Specific Binding of Free Apolipoprotein A-I to a High-Affinity Binding Site on HepG2 Cells: Characterization of Two High-Density Lipoprotein Sites

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ABSTRACT: In this paper, we present the first evidence that free apoA-I, without association with lipids, binds only to a high-affinity binding site (K_d = 1.8 μg/mL, B_{max} = 63.12 ng/mL). This is a new binding site of higher affinity (80–100 times) but of lower capacity than the binding sites already described for HDL. This is also the first evidence on HepG2 cells both of a high-affinity site (K_d = 0.685 μg/mL, B_{max} = 39.86 ng/mL) and of a low-affinity site (K_d = 55.65 μg/mL, B_{max} = 665.45 ng/mL) for HDL. ApoA-I–DMPC complexes also present two binding components comparable to the HDL_3 binding sites. This free apoA-I binding is specific, as shown by competition experiments, and allowed us to specifically study this high-affinity site, without interference of the low-affinity one. Kinetic rates of association/dissociation for the high-affinity site were faster than for the low-affinity site (10 and 20 min versus 40 and 30 min, respectively). The kinetic K_d values, derived from association and dissociation rate constants (K_d = 55.14 and 2.91 μg/mL), were of similar magnitude as the K_d values calculated by Scatchard analysis. These data confirm that HDL_3 binding sites characterized by saturation experiments follow the law of mass action, indicative of ligand–receptor interaction. In summary, HepG2 cells present high HDL_3 binding sites which are able to bind free apoA-I in contrast with the low-affinity HDL_3 binding sites.

High-density lipoproteins (HDL)[1] are of central importance in "reverse cholesterol transport," a process by which cholesterol is transported from peripheral tissues to the liver for reutilization or excretion as bile acids or sterols (Eisenberg, 1984). It appears likely that a number of different cellular mechanisms, possibly receptor-dependent and independent, are required to regulate this pathway (Eisenberg, 1984). However, the proposal that a receptor mediates cellular processing of HDL has not gained general acceptance, since there is no recognized, physiological function for such a protein. Physicochemical (Tabas & Tall, 1984; Mendel et al., 1988) and immunochemical (Leblond & Marcel, 1991) studies were interpreted to indicate that HDL binding may not necessarily involve protein–protein interaction. On the contrary, there is increasing biochemical evidence for HDL receptors which may play a role in the modulation of plasma or cellular cholesterol levels (Slotte et al., 1987; Oram et al., 1991). Various attempts have been made to characterize the ligands of the HDL binding sites, yet the nature of the ligand recognized by the receptor still remains controversial. ApoA-I seems to play a major role (Fidge et al., 1985; Jurgens et al., 1989; Rifici & Eder, 1984; Schmitz et al., 1985) although other HDL apolipoproteins (Fidge et al., 1985) and even HDL lipids (Mendel & Kunitake, 1988; Tabas & Tall, 1984) may be involved. The use of poorly labeled ligands for most studies on HDL binding could have masked the existence of different binding components. Hence, the study of a HDL binding displaying high-affinity binding but low capacity would have been almost impossible under such conditions. Recently, Morrison et al. (1992) reported the presence of high-affinity binding sites for HDL on liver plasma membranes. With these plasma membranes, the authors used the possibility to increase the signal and thus were able to characterize two HDL binding components. In our study, we used highly labeled HDL and apoA-I and thus showed, for the first time on intact hepatoma cells (HepG2), the presence of two HDL binding sites. Furthermore, free apoA-I, without any associated lipids, specifically bound to the high-affinity binding sites. Thus, lipid-free apoA-I represents a valuable tool to further characterize this HDL high-affinity binding site.

EXPERIMENTAL PROCEDURES

Materials. Dimyristoylphosphatidylcholine (DMPC), bovine serum albumin (BSA), Tricine, α_2-macroglobulin, and methylamine were obtained from Sigma (La Verpilliere, France). ^{125}INa was from ICN Biomedicals (Orsay, France). All other reagents used were analytical grade. α_2-Macroglobulin activated with methylamine was prepared according to Jensen et al. (1989).

