ml of LDL (containing 20 μM BHT, with a protein concentration of 1–3 mg/ml) in PBS (containing 10.1 mM NaP_i and 150 mM NaCl, pH 7.4) was added to 1 ml of a saturated solution of sodium acetate. Acetic anhydride was added in multiple small portions of 2 μl over a period of 1 h. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein used, the mixture was stirred for an additional 30 min. The solution was then dialysed against PBS saturated with N_2 . AcLDL with a relative (to native LDL) electrophoretic mobility (REM) of 1.90 and 1.50 was achieved by using acetic anhydride, which was diluted 10–50 times before addition, respectively.

LDL was modified oxidatively by incubating LDL (200 $\mu\text{g}/\text{ml}$ in PBS) with 10 μM CuSO_4 at 37 °C for 1, 3, 6, 9, 12 and 20 h. Oxidation was arrested by immediate cooling to 4 °C and subsequent addition of 200 μM EDTA. The preparations were concentrated using the Speed Vac (Savant Instruments), and subsequently dialysed against PBS/1 mM EDTA, under nitrogen at 4 °C. The mass-weighted particle size distribution of the modified lipoproteins (200 $\mu\text{g}/\text{ml}$ PBS/EDTA) was determined by photon correlation spectroscopy using the submicron particle analyser (Malvern 4700C, Malvern Instruments) at 25 °C and at a scattering angle of 90°. Moreover, it was established by particle-size analysis that the concentrated OxLDL preparations did not contain high-molecular-mass aggregates.

All lipoprotein preparations were stored in the presence of 40 μM BHT under nitrogen and used within 2 weeks. The electrophoretic mobility of modified lipoproteins was analysed on 0.75% agarose gels, using Tris/hippuric acid, pH 8.8. The electrophoretic mobility of the lipoprotein preparations did not increase during storage for 2 weeks. The extent of lysine modification of the lipoproteins was determined colorimetrically by the trinitrobenzenesulphonic acid (TNBS) method [17], using BSA as standard (60 lysine residues/mol [18]). LDL (50–100 μg of protein in PBS/EDTA) was mixed with 200 μl of 4% NaHCO_3 and 200 μl of 0.1% TNBS. After incubation for 2 h at 40 °C, 200 μl of 10% SDS and 100 μl of 1 M HCl were added and the absorbance at 335 nm was measured. The number of modified lysine residues per apolipoprotein B100 was calculated from the difference in values obtained for modified and unmodified lipoproteins, assuming apo B100 to have a molecular mass of 513 kDa [19]; 295 lysine residues were detected per mol of native LDL. OxLDL and AcLDL were radiolabelled according to the procedure of McFarlane [20] as modified by Bilheimer et al. [21]. The specific activity of the iodinated lipoproteins was 60–325 cpm/ng of protein.

Isolation of rat liver cells

For the isolation of liver endothelial and Kupffer cells, a collagenase procedure at 37 °C was performed as described previously [22]. Liver cells were separated by differential centri-

fugation and endothelial and Kupffer cells were subsequently purified by counterflow centrifugation [22]. The purity of endothelial and Kupffer cell preparations as monitored by peroxidase staining was at least 95 and 90%, respectively. The viability of the cells used for experiments *in vitro* was $\geq 90\%$ as judged by 0.2% Trypan Blue exclusion.

Binding of lipoproteins to liver endothelial and Kupffer cells *in vitro*

Saturation binding studies of ^{125}I -OxLDL and ^{125}I -AcLDL to endothelial and Kupffer cells were performed as follows. Kupffer cells (1.5×10^6 ; 150 μg of cell protein) or endothelial cells (2×10^6 ; 150 μg of cell protein) were incubated for 2 h at 4 °C with a variable concentration of radiolabelled ligand ranging from 0.5 to 60 $\mu\text{g}/\text{ml}$. Following incubation, cells were processed as described previously [22]. Non-specific binding was defined as the binding of radiolabelled ligand in the presence of 600 $\mu\text{g}/\text{ml}$ of unlabelled lipoprotein.

Displacement studies with endothelial and Kupffer cells were performed as previously described [22]. Kupffer and endothelial cells were incubated with ^{125}I -OxLDL (5 $\mu\text{g}/\text{ml}$) or ^{125}I -AcLDL (2 $\mu\text{g}/\text{ml}$) and a variable concentration of displacer, ranging from 0.5 to 700 $\mu\text{g}/\text{ml}$, for 2 h at 4 °C. Following incubation, cells were processed as described previously [22]. All incubations were carried out in Dulbecco's modified Eagle's medium, containing 2% (w/v) BSA in a total volume of 0.4 ml.

