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# Indium-111-Labeled Low-Density Lipoprotein Binds with Higher Affinity to the Human Liver as Compared to Iodine-123-Low-Density-Labeled Lipoprotein

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The interaction of  $^{111}\text{In}$ -low-density lipoprotein (LDL) and  $^{123}\text{I}$ -LDL with human liver-plasma membranes was investigated and compared. LDLs were isolated by sequential ultracentrifugation and radiolabeled either with  $^{123}\text{I}$  (using Iodogen or iodine-monochloride) each followed by purification with gel-chromatography or dialysis) or  $^{111}\text{In}$  (using cyclic DTPA-anhydride). LDL concentrations of 0.1 to 32  $\mu\text{g}$  protein/ml were used for direct binding assays investigating the specific binding of labeled LDL (in the presence of a 50-fold excess of unlabeled LDL) to human liver apoB-receptors. In separate experiments, displacement of bound  $^{111}\text{In}$ -( $^{123}\text{I}$ )-LDL by unlabeled LDL was studied. Human liver plasma membranes bound  $239 \pm 26$  ng protein of  $^{111}\text{In}$ -LDL/mg protein and  $148 \pm 18$  ng protein of  $^{123}\text{I}$ -LDL/mg protein specifically ( $p < 0.001$ ). The corresponding dissociation constants were  $0.6 \pm 0.2$  and  $1.2 \pm 0.7$   $\mu\text{g}$  protein/ml, respectively ( $p < 0.001$ ). The capacity of unlabeled LDL to displace bound  $^{111}\text{In}$ -LDL was four times higher than that for  $^{123}\text{I}$ -LDL ( $\text{IC}_{50}$ :  $1.7 \pm 0.7$  versus  $7.7 \pm 1.0$   $\mu\text{g}$  protein/ml). No significant differences among the different methods of iodination of LDL were found. The findings show that  $^{111}\text{In}$ -labeled lipoproteins might be a better ligand for lipoprotein-receptor binding studies as compared to radioiodinated lipoprotein products.

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**R**adiolabeled LDL [ $^{123}\text{I}$  (1-3);  $^{125}\text{I}$  (4);  $^{131}\text{I}$  (5) and  $^{99\text{m}}\text{Tc}$  (6-8)] have been used during the last years for the detection of atherosclerotic lesions in carotid and femoral arteries. Recently Rosen et al. (9) have shown the first in vivo application of  $^{111}\text{In}$ -labeled LDL in New Zealand white rabbits. Sacrifice at 6 days after injection revealed that the overall levels of uptake in all tissues, obtained from both normal and hypercholesterolemic rabbits, were several times higher with  $^{111}\text{In}$ -LDL than with  $^{125}\text{I}$ -LDL.

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However, their in vitro results on cultured fibroblasts suggested an increased nonsaturable component for  $^{111}\text{In}$ -LDL binding sites, indicating that while  $^{111}\text{In}$ -LDL is recognized by LDL receptors it may well behave like modified LDL at the same time. These results on fibroblasts were discordant with their in vivo findings of biodistribution. However, Vallabhajosula et al. (8) also reported an increased uptake for  $^{99\text{m}}\text{Tc}$ -LDL as compared to  $^{125}\text{I}$ -LDL and  $^{131}\text{I}$ -LDL and suggested that  $^{99\text{m}}\text{Tc}$ -LDL might act as an intracellularly trapped ligand.

The primary function of LDL receptors is the maintenance of cholesterol homeostasis (10). These receptors are located mainly in the liver (11), but are found also at many other cell types (for review see 12). Compared to the liver, vessels contain only minimal amounts of LDL receptors. LDL uptake by deendothelialized vessels of the rabbit (13) or morphologically proven atherosclerotic lesions in human in vivo (1-3) has not yet been proven to be LDL receptor-mediated. Hence, the optimal isotope for radiolabeling of LDL has not been established. Recently, we have shown that between the different labeling methods available for  $^{123}\text{I}$ -LDL no significant difference exists in radiolabeling results and binding of LDL to human liver plasma membranes (14). In this study, we investigated the binding of  $^{111}\text{In}$ -LDL to human liver plasma membranes and compared it to  $^{123}\text{I}$  binding results.

## METHODS

### LDL Isolation and Characterization

For isolation of human LDL, 36 ml of blood from normolipemic volunteers (8 males, 10 females, 25-35 yr) were drawn into four Monovette vials (Sarstedt, FRG) and anticoagulated 1:10 with 3.8% sodium citrate. Blood was always collected through siliconized needles after an overnight fast. Neither pooled plasma nor pooled LDL were used throughout. In all blood donors, routine plasmatic lipid concentrations were determined (cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides). Only blood of normolipemic volunteers was used for LDL preparation. LDL was prepared from fresh plasma by sequential

ultracentrifugation using KBr for density adjustment (15). Following an 18-hr ultracentrifugation (L5-75 ultracentrifuge, Beckman Instruments Inc., Palo Alto, CA, Rotor 40.3 Ti, 40,000 rpm, 10°C), the VLDL fraction was withdrawn and the pellet suspended in KBr solution ( $d = 1.063$  g/ml) ( $g$  KBr = plasma volume (ml)  $\times$  (1.063–1.019)  $\times$  0.94 (K)/1 – (0.295  $\times$  1.063)) and centrifuged against the density gradient for 18 hr (Rotor 40.3 Ti, 40,000 rpm, 10°C). The supernatant ( $d = 1.019$ –1.063 g/ml) contained the LDL fraction which was dialyzed against normal saline, pH 7.4, containing 0.1 mg/ml EDTA, and stored at 4°C. In the supernatant, the content of apoB100, apoCII, apoCIII, apoE and apoAI was measured by radial immunodiffusion techniques, which indicated the presence of apoB100 only.