Cells. The human hepatoblastoma-derived cell line HepG2 was obtained from the American Type Culture Collection (Rockville, MD). Cells were plated at (1–2) × 10^5 cells per well in 48-multiwell plates (Costar) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 and 95% air incubator. Medium was changed every 2 or 3 days, and the cells were subcultured every 7–8 days.

Lipoprotein, Apolipoprotein, and Proteoliposome Preparations. High-density lipoproteins (HDL_3) were isolated from the plasma of normolipodemic healthy human donors. Na_2EDTA, sodium azide, and phenylmethanesulfonyl fluoride were added to final concentrations of 1 mM, 0.05% (w/v), and 1 mM, respectively. The lipoproteins were obtained by sequential flotation ultracentrifugation. After being washed at their lower density limit (1.07 g/mL), total HDL were isolated (1.07–1.21 g/mL) and were further fractionated at

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[1] Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; apo, apolipoprotein; SDS, sodium dodecyl sulfate; DMPC, L-α-dimyristoylphosphatidylcholine.

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$d = 1.125$ g/mL into HDL_2 and HDL_3. HDL_3 were depleted of ApoE using heparin–sepharose chromatography as previously described (Weisgraber & Mahley, 1980). Using a specific radioimmunoassay, only trace amounts of apoE were detected. All HDL_3 preparations used in the present study were treated in the same way and are subsequently designated as HDL_3. HDL_3 were kept at 4 °C in the dark and under nitrogen and were extensively dialyzed against phosphate-buffered saline (PBS: 0.137 M NaCl, 4.3 mM sodium phosphate, 1.5 mM potassium phosphate, and 2.7 mM KCl, pH 7.4) before use.

Low-density lipoproteins (LDL) (1.006–1.063 g/mL) were depleted of any possible apoA-I by affinity chromatography on anti-apoA-I antibodies immobilized on a CNBr-activated Sepharose 4B gel. LDL were incubated overnight at 4 °C in PBS. Nonretained LDL were then eluted with PBS and were checked for the presence of apoA-I by Western blot. These apoA-I-free LDL were then used for competition experiments and designated as LDL.

ApoA-I and apoA-II were isolated from HDL_3 by ion-exchange chromatography as outlined before (Mezdour et al., 1987). The apoA-I and apoA-II preparations were checked on a HPLC apparatus using a reverse-phase C18 column and acetonitrile gradient. Furthermore, Western blot analysis (Towbin et al., 1979) was carried out using a cocktail of antibodies directed against human apoB, A-II, and C's for apoA-I analysis and against human apoB, A-I, and C's for apoA-II. In all cases, the apolipoprotein homogeneity was more than 99%.

Complexes containing apoA-I or apoA-II and DMPC were prepared by the cholate dialysis procedure as described by Chen and Albers (1982), at a phosphatidylcholine:protein molar ratio of 150:1. The Stokes radius of the complexes was estimated to be 100 ± 10 Å (Chen & Albers, 1982).

Labeling of apoA-I and HDL_3 with ^{125}I was performed by the *N*-bromosuccinimide method according to Sinn et al. (1988). Specific radioactivities ranged from 3000 to 5000 cpm/ng of protein. In the case of ^{125}I-apoA-I complexes, the apoA-I was labeled before insertion into the lipid complexes. The same preparation of ^{125}I-apoA-I was used either in a free form or complexed to DMPC in further binding experiments. The specific radioactivities ranged from 10 000 to 20 000 cpm/ng. More than 97% of the radioactivity was associated with protein.

Binding Assays. Binding of labeled lipoproteins and proteoliposomes to HepG2 cells was performed for 2 h at 4 °C as previously described (Steinmetz et al., 1990). Briefly, cell monolayers were incubated in PBS with labeled or unlabeled ligands and, then washed twice with ice-cold PBS (maximum washing time was 15 s). To determine the cell-associated radioactivity, 500 μL of 0.1 N NaOH was added to the washed monolayer. The NaOH digest was used for protein determination and radioactivity measurement. Non-specific binding was determined in the presence of a 100-fold excess (compared to the K_d value) of the corresponding unlabeled ligand; the values of nonspecific binding varied from 30 to 40% of total binding. The ratio of bound to free ^{125}I-apolipoprotein versus bound ^{125}I-apolipoprotein was plotted according to Scatchard (1949).