Figure 1 Saturation curves of ^{125}I -OxLDL-binding to liver endothelial and Kupffer cells

Isolated cells (a) endothelial and (b) Kupffer were incubated with increasing concentrations of ^{125}I -OxLDL in a total volume of 400 μl for 2 h at 4 °C. Non-specific binding was determined in the presence of 600 μg of OxLDL/ml. The total (●), specific (▲) and non-specific (○) binding values are expressed as ng of ^{125}I -OxLDL/mg of cell protein.

RESULTS

First we have performed saturation binding studies in order to define OxLDL and AcLDL binding to endothelial and Kupffer cells. ^{125}I -OxLDL-binding to both endothelial and Kupffer cells was saturable and of high affinity, having a Hill coefficient close to unity ($N^{\text{Hill}} = 1.11 \pm 0.13$ and 1.00 ± 0.17 , respectively) (Figure 1). The mean values of maximal specific binding (B_{max}) capacity of endothelial and Kupffer cells for OxLDL and AcLDL are shown in Table 1. The B_{max} for ^{125}I -OxLDL-binding to Kupffer cells appeared to be comparable with that for ^{125}I -OxLDL-

Table 1 Characteristics of ^{125}I -OxLDL- and ^{125}I -AcLDL-binding to liver endothelial and Kupffer cells

See Material and methods section for details. Non-specific binding of the lipoproteins was determined in presence of 600 $\mu\text{g}/\text{ml}$ of unlabelled lipoprotein. Maximal specific-binding and dissociation constants were determined by analysis of the binding isotherm using Graphpad ISI(R). Values represent mean $\pm$ S.E.M.

Cell type	B_{max} (ng/mg cell protein)	K_d ($\mu\text{g}/\text{ml}$)
^{125}I -OxLDL		
Endothelial cells ($n = 3$)	803 ± 21	5 ± 2
Kupffer cells ($n = 4$)	779 ± 155	6 ± 4
^{125}I -AcLDL		
Endothelial cells ($n = 3$)	156 ± 9	10 ± 3
Kupffer cells ($n = 7$)	45 ± 12	8 ± 2

Figure 2 Inhibition curves of ^{125}I -OxLDL-binding to liver endothelial and Kupffer cells by AcLDL and OxLDL

(a) Liver endothelial and (b) Kupffer cells were incubated with 5 $\mu\text{g}/\text{ml}$ of ^{125}I -OxLDL and either unlabelled 20 h OxLDL ($\circ$) or AcLDL ($\bullet$) in a total volume of 400 μl for 2 h at 4 $^{\circ}\text{C}$. The 100% binding value was determined in triplicate in the absence of competitors. Values represent the mean of two experiments. Non-specific binding of ^{125}I -OxLDL to endothelial and Kupffer cells was 7.6 and 8.4%, respectively.

binding to endothelial cells. The maximal binding-capacity of Kupffer cells for OxLDL showed a greater variation than the OxLDL binding-level found with endothelial cells. The dissociation constants of OxLDL binding to Kupffer and endothelial cells were similar ($K_d = 5\text{--}6$ $\mu\text{g}/\text{ml}$). The maximal binding capacity of endothelial cells for ^{125}I -AcLDL was 5-fold smaller than that for ^{125}I -OxLDL ($B_{\text{max}} = 156 \pm 9$ ng/mg and $B_{\text{max}} = 803 \pm 21$ ng/mg, respectively). The B_{max} of ^{125}I -AcLDL-binding to Kupffer cells was considerably lower than that to endothelial cells ($B_{\text{max}} = 45 \pm 12$ ng/mg), which is about 17-fold lower than the binding-capacity to Kupffer cells for ^{125}I -OxLDL.