### Radiolabeling of LDL

For each series of experiments, LDL of one normolipemic subject was used. One series consisted of the Iodogen or iodine monochloride method of labeling LDL with  $^{123}\text{I}$  and of  $^{111}\text{In}$ -labeling of LDL. Subsequent binding studies with each of these radiolabels were carried out just after the labeling procedure.

**Iodogen Method.** Iodine-123-labeling of LDL with the Iodogen method was performed according to Fraker et al. (16). Briefly, in a microvial 500  $\mu\text{l}$  of chloroform solution of 30  $\mu\text{g}$  Iodogen were evaporated with a stream of nitrogen, redissolved and blown dry again to produce homogenous surface coating. To the Iodogen-coated vial, approximately 1 mg (protein) of LDL in saline, 0.01 M phosphate buffer pH 7.5, 0.15  $\mu\text{g}$  NaI-carrier/5  $\mu\text{l}$  and about 1 mCi  $^{123}\text{I}$ -NaI/10  $\mu\text{l}$  (IRE, Belgium, in 0.01 M NaOH, >100 mCi/ml) were added. The specific activity of  $^{123}\text{I}$ -NaI used was <0.08 nmol/mCi. To achieve satisfactory reproducible labeling yields and a constant molar ratio of I/LDL with varying radioactivity, 1 nmol of NaI carrier was added. At the resulting molar ratio I/LDL of about 0.5, no denaturation of the protein is to be expected. The reaction mixture (500  $\mu\text{l}$ ) was stirred slowly at 4°C for 10 min and applied to a Sephadex G25M-column (bed size 9  $\times$  100 mm), which had been pre-eluted with identical unlabeled LDL. The  $^{123}\text{I}$ -LDL peak was collected from about 2.5 to 4.5 ml eluate (phosphate-buffered saline (PBS) pH 7.5) using a radioactivity and a UV detector, stabilized by addition of 20 mg human serum albumin/ml (HSA; 20 mg/ml product solution) and finally sterilized by 0.2  $\mu\text{m}$  membrane filtration. Alternatively, the reaction mixture was sterile filtered into a dialysis bag that was kept in dialysis buffer (0.15 M NaCl, 0.01 M  $\text{PO}_4$ , pH 7.5, 0.2 mM EDTA) until application for in vitro binding studies.

Radiochemical purity was determined by:

1. Trichloroacetic acid (TCA) protein precipitation.
2. Cellulose acetate electrophoresis (CA-EP): 0.05 M barbital buffer pH 8.6, containing 1 mM EDTA and 1% HSA, horizontal zone electrophoresis at 300 V for 10 min.
3. Polyacrylamide gel electrophoresis (PAGE; gradient gel (T = 8%–18%; gel buffer 0.12 M Tris, 0.12 M acetate and 0.1% SDS, pH 6.4; 200 V/25 mA for 20 min, then 600 V/25 mA for 60 min).

**Iodine-Monochloride (ICI) Method.** Iodine-123-labeling of LDL with the ICI method was performed according to McFarlane (17) as modified for lipoproteins (18). An ICI stock solution (34  $\mu\text{mol/ml}$  6 M HCl) was purified before labeling by three extractions with  $\text{CHCl}_3$  and diluted 1:100 with aqueous 2 M NaCl. To a microvial kept at 4°C, approximately 1 mg (protein) of LDL (100  $\mu\text{l}$ ), 200  $\mu\text{l}$  of 1 M glycine buffer pH 10, about 1 mCi  $^{123}\text{I}$ -

NaI/10  $\mu\text{l}$  (IRE, Belgium, in 0.01 M NaOH, >100 mCi/ml, <0.08 nmol I/mCi) and freshly diluted ICI solution were added to give a molar ratio ICI/apoprotein of 10/1. The reaction mixture (0.5–1 ml) was slowly stirred for 10 min at 4°C and sterile filtered into a dialysis bag that was kept in dialysis buffer (0.15 M NaCl, 0.01 M  $\text{PO}_4$ , pH 7.5, 0.2 mmol EDTA) until application for in vitro studies. Alternatively, the reaction mixture was purified by Sephadex chromatography as described above. Analysis for radiochemical purity was performed in a manner identical to that for the Iodogen method.