Competition Assays. As for the binding assays, the competition experiments were performed at 4 °C for 2 h. A concentration of 0.5 μg/mL apoA-I was used, and increasing concentrations of unlabeled competitors were added. Washing and radioactivity measurements were performed as above. Data were expressed as the percent of the specific binding measured in the absence of competitor versus the log of proteoliposome concentration (in micrograms of protein per milliliter). The amounts of DMPC liposomes used were identical to those present in apoA-I–DMPC complexes.

Kinetic Assays. The association rate constant (k_{+1}) of different ligands was determined by measuring the amount of protein *specifically* bound on HepG2 cells at 4 °C and at various times. The concentration of the radioactive ligand was constant during all experiments. Nonspecific binding was determined with a 100-fold excess of the corresponding nonradioactive ligand. All the washing and radioactivity measurements were performed as for the binding assays. The pseudo-first-order method was used for k_{+1} calculation: considering L as the concentration of the labeled ligand and B_e as the equilibrium binding value, a plot of $\ln[B_e/(B_e - B)]$ versus time will have a slope of k_{ob} (k_{ob} is the observed association rate constant) (Williams & Lefkowitz, 1978; Bennett, 1978). If k_{-1} (dissociation rate constant) is known from independent experiments (i.e., dissociation experiments—see below), then

$$k_{+1} = (k_{ob} - k_{-1})/L$$

Dissociation experiments were carried out on HepG2 cells at 4 °C using a single concentration of labeled ligand. After the association equilibrium binding was reached (i.e., 2 h for HDL_3 or 30 min for free apoA-I), a 100-fold excess of unlabeled ligand was added, or, in some cases, after two washes in ice-cold PBS, fresh medium was added to the cells (called dissociation by "dilution"; De Meyts et al., 1973). Under these conditions, we can use a first-order equation to calculated k_{-1}. Thus, a plot of $\ln(B/B_0)$ versus time (where B_0 is the binding value at time = 0, i.e., at equilibrium) will have a slope of $-k_{-1}$. Alternatively, k_{-1} can be estimated as $k_{-1} = 0.693/t_{1/2}$, where $t_{1/2}$ is the time when $B = 0.5B_0$ (Bylund, 1980).

Data Analysis. Binding and competition data were analyzed using a weighted nonlinear curve-fitting program, based on the LIGAND analysis program (Munson & Rodbard, 1980). In competition experiments, the sigmoidal curves were transformed into a linear relationship, using the logit transformation (Rodbard & Frasier, 1975). If P is the percentage bound, then

$$\text{logit(P)} = \ln[\text{P}/(100 - \text{P})]$$

The data are represented in a logit–log(proteoliposomes) plot. The IC_{50} (i.e., the concentration that inhibits 50% of the binding), which corresponds to a logit value of 0, was determined by linear regression analysis.

Electrophoresis. Samples, in an SDS sample buffer, were run on Tricine/SDS/polyacrylamide (0.1 M/0.1%/12%, w/v) gel electrophoresis as described (Schägger & von Jagow, 1987). Under these conditions, all the major HDL_3 apolipoproteins were visualized by silver staining (Merril et al., 1984). For Western blot experiments, proteins were transferred using a semidry apparatus (Pharmacia) during 45 min with an intensity of 5.5 mA/cm^2. Nitrocellulose was incubated as previously described (Towbin et al., 1979) with a 2000-fold dilution of each antiserum followed by a second incubation with specific ^{125}I-labeled $F(ab')_2$ fragments (1 μCi/nitrocellulose). At least specific proteins were visualized after overnight autoradiography at −70 °C.

Analytical Procedures. The protein concentration was determined by the method of Bradford (1976), using the Bio-Rad protein assay dye and bovine serum albumin (BSA) as a standard.