In a previous study [7] we reported that AcLDL, at concentrations up to 500 $\mu\text{g}/\text{ml}$, inhibited only partly the cell association of ^{125}I -OxLDL to both Kupffer and endothelial cells at 37 $^{\circ}\text{C}$. To study the binding characteristics of the OxLDL- and AcLDL-binding sites of Kupffer and endothelial cells more quantitatively all experiments described in this study were performed at 4 $^{\circ}\text{C}$. Figure 2 shows the competition of ^{125}I -OxLDL-binding to endothelial and Kupffer cells with increasing concentrations of AcLDL and OxLDL. OxLDL completely inhibited ^{125}I -OxLDL-binding to both endothelial and Kupffer cells, whereas ^{125}I -OxLDL-binding was inhibited only partly by AcLDL. From the competition curves we can calculate that part of ^{125}I -OxLDL-binding that can be inhibited by an excess of AcLDL (% of total binding, B_x) and the apparent inhibition constants of AcLDL (K_i in $\mu\text{g}/\text{ml}$). The B_x and K_i for the inhibition of ^{125}I -OxLDL-binding to endothelial cells by AcLDL were $52 \pm 5\%$ and 29 ± 1 $\mu\text{g}/\text{ml}$, respectively, whereas for Kupffer cells, the B_x was $25 \pm 9\%$ with a K_i of 26 ± 6 $\mu\text{g}/\text{ml}$ (Figure 2). Thus, OxLDL is bound to an OxLDL-specific binding-site and to an Ac/OxLDL binding-site on liver endothelial and Kupffer cells, which is consistent with our previous studies at 37 $^{\circ}\text{C}$ *in vitro* [7].

Displacement of ^{125}I -AcLDL binding to liver endothelial cells by modified LDL

Previously, we have demonstrated that ^{125}I -AcLDL-binding to the endothelial cells can be inhibited completely by excess AcLDL as well as OxLDL [7]. It has been proposed that the net negative charge is the major determinant for high-affinity recognition by this receptor [23]. In order to assess the effect of the net charge of modified lipoproteins, as evaluated from the electrophoretic mobility, on the affinity for the Ac/OxLDL binding-site on endothelial cells, we performed displacement studies, using ^{125}I -AcLDL as ligand. As competitor we used LDL, acetylated to various extents, with REM values of 1.50, 1.94 and 2.50. All AcLDL preparations displayed monophasic inhibition of ^{125}I -AcLDL, with a Hill coefficient of 1.00 ± 0.10 (mean $\pm$ S.E.M.) (data not shown) (Table 2). Clearly, the affinity of AcLDL for the Ac/OxLDL binding-site on endothelial cells depends strongly on the relative electrophoretic mobility of the lipoprotein. AcLDL having an REM of 2.50 has a 115-fold higher affinity than AcLDL with an REM of 1.50. In analogy to AcLDL, the ability of OxLDL to inhibit AcLDL binding is dependent on the net charge of OxLDL. OxLDL with an REM of 2.43 is a 300-fold more potent inhibitor of AcLDL-binding than OxLDL with an REM of 1.57. However, over the whole range of REM values, OxLDL possessed a significantly higher affinity for the Ac/OxLDL site than AcLDL. Fully oxidized LDL (REM = 2.43) was a potent inhibitor of ^{125}I -AcLDL-binding to the endothelial cells, with an inhibition constant (K_i) which was 4-fold lower than that of AcLDL (REM = 2.50) itself (Table 2). Even oxidized OxLDL, with a slightly enhanced electrophoretic mobility (REM 1.57–1.76), was a better competitor for the ^{125}I -AcLDL binding-site than AcLDL with comparable electrophoretic behaviour.

Table 2 Inhibition constants of ^{125}I -AcLDL binding to liver endothelial cells by AcLDL and OxLDL

For experimental details see the Materials and methods section. Inhibition constants were determined by analysis of the competition curve using Graphpad ISI(R). The 100% value for binding of ^{125}I -AcLDL to endothelial cells was 60 ± 16 ng/mg of cell protein. Values are the mean of two independent experiments. REM, relative (to native LDL) electrophoretic mobility.

Modified LDL	REM	K_i ($\mu\text{g/ml}$)
AcLDL	1.50	460
	1.94	313
	2.50	4
OxLDL	1.57	287
	1.76	95
	2.43	1

Table 3 Inhibition constants of ^{125}I -OxLDL-binding to liver endothelial (EC) and Kupffer cells (KC) by LDL, oxidized to different extents

For experimental details see the Materials and methods section. Inhibition constants were determined by analysis of the competition curve using Graphpad ISI(R). **Displacement studies with LDL, oxidized for 1 and 6 h, were performed in duplicate; *as determined with the trinitrobenzenesulphonic acid assay [19]; REM, relative (to native LDL) electrophoretic mobility. The 100% values for binding of ^{125}I -OxLDL to Kupffer and endothelial cells were 199 ± 34 and 112 ± 18 ng/mg of cell protein, respectively. N.D., not determined.