**Indium-111-Labeling of LDL.** This was performed according to the recently described method of Rosen et al. (9). To a microvial equipped with a magnetic stirrer, 1 mg (protein) LDL in 200  $\mu\text{l}$  PBS, pH 7.5, 20  $\mu\text{l}$  0.5 M  $\text{NaHCO}_3$  and 36  $\mu\text{g}$  cyclic diethylene-triaminepentaacetic acid-anhydride (cDTPAA) in 18  $\mu\text{l}$  of dry dimethylsulfoxide (DMSO) were added. This mixture was slowly stirred for 1 hr and applied to a Sephadex G50F column (5  $\times$  40 mm) equilibrated in metal-free acetate-buffered saline (ABS), pH 5.5. The column was eluted with ABS and the protein fraction (240  $\mu\text{l}$ ) was collected into a microvial. Six hundred microcuries of  $^{111}\text{In-Cl}_3$  in 40  $\mu\text{l}$  of 0.04 M HCl were added to this and gently mixed. After 1 hr at room temperature, the reaction mixture was applied onto a second ABS-equilibrated Sephadex G50F column. The  $^{111}\text{In}$ -labeled protein fraction (350  $\mu\text{l}$ ) was collected and mixed with twice its volume of 1 mmol DTPA in PBS to give the final product solution. Analysis for radiochemical purity was performed by TLC (Merck SG, MeOH:10%  $\text{HCOONH}_4$ :0.5 citric acid, 20:20:40), CA-EP and PAGE as described above for the Iodogen method.

**Determination of the Average Number of DTPA Groups Bound per LDL Molecule.** Carefully measured amounts of cDTPAA and LDL (protein) were conjugated in a molar ratio of 50:1. Without preparation of unbound DTPA, the mixture, buffered at pH 5.5 with ABS, was labeled with  $^{111}\text{In}$ -chloride. The percentage of  $^{111}\text{In}$ (DTPA)-LDL and  $^{111}\text{In}$ -DTPA was quantitatively determined by TLC. Using this and the known molar amounts of reactants, the number of DTPA groups bound per LDL were calculated.

### Preparation of Liver Membranes

Liver tissue samples were obtained from 15 male normolipemic patients aged 38–68 yr during gastrointestinal surgery. Liver tissue was kept at 4°C until preparation of liver plasma membranes. Routine morphology was assessed by hematoxylin-eosin staining. Only tissue samples morphologically proven to show normal structure were used for binding assaying.

Liver membranes were prepared according to Neville (19) as modified by us (20) on the day of tissue removal. The membranes were taken up in assay buffer containing 50 mmol/liter Tris-HCl, pH 7.5, 5 mmol/liter  $\text{CaCl}_2$  and 5 mmol/liter  $\text{MgCl}_2$  at a protein concentration of 500  $\mu\text{g/ml}$  assayed by dye binding using the assay kit provided by BIORAD Laboratories (Commassie Blue Reagents, G20, Richmond, CA). The membranes were stored at  $-80^\circ\text{C}$  for not longer than 2 wk.

### Binding Studies

In order to evaluate ligand binding to the LDL-apoB-receptor of liver plasma membranes, direct binding experiments were carried out. All experiments were performed in duplicate and consisted of two incubation series: total binding (determined through the concentration of  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL which amounted to 1  $\mu\text{g}$  protein/ml in competition experiments and ranged from

0.1 to 32  $\mu\text{g}$  protein/ml in saturation experiments) and nonspecific binding (determined in the presence of 50  $\mu\text{g}$  protein of unlabeled LDL/ml in saturation experiments and 0.1 to 50  $\mu\text{g}$  protein/ml in competition experiments). Specific binding was expressed as the difference of total and nonspecific binding. The concentration of liver membranes used throughout amounted to 500  $\mu\text{g}$  protein/ml. In initial experiments, the time course of specific binding as well as the dependency on temperature were studied. Based on the results of these experiments all further incubations were performed at 22°C for 45 min. After incubation, the tubes were centrifuged (1800 rpm, 10 min, 4°C) to separate free from membrane-bound radioligand. After twice washing (6000 rpm, 10 min, 4°C) in 50 mmol Tris-HCl buffer pH 7.5, the pellet was counted in a gamma counter for 1 min. Vials retained less than 3% of total radioactivity (blank value = without liver membranes). In typical experiments, nonspecific binding amounted to less than 10% of total binding ( $\text{SB} = \text{B} - \text{NSB} = 100 - (<10) = >90$ ).

### Statistical Analysis

Binding data were analyzed according to Scatchard (21). Values are presented as means  $\pm$  standard deviation. Significance was calculated by the Student's t-test.

## RESULTS

Human LDL ( $d = 1.019\text{--}1.063$  g/ml) was isolated by density-gradient ultracentrifugation in KBr and labeled with either  $^{125}\text{I}$  or  $^{111}\text{In}$  to specific activities of 0.7–0.9 or 0.16–0.24 mCi/mg of protein, respectively. The molar ratios  $^{125}\text{I}/\text{LDL}$  and  $\text{cDTPAA}/\text{LDL}$  used for labeling, typical radiochemical yields, specific activities and activity concentrations of final products are summarized in Table 1. Radiochemical purity of the  $^{125}\text{I}$ -labeled preparations showed slightly better results for the ICI method as measured by TCA precipitation and electrophoresis at 5 min and 2 hr after purification. Indium-111-labeled LDL, as shown by TLC and CA-EP, remained at the application point and contained typically less than 1% of  $^{111}\text{In}$ -DTPA and free  $^{111}\text{In}$  ion (Table 2, Figs. 1 and 2). On PAGE,  $^{125}\text{I}$ -LDL and  $^{111}\text{In}$ -LDL displayed identical bands as compared to unlabeled LDL.