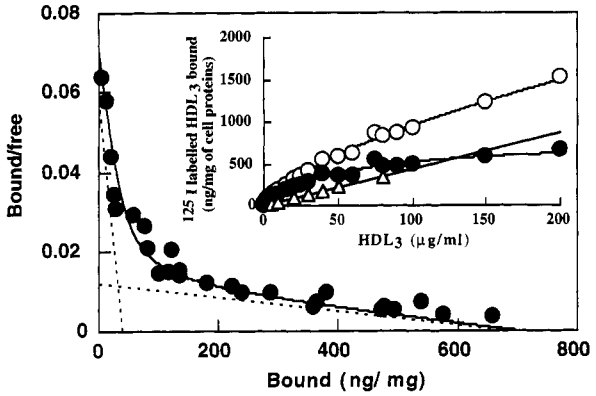

FIGURE 1: Scatchard representation and binding isotherm of ^{125}I-labeled HDL_3 to HepG2 cells. Specific binding of ^{125}I-labeled HDL_3 was measured on duplicate wells after 2 h of incubation at 4 °C. Nonspecific binding was assessed as described under Experimental Procedures and represented 40% of the total binding. The solid line represents the computer-generated line of best fit, composed of high- and low-affinity binding sites (dashed lines). The mean values from duplicate wells are reported (±10% from the mean) and are representative of three independent experiments performed on three different series of cells. Inset: Corresponding binding isotherm of ^{125}I-labeled HDL_3 to HepG2 cells: total binding of ^{125}I-labeled HDL_3 (○); specific binding (●); nonspecific binding (△).

Table 1: Binding Parameters of ^{125}I-Labeled HDL_3, ^{125}I-Labeled ApoA-I–DMPC Complexes, and ^{125}I-Labeled Free ApoA-I to HepG2 Cells[a]

	high affinity		low affinity	
	K_d (µg/mL)	B_{max} (ng/mg)	K_d (µg/mL)	B_{max} (ng/mg)
HDL_3	0.685	39.86	55.65	664.45
apoA-I–DMPC	0.190	6.67	35.02	413.12
apoA-I	1.8	63.12		

[a] Data are obtained from Scatchard plots of Figures 1, 3, and 4 and are representative of four, three, and two independent experiments performed on four, three, and two series of cells, respectively.

RESULTS

All the saturation, kinetic, and competition studies were performed at 4 °C in PBS with HepG2 cell monolayers. Preliminary experiments have shown that, in such conditions, the cells remain intact (as described previously) after over 6-h incubation. In order to study multicomponent HDL_3 binding, we used a very high specific radioactivity for HDL_3 and apoA-I. The average of iodine atoms fixed per molecule of apoA-I was 0.2 and 0.1 for HDL_3 and apoA-I, respectively. Previous experiments were carried out to compare these highly labeled ligands versus lower, classically labeled ligands and showed no significantly different low-affinity binding values (data not shown). Only highly labeled ligands were further used in this study.

A Scatchard representation of HDL_3 binding to HepG2 cells clearly shows two binding components (Figure 1). The calculated binding parameters (from Figure 1) are presented in Table 1. The HDL_3 K_d value for the high-affinity binding site was considerably lower (80-fold) than the K_d value for the low-affinity binding site. However, this difference was not great enough to allow us to measure the kinetic and competition parameters of the high-affinity binding sites simply by using a low concentration of labeled HDL_3.

Thus, kinetic experiments of HDL_3 binding (Figure 2) were performed using 30 µg/mL HDL_3, and the measured parameters thus correspond to the contribution of both the high- and low-affinity binding sites (Figure 2 and Table 2).