Oxidation period (h)	REM	Mol Lys modified mol apo B100*	KC K_i ($\mu\text{g/ml}$)	EC K_i ($\mu\text{g/ml}$)
1**	1.09	N.D.	> 1000	> 1000
6**	1.72	64	440	> 1000
9	2.09	N.D.	38	87
12	2.17	N.D.	40	31
20	2.26	112	14	8

Effect of the degree of oxidation of LDL on the potency to inhibit ^{125}I -OxLDL-binding to the OxLDL-specific binding-site on liver endothelial and Kupffer cells

In order to analyse the effect of the net charge of LDL on its binding to endothelial and Kupffer cells, various degrees of oxidation were used (1–20 h). The relative (to LDL) electrophoretic mobility and the extent of lysine-modification of apolipoprotein B100 were measured (Table 3). Oxidation of LDL is accompanied by an increase in electrophoretic mobility (relative to native LDL) on agarose gels and a concomitant increase in the amount of modified lysine residues of apolipoprotein B100. After incubation of LDL with CuSO_4 for 20 h, 38% of a total of 295 reactive lysine residues per mol of LDL were modified as detected with the TNBS assay (Table 3).

In the displacement studies *in vitro*, the OxLDL-specific binding site on endothelial and Kupffer cells was studied in the presence of an excess of AcLDL, in order to block the Ac/OxLDL binding site on endothelial and Kupffer cells.

Figure 3 shows the competition studies for ^{125}I -OxLDL-binding to Kupffer and endothelial cells (under the aforementioned conditions) using native LDL and LDL, oxidized for 1, 6, 9, 12 and 20 h (referred to as 1 h OxLDL etc.) as inhibitor. Native LDL was not capable of displacing ^{125}I -OxLDL from the OxLDL-specific binding site on both endothelial and Kupffer cells at concentrations up to $700 \mu\text{g/ml}$. The binding of ^{125}I -OxLDL to endothelial cells was not significantly inhibited by 1 h OxLDL or 6 h OxLDL ($700 \mu\text{g/ml}$). Oxidation of LDL for 1

Figure 3 Displacement of ^{125}I -OxLDL-binding by LDL oxidized to various degrees

(a) Liver endothelial and (b) Kupffer cells were incubated with $5 \mu\text{g/ml}$ of ^{125}I -OxLDL in the presence of 125 and $100 \mu\text{g/ml}$ of AcLDL, respectively. Inhibition of the binding of ^{125}I -OxLDL was determined with increasing concentrations of native LDL ($\square$) and LDL, oxidized for 6–20 h with CuSO_4 at 37°C , in a total volume of $400 \mu\text{l}$, for 2 h at 4°C . The different OxLDL preparations were: 1 h OxLDL ($\blacktriangledown$), 6 h OxLDL ($\bullet$), 9 h OxLDL ($\blacklozenge$), 12 h OxLDL ($\triangle$) and 20 h OxLDL ($\circ$). The 100% binding value in the presence of AcLDL was 120 ± 21 and 224 ± 20 ng of ^{125}I -20 h OxLDL/mg of cell protein for endothelial and Kupffer cells, respectively ($n = 3$, mean $\pm$ S.D.).

(REM = 1.09) or 6 h [REM = 1.72; 64 (22%) modified lysine residues/mol apo B100] displayed an affinity for the OxLDL binding site on the Kupffer cells, while recognition by the endothelial cells became more apparent at oxidation times exceeding 9 h (REM = 2.09). 9 h OxLDL displaced ^{125}I -OxLDL with a K_i of $38 \mu\text{g/ml}$, while the OxLDL binding-site on endothelial cells needed a higher concentration (K_i of $87 \mu\text{g/ml}$). 12 h OxLDL displayed ^{125}I -OxLDL-binding to Kupffer and endothelial cells with a K_i of 31 and $40 \mu\text{g/ml}$, respectively, while 20 h OxLDL was the most potent inhibitor for OxLDL-binding to both cell types.

DISCUSSION

In this study we have compared the binding characteristics of the scavenger receptor, recognizing both AcLDL and OxLDL on liver endothelial and Kupffer cells (Ac/OxLDL site), and the specific OxLDL recognition-site on Kupffer cells and endothelial cells. Recent blotting-data indicated that these sites may correspond with a 220 kDa protein on endothelial and Kupffer cells (Ac/OxLDL recognition-site) and a 95 kDa protein on Kupffer cells (recognition-site specific for OxLDL) [14].