In order to determine the percentage of total radioactivity bound to lipoprotein of identical density, aliquots of labeled LDL were added to unlabeled LDL and analyzed by ultracentrifugation at KBr density of 1.063 g/ml. The radioactivity found amounted to  $94\% \pm 3\%$  for  $^{111}\text{In}$ -LDL and to  $92\% \pm 4\%$  for  $^{125}\text{I}$ -LDL in the LDL-specific fraction, indicating that nearly all of the radioactivity bound to a product with the density of native LDL.

In the absence of liver membranes, the application of 32  $\mu\text{g}$  of protein of  $^{111}\text{In}$ -LDL ( $^{125}\text{I}$ -LDL) resulted in the recovery of less than 1  $\mu\text{g}$  of protein of  $^{111}\text{In}$ -LDL ( $^{125}\text{I}$ -LDL) in the tip of the tube after centrifugation (<3%). This amount was identical for incubations of total and nonspecific binding.

In initial experiments, the labeled LDL was assayed for retention of natural biologic activity using isolated liver plasma membranes. The ability of  $^{111}\text{In}$ -LDL to competitively inhibit binding of  $^{125}\text{I}$ -LDL to LDL receptors was compared with that of unlabeled LDL. Indium-111-LDL significantly inhibited binding of  $^{125}\text{I}$ -LDL causing 85% inhibition with 50  $\mu\text{g}$  of protein/ml (Fig. 3). The corresponding  $\text{IC}_{50}$  value for  $^{111}\text{In}$ -LDL to displace  $^{125}\text{I}$ -LDL amounted to  $8.4 \pm 1.2$   $\mu\text{g}$  of protein/ml. Unlabeled LDL caused significant inhibition of both,  $^{111}\text{In}$ -LDL and  $^{125}\text{I}$ -LDL binding to liver membranes. The corresponding  $\text{IC}_{50}$  values for unlabeled LDL were  $1.7 \pm 0.7$   $\mu\text{g}$  of protein/ml for  $^{111}\text{In}$ -LDL binding and  $7.7 \pm 1.0$   $\mu\text{g}$  of protein/ml for  $^{125}\text{I}$ -LDL binding ( $p < 0.001$ ).

In initial experiments, the interaction of LDL with washed liver plasma membranes was assessed as a function of time and temperature. As shown in Figure 4, each ligand bound to the liver membranes at 22°C, and the time course of the binding reaction was similar for  $^{111}\text{In}$ -LDL and  $^{125}\text{I}$ -LDL. However, during the first 20 min (this is the time span when association of the ligand-receptor complex takes place), the interaction of  $^{111}\text{In}$ -LDL with liver membranes was significantly faster ( $p < 0.01$ ) as compared to  $^{125}\text{I}$ -LDL. Binding of  $^{111}\text{In}$ -labeled LDL in the presence of an excess of nonlabeled LDL (50  $\mu\text{g}$

TABLE 1  
Radiochemical Yields and Final Activity

| Labeling method             | I/LDL<br>molar ratio             | Radiochem. yield<br>(%) | Specific activity<br>(mCi/mg) | Activity<br>concentration<br>( $\mu\text{Ci}/\text{ml}$ ) |
|-----------------------------|----------------------------------|-------------------------|-------------------------------|---|
| Iodogen-Sephadex            | $0.5 \pm 0.1$                    | $88 \pm 4$              | 0.88                          | 50  |
| Iodogen-Dialysis            | $0.5 \pm 0.1$                    | $92 \pm 5$              | 0.92                          | 50  |
| ICI-Sephadex                | $10 \pm 1.2$                     | $69 \pm 4$              | 0.69                          | 50  |
| ICI-Dialysis                | $10 \pm 1.6$                     | $72 \pm 6$              | 0.72                          | 50  |
| $^{111}\text{In}$ -labeling | <u>molar ratio</u><br>cDTPAA/LDL |                         |                               |   |
| Reactants                   | 50                               |                         |                               |   |
| Bound                       | 5.5                              | $72 \pm 9$              | 0.16–0.24                     | $360 \pm 90$  |

mean  $\pm$  s.d.;  $n = 6$ .

**TABLE 2**  
Radiochemical Purity of Labeled LDL After Purification

| Labeling method            | TCA-precipitate (%) |        | Electrophoresis (% I <sup>-</sup> ) |           | TLC (%)   |            |            |
|----------------------------|---------------------|--------|-------------------------------------|-----------|-----------|------------|------------|
|                            | 5 min               | 2 hr   | 5 min                               | 2 hr      | 18 hr     | 2 hr       | 18 hr      |
| Iodogen-Sephadex           | 91 ± 5              | 80 ± 4 | 3.5 ± 2.4                           | 8.6 ± 3.2 |           |            |            |
| Iodogen-Dialysis           | 90 ± 4              | 80 ± 5 | 2.6 ± 1.2                           | 6.0 ± 2.3 |           |            |            |
| ICI-Sephadex               | 94 ± 2              | 85 ± 3 | 1.2 ± 0.3                           | 5.1 ± 2.2 |           |            |            |
| ICI-Dialysis               | 95 ± 4              | 84 ± 5 | 0.6 ± 0.2                           | 4.1 ± 2.3 |           |            |            |
| <sup>111</sup> In-labeling |                     |        | % free In                           | 0.3 ± 0.1 | 0.7 ± 0.3 | 99.5 ± 0.2 | 99.2 ± 0.3 |

mean ± s.d.; n = 6.