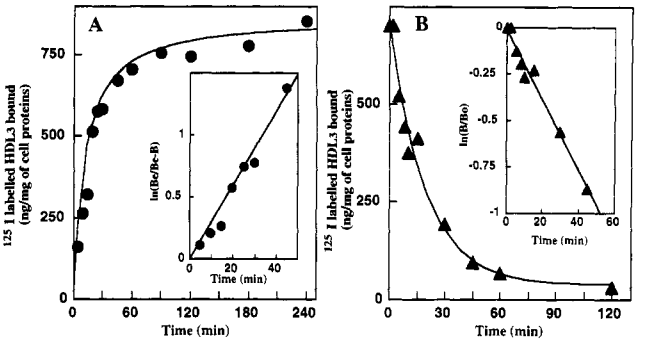

FIGURE 2: Kinetics of specific association (A) and specific dissociation (B) at 4 °C of ^{125}I-labeled HDL_3 onto HepG2 cells. A concentration of 30 µg/mL was used for specific association and specific dissociation experiments. A pseudo-first-order equation was used to calculate the association rate constant (k_{+1}) of the association. A plot of $\ln[B_e/(B_e - B)]$ versus time (A, inset) enables calulation of the association rate constant as described under Experimental Procedures. After 90 min, a concentration of 3 mg/mL unlabeled HDL_3 was added for dissociation experiments (B). Thus, a first-order equation can be used to calculate the dissociation rate constant (k_{-1}). As shown in the inset of panel B, a plot of $\ln(B/B_0)$ versus time has a slope of $-k_{-1}$ (see Experimental Procedures for details). The curves represent the specific binding calculated as described under Experimental Procedures. Nonspecific binding averaged 40% of the total binding. The curves are representative of two experiments performed on two independent series of cells.

Table 2: Kinetic Association and Dissociation Parameters

	HDL_3	apoA-I
k_{-1} (min^{-1})	0.0193	0.1602
k_{+1} [$min^{-1}\cdot(\mu g/mL)^{-1}$]	0.00035	0.055
$t_{1/2,diss}$ (min)	35.90	4.32
K_d[a] (µg/mL)	55.14	2.91

[a] Calculated $K_d = k_{-1}/k_{+1}$.

Calculation of the association and dissociation rate constants for HDL_3 allowed us to estimate a K_d value. This calculated K_d value was very comparable to the K_d value of the low-affinity binding sites obtained from isotherm binding experiments (Table 2). It is noteworthy that the dissociation rates were the same when dissociation was carried out by dilution (De Meyts et al., 1973), indicating that the two families of sites are independent.

Since HDL_3 is heterogeneous in term of apolipoprotein composition, we repeated binding isotherm experiments using apoA-I–DMPC complexes as a ligand (Figure 3). The data clearly showed similar binding parameters as compared to HDL_3, and evidenced again two binding components (Figure 3 and Table 1). Previous competition experiments have shown that labeled apoA-I–DMPC complexes are efficiently displaced by HDL_3, strongly suggesting the apoA-I–DMPC complexes bind to the same binding site as HDL_3 (not shown).

Thus, neither the competition nor the kinetic experiments enabled us to study the high-affinity binding site alone. Then, we have followed the binding of free apoA-I without any association with lipids. As illustrated in Figure 4, the Scatchard representation of the binding experiments using ^{125}I-labeled free apoA-I as a ligand clearly showed a single component comparable to the high-affinity site described above for HDL_3 (Table 1 and Figure 4). Using free apoA-I, no binding through the low-affinity binding site of HDL_3 and apoA-I–DMPC complexes was observed. Kinetic studies of apoA-I binding (Figure 5) showed very different parameters compared to HDL_3 (Figures 2 and 5 and Table 2). The association and dissociation equilibrium occurred in 10 and 20 min, respectively, compared to 50 and 60 min for HDL_3.

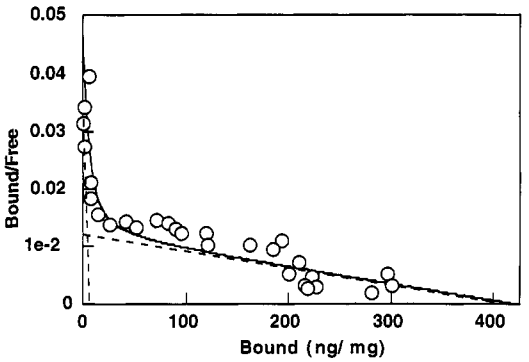

FIGURE 3: Scatchard representation of the binding isotherm of ^{125}I-labeled apoA-I–DMPC complexes to HepG2 cells. Specific binding of ^{125}-labeled apoA-I–DMPC complexes was measured on duplicate wells after 2-h incubation at 4 °C. Nonspecific binding was assessed as described under Experimental Procedures and represented 30% of the total binding. The solid line represents the computer-generated line of best fit, composed of high- and low-affinity binding sites (dashed lines). The mean values from duplicate wells are reported (±10% from the mean) and are representative of two independent experiments performed on two different series of cells.