Saturation binding-data showed that the binding of OxLDL and AcLDL to both liver-cell types is monophasic (Hill coefficients close to unity), saturable and of high affinity. The capacity of the Kupffer cells to bind OxLDL was comparable to that observed for endothelial cells. In addition, the affinity of OxLDL for the specific OxLDL recognition-site on endothelial and Kupffer cells were essentially similar.

In accordance with previous uptake studies *in vitro* [7], approximately 52% of the ^{125}I -OxLDL binding to endothelial cells could be inhibited by AcLDL, while only 25% of ^{125}I -OxLDL binding to Kupffer cells was inhibited by AcLDL, suggesting a difference in the amount of OxLDL binding sites on endothelial and Kupffer cells. Ligand-blotting studies have clearly demonstrated the presence of a 220 kDa protein recognizing both OxLDL and AcLDL (the classical scavenger-receptor) on both cell types [14]. In agreement with the above binding studies, OxLDL-binding to the blotted 220 kDa protein could be completely inhibited by a 20-fold excess OxLDL (80%) but only partly by a 20-fold excess of AcLDL ($36 \pm 5\%$) (Y. B. De Rijke and T. J. C. van Berkel, unpublished work). The same study has revealed the presence of a major 95 kDa protein recognizing only OxLDL and a minor 220 kDa protein recognizing both OxLDL and AcLDL on Kupffer cells [14]. Moreover, Naito et al. [24] have demonstrated the expression of a 220 kDa scavenger receptor in bovine Kupffer cells by immunohistochemistry. Therefore, we may conclude that the endothelial cells contain only a single Ac/OxLDL binding-protein (220 kDa), whereas Kupffer cells express both a 95 kDa OxLDL-specific binding site at a level of approximately 550 ng/mg and a 220 kDa Ac/OxLDL binding-protein at a level of 230 ng/mg (assuming the binding ratio of OxLDL to AcLDL for the Ac/OxLDL binding-proteins on endothelial and Kupffer cells to be identical). The binding of ^{125}I -AcLDL to both endothelial and Kupffer cells is completely and efficiently inhibited by AcLDL and OxLDL. It was observed that OxLDL possesses a significantly higher affinity for the Ac/OxLDL binding-site ($K_i = 1 \mu\text{g/ml}$) than for the OxLDL-specific binding-site on endothelial cells ($K_i = 8 \mu\text{g/ml}$). This concurs with the observation that the total ^{125}I -OxLDL binding to endothelial cells was inhibited by AcLDL at a lower affinity than that for the inhibition of ^{125}I -AcLDL-binding by AcLDL.

It has been suggested that the net charge is an important determinant of chemically modified LDL for its recognition by scavenger receptors [23]. We have compared the effect of negative charge of LDL on the affinity of LDL for the Ac/OxLDL binding-site and the OxLDL binding-site on both cell types. These binding sites share the property of being completely inhibited by polyinosinic acid [7]. It appears that the affinity of modified LDL for the Ac/OxLDL binding-site on the endothelial and Kupffer cells is greatly increased with an increasing negative charge. The extent of oxidation of LDL similarly affected the affinity for the OxLDL- and Ac/OxLDL-binding-site. However, it appeared that oxidatively modified LDL was a more potent competitor for ^{125}I -AcLDL-binding to endothelial cells than AcLDL having a comparable electrophoretic mobility. These results are in concert with data from mutagenesis experiments recently published by Doi et al. [12]. These authors concluded that only a narrow region of the scavenger receptor is involved in the binding of AcLDL, while the binding domain of OxLDL spans a wider region. It can be anticipated that binding to a wider region will lead to an increased affinity for OxLDL as compared with AcLDL.

The OxLDL-specific binding site on endothelial and Kupffer cells was studied in the presence of an excess of AcLDL. OxLDL possessed a lower affinity for the OxLDL-specific binding site than for the Ac/OxLDL-binding site. In general, however, the OxLDL-binding sites on endothelial and Kupffer cells only marginally differ regarding the effect of the extent of oxidation of LDL to compete for ^{125}I -OxLDL binding. It appeared that the OxLDL binding-site on endothelial cells did not recognize LDL

oxidized for 1 to 6 h, while the OxLDL binding-sites on Kupffer cells tend to be more sensitive to mildly oxidized LDL. Haberland et al. [25] indicated that for the uptake of malondialdehyde-modified LDL by the scavenger receptor of human monocyte macrophages, a modification of at least 64 mol lysine residues/mol of LDL was required, which is in the same range as required for the binding of OxLDL to the OxLDL-specific binding sites on Kupffer cells.