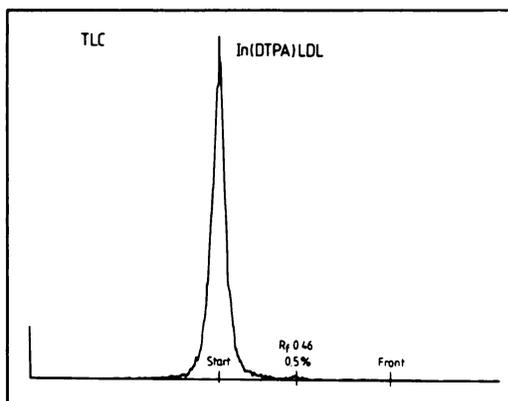
protein/ml) was less than 10% of the total binding observed in the absence of nonlabeled LDL. Equilibration was stable for at least 120 min. Once specifically bound, <sup>123</sup>I-LDL (<sup>111</sup>In-LDL) was fully displaceable by addition of an excess amount of unlabeled LDL within 30 min after equilibration.

The interactions were only slightly dependent on temperature (Fig. 5). At 22°C, binding of <sup>111</sup>In-LDL (<sup>123</sup>I-LDL) at 45 min was 98% (95%) of that observed at 4°C and at 37°C it was 96% (94%). In all subsequent experiments, LDL binding was measured at 22°C and a 45-min incubation time was chosen to ensure equilibrium. The capacity to saturate the liver binding sites for LDL was assessed by incubating increasing concentrations of <sup>111</sup>In-LDL or <sup>123</sup>I-LDL in absence and presence of unlabeled LDL (50 µg protein/ml). Specific binding was defined by subtraction of the binding observed in the presence from that observed in the absence. Specific binding of both <sup>111</sup>In-LDL and <sup>123</sup>I-LDL to washed liver membranes was saturable and indicated a high affinity binding site (see Discussion) capable of binding 239 ± 26 ng of protein of

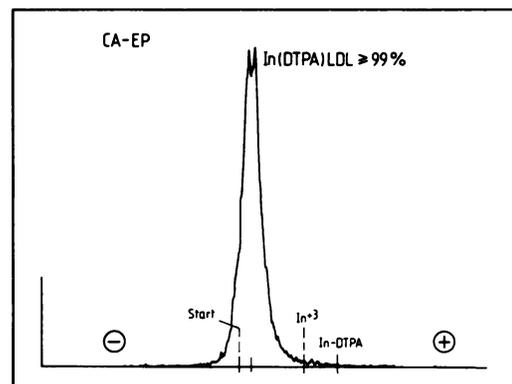
<sup>111</sup>In-LDL/mg liver plasma membrane protein and 148 ± 18 ng of protein of <sup>123</sup>I-LDL/mg liver plasma membrane protein (p < 0.001). The corresponding dissociation constants (K<sub>d</sub>) were 0.6 ± 0.2 µg protein/ml for <sup>111</sup>In-LDL and 1.2 ± 0.7 µg protein/ml for <sup>123</sup>I-LDL (p < 0.01). Representative saturation curves are shown in Figure 6A. In concentrations above 15 µg of protein/ml, saturation was obtained for <sup>123</sup>I-LDL binding, and saturation was obtained in concentrations above 5 µg of protein/ml for <sup>111</sup>In-LDL binding. Scatchard plots (Fig. 6B) indicate single straight lines with correlation coefficients ranging from r = -0.92 to -0.98.

## DISCUSSION

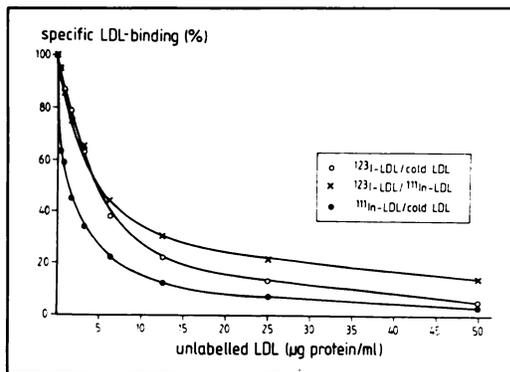
The liver is the optimal organ for comparative ligand studies because of the presence of the highest amount of LDL receptors which determine the clearance of cholesterol from plasma (22). Approximately two-thirds of plas-matic cholesterol are transported by LDL and internalized by endocytosis through high-affinity apoB,E-receptor binding (11). Insufficient LDL receptor activity or com-



**FIGURE 1.** Thin-layer chromatography of <sup>111</sup>In-LDL. One microliter was spotted on Merck silica-gel plates and developed in a solvent mixture of MeOH: 10% HCOONH<sub>4</sub>: 0.5 M citric acid (20:20:10). Indium-111-LDL remained at the application point, while In<sup>3+</sup> and In-DTPA migrated with a R<sub>f</sub> of 0.15 and 0.45, respectively. Radiochemical purity was more than 99% at 20 hr after labeling.