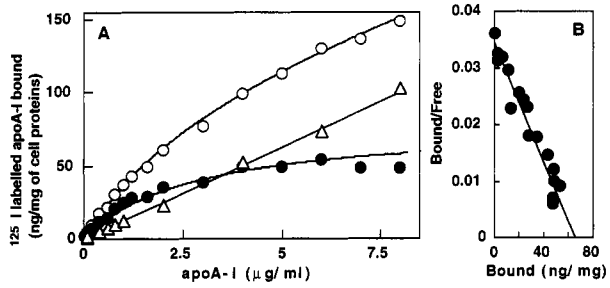

FIGURE 4: Binding isotherm of ^{125}I-labeled apoA-I to HepG2 cells. (A) The total binding of ^{125}I-labeled apoA-I (○) was measured on duplicate wells after 90 min of incubation at 4 °C. The specific binding (●) was measured as described under Experimental Procedures. The nonspecific binding (△) represented 40% of the total binding. (B) The Scatchard plot of the specific binding is displayed and represents the computer-generated line of best fit, showing a single independent family of sites corresponding to the high-affinity binding sites of HDL_3 and apoA-I–DMPC complexes (see Figures 1 and 3). The mean values from duplicate wells are reported (±10% from the mean) and are representative of two independent experiments performed on two different series of cells.

The specificity of this binding of free apoA-I to HepG2 cell was tested by competition experiments (Figure 6). The data showed that this high-affinity binding of apoA-I was specific and corresponded to the high-affinity site for HDL_3 and apoA-I–DMPC complexes. LDL, DMPC liposomes, and α_2-macroglobulin were unable to displace this binding, ruling out the binding of free apoA-I to the apoB/E receptor or the LDL receptor-related protein (LRP) or interactions between membrane lipids and apoA-I. The apparent K_d value calculated for free apoA-I was 4.89 μg/mL and was comparable to the K_d value for free apoA-I obtained in binding isotherm experiments, indicating that ^{125}I labeling of free apoA-I does not interfere with the binding to HepG2 cells. The logit representation (Figure 6, right panel), in which the slopes are equivalent in each experiment, suggests simple competition curves (indicative of a single class of noninteracting binding sites). The only exception is for the logit representation of the competition by apoA-II–DMPC complexes which also display a lesser efficiency, suggesting a different interaction with apoA-I binding sites.

DISCUSSION

The present study shows that the hepatoma cell line HepG2,

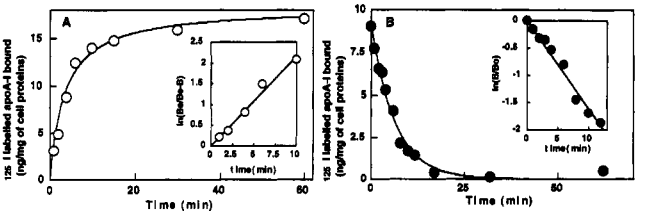

FIGURE 5: Kinetics of specific association (A) and specific dissociation (B) at 4 °C of ^{125}I-labeled apoA-I onto HepG2 cells. A concentration of 1 μg/mL was used for specific association and specific dissociation experiments. A pseudo-first-order equation was used to calculate the association rate constant (k_{+1}) of the association. A plot of $\ln[B_e/(B_e - B)]$ versus time (A, inset) enables calculation of the association rate constant as described under Experimental Procedures. After 60 min, a concentration of 100 μg/mL unlabeled apoA-I was added for dissociation experiments (B). Thus, a first-order equation is used to calculate the dissociation rate constant (k_{-1}). As shown in the inset of panel B, a plot of $\ln(B/B_0)$ versus time has a slope of k_{-1} (see Experimental Procedures for details). The curves represent the specific binding calculated as described under Experimental Procedures. Nonspecific binding averaged 40% of the total binding. The curves are representative of two experiments performed on two independent series of cells.