We conclude that the recognition of OxLDL by the OxLDL-specific binding-site as well as the recognition of OxLDL by the Ac/OxLDL binding-site depends on the net negative charge of modified LDL. In view of the putative role of scavenger receptors in the atherogenesis, it will be of great interest to resolve the molecular nature of the 95 kDa protein so that the structural elements involved in the binding of OxLDL can be elucidated.

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REFERENCES

- Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) *New Engl. J. Med.* **320**, 915–924
- Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333–337
- Fogelman, A. M., Schlechter, I., Saeger, J., Hokom, M., Chold, J. S. and Edwards, P. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2214–2218
- Parthasarathy, S., Steinbrecher, U. P., Barnett, J., Witztum, J. L. and Steinberg, D. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3000–3004
- Nagelkerke, J. F., Barto, K. P. and Van Berkel, Th. J. C. (1983) *J. Biol. Chem.* **208**, 12221–12227
- Pitas, R. E., Boyles, J., Mahley, R. W. and Bissell, D. M. (1985) **100**, 103–107
- van Berkel, Th. J. C., de Rijke, Y. B. and Kruijt, J. K. (1991) *J. Biol. Chem.* **266**, 2282–2289
- Ylä-Herttuala, Rosenfeld, M. E., Parthasarathy, S., Sigal, E., Särkioja, T., Witztum, J. L. and Steinberg, D. (1991) *J. Clin. Invest.* **87**, 1146–1152
- Rosenfeld, M. E., Khoo, J. C., Miller, E., Parthasarathy, S., Palinski, W. and Witztum, J. L. (1991) *J. Clin. Invest.* **87**, 90–99
- Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. and Krieger, M. (1990) *Nature (London)* **343**, 531–535
- Rohrer, L., Freeman, M., Kodama, T., Penman, M. and Krieger, M. (1990) *Nature (London)* **343**, 570–572
- Doi, T., Higashino, K., Kurihara, Y., Wada, Y., Miyazaki, T., Nakamura, H., Uesugi, S., Imanishi, T., Kawabe, Y., Itakura, H., Yazaki, Y., Matsumoto, A. and Kodama, T. (1993) *J. Biol. Chem.* **268**, 2126–2133
- Acton, S., Resnick, D., Freeman, M., Ekkel, L., Ashkenas, J. and Krieger, M. (1993) *J. Biol. Chem.* **268**, 3530–3537
- De Rijke, Y. B. and Van Berkel, Th. J. C. (1994) *J. Biol. Chem.* **269**, 824–827
- Redgrave, T. G., Roberts, D. C. K. and West, C. E. (1975) *Anal. Biochem.* **65**, 42–49
- Basu, S. K., Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3178–3182
- Habeeb, A. F. S. A. (1966) *Anal. Biochem.* **14**, 328–336
- Jansen, R. W., Kruijt, J. K., Van Berkel, Th. J. C. and Meijer, D. K. F. (1993) *Hepatology* **18**, 146–152
- Chen, S.-H., Yang, C.-Y., Chen, P.-F., Setzer, D., Tanimura, M., Li, W.-H., Gotto, A. M. and Chan, L. (1986) *J. Biol. Chem.* **261**, 12918–12921
- McFarlane, A. S. (1958) *Nature (London)* **812**, 53
- Bilheimer, D. W., Eisenberg, S. and Levy, R. I. (1972) *Biochim. Biophys. Acta* **260**, 212–221
- De Rijke, Y. B., Jürgens, G., Hessels, E. M. A. J., Hermann, A. and van Berkel, Th. J. C. (1992) *J. Lipid Res.* **33**, 1315–1325
- Brown, M. S., Basu, S. K., Falck, J. R., Ho, Y. K. and Goldstein, J. L. (1980) *J. Supramol. Struct.* **13**, 67–81
- Naito, M., Kodama, T., Matsumoto, A. and Takahashi, K. (1991) *Kupffer Cell Foundation: Cells of the Hepatic Sinusoid* (Wisse, E., Knook, D. L. and McCuskey, R. S. eds.), Vol. 3, pp. 497–501, Kupffer Cell Foundation, Rijswijk, The Netherlands
- Haberland, M., Fogelman, A. and Edwards, P. A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1712–1716