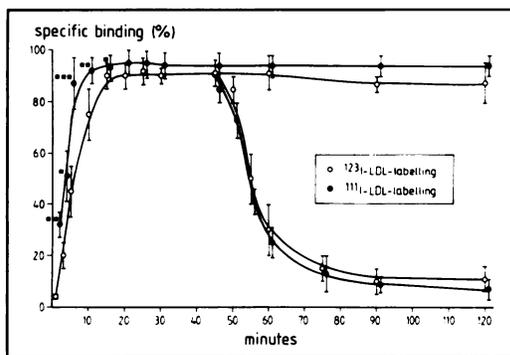


**FIGURE 2.** Cellulose acetate electrophoresis of <sup>111</sup>In-LDL. One microliter was spotted on rehydrated CA-strips that were subject to horizontal zone electrophoresis in 0.05 M barbital buffer pH 8.6 containing 1 mM EDTA and 1% HSA at 300 V for 20 min. Indium-111-LDL migrated only about 5 mm, while In<sup>3+</sup> and In-DTPA migrated 25 and 40 mm, respectively. Radiochemical purity was better than 99% at 20 hr after labeling.

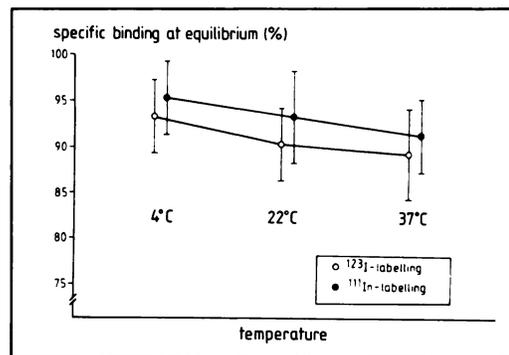


**FIGURE 3.** Ability of unlabeled human LDL and  $^{111}\text{In}$ -LDL from normolipemic subjects to compete with human  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL for binding to human liver membranes. Each assay tube contained human  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL ( $1\ \mu\text{g}$  of protein/ml; 625 cpm/ng of protein for  $^{123}\text{I}$ -LDL, 1000 cpm/ng protein for  $^{111}\text{In}$ -LDL) and the indicated concentrations of unlabeled LDL or  $^{111}\text{In}$ -LDL. The 100% control value for  $^{123}\text{I}$ -LDL binding in the absence of unlabeled LDL was  $136 \pm 19$  ng of protein bound/mg of membrane protein. The 100% control value for  $^{111}\text{In}$ -LDL binding in the absence of unlabeled LDL was  $241 \pm 21$  ng of protein bound/mg of membrane protein. The corresponding  $\text{IC}_{50}$  value for  $^{111}\text{In}$ -LDL to displace  $^{123}\text{I}$ -LDL amounted to  $8.4 \pm 1.2\ \mu\text{g}$  protein/ml, for unlabeled LDL to displace  $^{111}\text{In}$ -LDL:  $1.7 \pm 0.7\ \mu\text{g}$  protein/ml, and to displace  $^{123}\text{I}$ -LDL:  $7.7 \pm 1.0\ \mu\text{g}$  protein/ml. Each point represents the mean  $\pm$  s.d. of six independent experiments with liver membranes from different patients.

plete lack of LDL receptors has been evidenced (11). Patients with such LDL receptor defects suffer from coronary artery disease at a young age, a fact based upon the clear association between plasmatic LDL-cholesterol and progression of atherosclerosis. Knowledge about LDL receptor status could be of value for the diagnosis of familial hypercholesterolemia. Such a methodology would be the quantitative determination of liver receptors *in vivo*

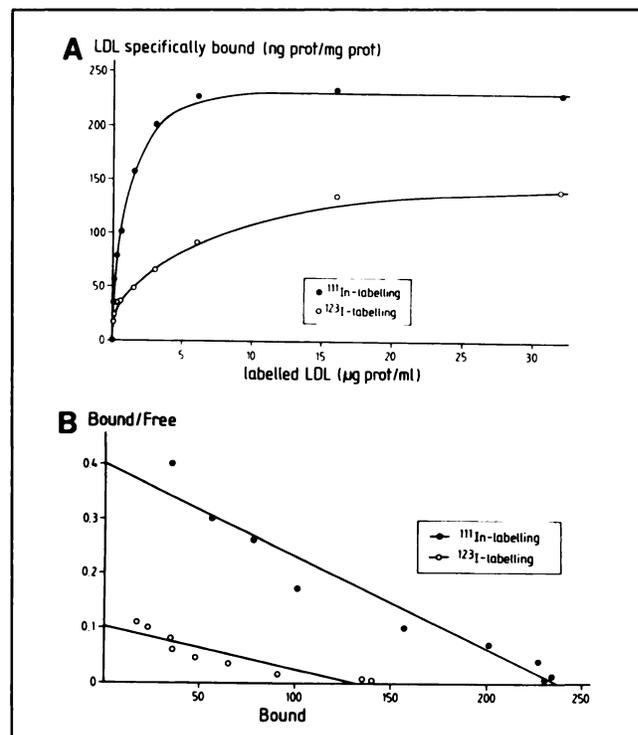


**FIGURE 4.** Time course of specific  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL binding to human liver membranes. Association:  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL ( $1\ \mu\text{g}$  protein/ml) was incubated with liver membranes ( $500\ \mu\text{g}$  protein/ml) in absence (total binding) and presence (nonspecific binding) of unlabeled LDL ( $50\ \mu\text{g}$  protein/ml) for the time intervals indicated. Dissociation: at equilibrium an excess of unlabeled LDL ( $50\ \mu\text{g}$  protein/ml) was added at the times indicated. Each point represents the mean  $\pm$  s.d. of six independent experiments with liver membranes prepared from different patients.