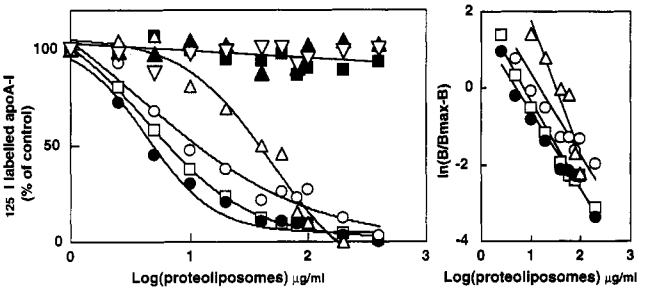

FIGURE 6: Competitive inhibition of the binding of ^{125}I-labeled apoA-I to HepG2 cells. HepG2 cells were incubated for 2 h at 4 °C in the presence of 0.5 μg/mL ^{125}I-labeled apoA-I and increasing concentrations of unlabeled HDL_3 (□), apoA-I (●), apoA-I–DMPC complexes (○), apoA-II–DMPC complexes (△), activated α_2-macroglobulin (▽), LDL (▲), and DMPC liposomes (■), respectively. DMPC liposome concentrations were calculated as described under Experimental Procedures. 100% specific binding corresponds to 34.54 ng/mg of cellular protein. Nonspecific binding represented 38% of the total binding. Right panel: A logit plot (as described under Experimental Procedures) of each experiment is shown. The results are representative of two independent experiments performed on two different series of cells. The mean values of duplicate wells are reported (±10%).

at 4 °C, presents high-affinity binding sites for free apoA-I. It has been previously reported that apoA-I alone, without any lipid association (referred to as free apoA-I), in a "classical range" of concentrations (between 10 and 400 μg/mL), does not bind to HepG2 cells (Barbaras et al., 1986). Using higher specific radioactivity and lower concentrations, we were able to characterize a specific interaction with parameters comparable to the HDL_3 high-affinity binding site. Furthermore, competition experiments showed the specificity of this free apoA-I binding to HepG2 cells. This process does not involve an apoB/E receptor nor an LDL receptor-related protein, as demonstrated by the inability of LDL and α_2-macroglobulin to compete for free apoA-I binding. It is very important to remember, that at the low concentrations of free apoA-I that were used (< 200 μg/mL), no apoA-I aggregation occurs, as previously reported by Stone and Reynolds (1975), and that all the experiments were carried out at 4 °C in order to impede any protein and/or lipid exchanges. In summary, the **same** labeled apoA-I, with or without association to DMPC complexes, displays very different behaviors: (i) free apoA-I only binds to the high-affinity site. (ii) The apoA-I–DMPC

complexes bind to the high-affinity **and** the low-affinity sites. However, the lipid moiety of the complexes does not support the low- and high-affinity interactions, since DMPC liposomes did not displace these bindings. This observation is good evidence for the importance of the apoA-I conformation. Indeed, at low concentrations, free apoA-I may have a conformation exposing a ligand domain, only available for specific binding to the high-affinity site. Conversely, the low-affinity site may require a particular conformation, only found in apoA-I lipid particles. Or, more simply, the aggregation of free apoA-I at higher concentrations may mask the ligand domain for binding to the low-affinity site.

To our knowledge, this is also the first HDL multibinding characterization on HepG2 cells. For this study, we used a higher specific radioactivity for HDL_3 and apoA-I than usually used (at least 10 times). Preliminary experiments did show that the binding parameters of HDL_3 and apoA-I were not affected by this high labeling. The affinity difference of these high- and low-affinity binding sites is around 80 times (Table 1), and on a molar basis, it represent a 10^{-7}–10^{-9} M range, considering a HDL_3 molecular mass of 200 kDa. Two other possibilities could be compatible with a concave-upward Scatchard plot, namely, a negative cooperativity between the two families of sites or a lattice effect. We have performed dissociation experiments both with an excess of HDL_3 and by simple dilution in fresh medium with no lipoproteins. Both dissociation experiments displayed the same rate constants, suggesting that the two families of sites are independent (De Meyts et al., 1973). Furthermore, we also performed dissociation experiments with different degrees of site occupancy (1 – 30 μg/mL, data not shown). Again, we found that the dissociation rate was not dependent of the degree of occupancy. All these observations allow us to exclude negative cooperativity. The lattice effect is a very complex event to evaluate, and so far we cannot totally rule out this possibility as an explanation of the Scatchard plot. Nevertheless, if we compare the parameters of LDL binding (Chappell et al., 1991, 1992), we find some differences in the HDL_3 characteristics: (i) The differences in K_d and B_{max} values of HDL_3 binding between the two families of sites are too great (80- and 16-fold, respectively) to be due to a lattice effect only. (ii) The HDL_3 association is clearly saturable, so that no delay is needed for a repositioning of the bound ligand to maximize the accessibility of more binding sites for further binding as suggested by Chappell et al. in the case of LDL (Chappell et al., 1991, 1992). (iii) The dissociation experiment fits (Figure 2B) were monoexponential, suggesting simple bimolecular binding.