**FIGURE 5.** Temperature dependency of specific  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL-binding to human liver membranes.  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL ( $1\ \mu\text{g}$  protein/ml) was incubated with liver membranes ( $500\ \mu\text{g}$  protein/ml) in absence (total binding) and presence (nonspecific binding) of unlabeled LDL ( $50\ \mu\text{g}$  protein/ml) for 45 min at 4, 22, and 37°C. Each point represents the mean  $\pm$  s.d. from six independent experiments with liver membranes from different patients.

(23) via gamma camera imaging with an isotope suitable for long-term studies. The application of iodinated LDL to humans brings about the problem of hepatic deiodination which limits quantitative determinations. Such determinations would be possible by use of the recently developed glycoprotein receptor-specific program of Vera et al.



**FIGURE 6.** Saturation curve (A) and Scatchard analysis (B) of specific  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL binding to human liver apoB-receptors. Each assay tube contained the indicated concentrations of  $^{123}\text{I}$ -( $^{111}\text{In}$ )-LDL (625 cpm/ng of protein for  $^{123}\text{I}$ -LDL, and 1000 cpm/ng protein for  $^{111}\text{In}$ -LDL). Specific binding was calculated by subtracting the amount of  $^{123}\text{I}$ -LDL bound in the presence of excess of unlabeled LDL ( $50\ \mu\text{g}$  protein/ml) from that bound in its absence.

**TABLE 3**  
Binding Data for LDL Receptor Binding to Human Liver Plasma Membranes

| Labeling Method            | IC <sub>50</sub><br>( $\mu\text{g protein/ml}$ ) | B <sub>max</sub><br>(ng protein/<br>mg protein) | K <sub>d</sub><br>( $\mu\text{g protein/ml}$ ) |
|----------------------------|--|---|--|
| Iodogen-Sephadex           | 7.6 $\pm$ 1.1                                    | 144 $\pm$ 23                                    | 1.3 $\pm$ 0.8                                  |
| Iodogen-Dialysis           | 7.8 $\pm$ 0.9                                    | 151 $\pm$ 18                                    | 1.2 $\pm$ 0.7                                  |
| ICI-Sephadex               | 8.1 $\pm$ 1.0                                    | 139 $\pm$ 14                                    | 1.2 $\pm$ 0.8                                  |
| ICI-Dialysis               | 7.3 $\pm$ 1.1                                    | 159 $\pm$ 19                                    | 1.0 $\pm$ 0.7                                  |
|                            | 7.7 $\pm$ 1.0                                    | 148 $\pm$ 18                                    | 1.2 $\pm$ 0.7                                  |
| <sup>111</sup> In-labeling | 1.7 $\pm$ 0.7                                    | 239 $\pm$ 26                                    | 0.6 $\pm$ 0.2                                  |

mean  $\pm$  s.d.; n = 6.

(24), since glycoproteins maintain a similar intrahepatic pathway to lipoproteins. Also, the use of SPECT to determine the liver volume and whole-body scanning to measure the activity trapped by the liver could allow quantitative uptake measurements as a reliable parameter for follow-up studies.

As recently outlined (14), we found no significant difference in the binding of <sup>123</sup>I-LDL to human liver plasma membranes after labeling by the Iodogen (16) or ICI method (17). In addition, the differences in radiochemical purity and stability between the four labeling purification methods for <sup>123</sup>I labeling were rather small, although there were somewhat better results for the ICI method (Tables 1 and 2). Compared to <sup>123</sup>I-labeling of LDL, <sup>111</sup>In labeling showed significantly better results of radiochemical purity and in vitro product stability. As shown by TLC and CAEP, <sup>111</sup>In-labeled LDL contained less than 1% of <sup>111</sup>In-DTPA and free <sup>111</sup>In ion, whereas <sup>123</sup>I-labeled LDL contained about 5% free <sup>123</sup>I ion 2 hr after application. However, on PAGE, <sup>123</sup>I-LDL and <sup>111</sup>In-LDL displayed identical bands as compared to unlabeled LDL.

Using cultured human fibroblasts, Rosen et al. (9) have recently shown that <sup>111</sup>In-LDL binding demonstrated lower affinity and an increased nonsaturable component, whereas <sup>125</sup>I-LDL binding was fully saturable. However, these observations were discordant with their in vivo findings of biodistribution in rabbits. These in vitro studies on cultured fibroblasts are in contrast to our observations with human liver and lymphocytes (paper in preparation), which suggest that <sup>111</sup>In-labeled LDL might be a better ligand for the LDL receptor than <sup>123</sup>I-LDL. Indium-111-LDL is about two times more tightly bound than <sup>123</sup>I-LDL, and also significantly more <sup>111</sup>In-labeled LDL is specifically bound as compared to <sup>123</sup>I-LDL.

The reason for the significant difference in affinity for the LDL receptor might to some extent be due to the higher in vitro stability of the <sup>111</sup>In-labeled ligand with a dissociation constant of 0.6  $\pm$  0.2  $\mu\text{g}$  of protein/ml, whereas <sup>123</sup>I-LDL binds with half of the affinity (K<sub>d</sub> 1.2  $\pm$

0.7  $\mu\text{g}$  protein/ml). This, however, does not explain the higher number of LDL receptors identified (239  $\pm$  26 ng protein/mg protein for <sup>111</sup>In-LDL versus 148  $\pm$  18 ng protein/mg protein for <sup>123</sup>I-LDL). Furthermore, unlabeled LDL appears to compete significantly more easily with binding of <sup>111</sup>In-LDL than of <sup>123</sup>I-LDL (IC<sub>50</sub> = 1.7 and 7.7  $\mu\text{g}$  protein/ml, respectively), even though the latter obviously is less strongly bound.

When labeled and nonlabeled ligands compete for binding to a homogeneous population of saturable binding sites, the IC<sub>50</sub> for the unlabeled ligand can be easily shown to be:

$$\text{IC}_{50} = K_d \cdot (1 + L^*/K_{d^*}), \quad \text{Eq. 1}$$

where K<sub>d</sub> and K<sub>d</sub>\* are the dissociation constants for binding of nonlabeled and labeled ligand, respectively, and L\* is the total concentration of labeled ligand. In this equation, the total binding has been set equal to the corresponding concentrations of unbound ligands, which is a safe approximation under the experimental conditions.

When applying Equation 1 to the experiments with <sup>111</sup>In-labeled LDL, the apparent K<sub>d</sub> for unlabeled LDL is calculated to be 0.64  $\mu\text{g}$  protein/ml. This value is nearly identical with the corresponding value obtained for <sup>111</sup>In-LDL, indicating that the binding affinity of LDL is virtually unaffected by labeling with <sup>111</sup>In as described. From the competition experiments with <sup>123</sup>I-LDL, however, a significantly lower apparent affinity is obtained for unlabeled LDL (K<sub>d</sub> = 4.2  $\mu\text{g}$  protein/ml), or, in other words, the IC<sub>50</sub> appears to be "too high" in this case.

The least complex explanation for the observed differences in binding behavior of the two differently labeled LDL preparations (with respect to K<sub>d</sub>, B<sub>max</sub>, IC<sub>50</sub>) is to assume some microheterogeneity of LDL binding sites. This may be partly due to the loss of cellular integrity or may be an inherent feature of the LDL receptor known to possess multiple (interacting or not) binding sites.

We assume that both unlabeled and <sup>111</sup>In-labeled LDL can bind to the whole range of these receptor sites with an apparent macroscopic (average)—K<sub>d</sub> of 0.6  $\mu\text{g}$  protein/ml. On the other hand, binding of <sup>123</sup>I-LDL appears to be restricted to the lower affinity subfractions (average K<sub>d</sub>: 1.2  $\mu\text{g}$  protein/ml). Due to their modification, access to those sites which exhibit higher affinity towards native LDL is severely hindered. Consequently, unlabeled LDL will first saturate all the sites with higher binding affinities before competing with the binding of <sup>123</sup>I-LDL. Hence, IC<sub>50</sub> in this case will markedly exceed the value calculated from Equation 1.

From this general model, IC<sub>50</sub> can no longer be explicitly expressed (25,26), but it can be numerically calculated by iteration to fit Equation 2:

$$\left| \frac{\Lambda}{\sum n_i} \sum \frac{n_i \text{IC}_{50}}{K_d + \text{IC}_{50}} \cdot \left( \Lambda - \frac{\Lambda}{2 \sum n_j^*} \sum \frac{n_j^* L^*}{K_{d_j^*} + L^*} \right) - \frac{\Lambda}{2} \right| = 0,$$

where n<sub>i</sub> is the number of binding sites/mg membrane protein of class i, binding unlabeled ligand with K<sub>d</sub><sub>i</sub>, and

$n_j$  is the number of binding sites/mg membrane protein of class  $j$ , binding labeled ligand with  $K_{d_j}$ .

The following calculation (not shown in detail) is based on the assumption of a Gaussian distribution of receptor-site affinities (95% of the sites ranging from  $K_d$  0.3 to 1.8  $\mu\text{g}$  protein/ml). Binding to these sites should yield a binding isotherm which, within experimental error, is almost indistinguishable from binding to a uniformly behaving receptor-site population with  $K_d$  0.6  $\mu\text{g}$  protein/ml. The apparent average  $K_d$  for nonlabeled LDL competing with  $^{123}\text{I}$ -LDL ( $\text{IC}_{50} = 7.7 \mu\text{g}$  protein/ml) then turns out as 2.4  $\mu\text{g}$  protein/ml, a value fairly close to the experimental value obtained for  $^{123}\text{I}$ -LDL binding to the same subset of sites.

In conclusion, these findings show that methods for  $^{123}\text{I}$ -labeling of lipoproteins can be recommended as equivalent techniques for production of tracers for in vitro receptor evaluation. Iodine-123 techniques are sufficiently good for in vitro studies, however,  $^{111}\text{In}$ -LDL might be a more powerful radiolabel with respect to in vitro stability and binding affinity. Furthermore, its half-life of 2.83 days has a theoretical advantage over  $^{123}\text{I}$ -LDL or  $^{99\text{m}}\text{Tc}$ -LDL.

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