Recently, two classes of HDL binding sites with different affinities were described in liver plasma membranes by Morrison et al. (1992). While their binding parameters were comparable to those obtained on HepG2 cells, the kinetic data were quite different, especially for the low-affinity binding site, with association equilibrium times exceeding 8 h. This discrepancy in the kinetic parameters can be due to the use of plasma membrane instead of intact cells. It is noteworthy that the binding parameters of the low-affinity site are of similar magnitude as those reported by different authors [Pitas et al., 1979; for a review, Johnson et al. (1991)]. So, until now, the HDL_3 binding studies mostly concerned the low-affinity binding site. The evidence of a new high-affinity site may enable another approach of the specific interactions between HDL and cells.

Between the two sites, the K_d difference is too small and the difference in binding capacity (B_{max}) too high (Table 1) to allow the specific study of each binding site using competition and kinetic experiments, in choosing the appropriate concentration of labeled ligand (Burt, 1980). Thus, the binding observed in competition and kinetic experiments mostly results from the low-affinity binding sites or, more precisely, from the sum of the high- and low-affinity sites. Using different ligands, we could perform kinetic experiments specific of each binding site and thus compare them. Kinetics analysis showed a very short time of association and dissociation of free apoA-I compared with HDL_3 or apoA-I–DMPC complexes (Table 2). Furthermore, the kinetic K_d values, derived from the association and dissociation rate constants, were in the same range as the K_d values calculated from the Scatchard analysis. This similarity between these different K_d values confirms that the binding sites characterized by saturation experiments follow the law of mass action, indicative of a ligand–receptor interaction.

Interaction between free apoA-I and cells has been recently described. Indeed, Hara and Yokoyama (1991, 1992) have recently shown that incubation of macrophages with free apoA-I induced the formation of lipid–apoA-I complexes, with an electrophoretic migration similar to the pre-β-HDL particles described by Castro and Fielding (1988). Thus, free apoA-I could be at the origin of the formation of these pre-β-HDL which are supposed to be the first cholesterol acceptor in the HDL metabolism pathway. It is noteworthy that it has recently been described that the cholesterol efflux promoted by pre-β-HDL is partly (50%) dependent on a protein–protein-specific interaction (Kawano et al., 1993).

In conclusion, two families of HDL_3 binding sites were described on HepG2 cells. The physiological meaning of these binding sites of different affinity is unknown, and needs further investigation. One hypothesis might be that each binding component corresponds to a specific and distinct subpopulation of HDL_3. The difference between these subpopulations might concern the relative exposure of apoA-I domains, corresponding to a change in the apoA-I conformation due to lipid (Collet et al., 1991; Curtis & Smith, 1988) or to association with other apolipoproteins (Leblond & Marcel, 1991). Thus, each binding site could drive a distinct metabolic pathway. Another hypothesis is that the high-affinity binding sites may regulate the low-affinity ones. Indeed, in contrast with the high-affinity sites, the low-affinity binding seems to have a broad range of interaction with different ligands (i.e., apoA-IV, trypsinized HDL, etc.; Morrison et al., 1992). Thus, a very specific, high-affinity site could trigger cholesterol uptake through a less specific, low-affinity site. This triggering, induced by a low amount of highly specific HDL_3 particles, might strongly amplify HDL_3 cellular uptake via the low-affinity site. Whatever the hypothesis, it will ve very interesting to further characterize (i.e., apolipoprotein and lipid compositions) the ligands for the high-affinity binding site.

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