Specific Sorbent of Apolipoprotein B-Containing Lipoproteins for Plasmapheresis

Characterization and Experimental Use in Hypercholesterolemic Rabbits

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Dextran sulfate was covalently bound to cellulose beads (LA01) and used as the specific sorbent of apolipoprotein B-containing lipoproteins. The binding profiles of very low density, low density, and high density lipoproteins to the material were consistent with simple Langmuir adsorption isotherms. Although the affinities of all lipoproteins for the sorbent were roughly similar, the surface area available for high density lipoprotein binding was much smaller than that for very low and low density lipoproteins (29, 927, and 1934 m²/liter of swollen beads, respectively). When human plasma was passed through a sorbent column, very low and low density lipoproteins were selectively adsorbed, while almost all the high density lipoproteins were recovered from the column together with the other major plasma components. An extracorporeal circulation system with this sorbent was used for the treatment of hypercholesterolemic rabbits (diet-induced and homozygous WHHL). With a 25 ml sorbent column, very low and low density lipoproteins were selectively removed from rabbit plasma, resulting in a reduction of plasma cholesterol concentration by 300 mg/dl. (Arteriosclerosis 4:276-282, May/June 1984)

Familial hypercholesterolemia is an inherited metabolic disorder characterized by an increased level of plasma low density lipoprotein (LDL) which is attributed to a genetic deficiency in the specific cell surface receptor of LDL.1 Homozygous patients develop fatal premature atherosclerotic vascular lesions in their first decade of life, and heterozygotes develop these lesions in the third decade. Some oral drugs may reduce the plasma cholesterol levels of heterozygotes below 250 mg/dl.2-6 For homozygotes, such treatments can only keep plasma cholesterol level at approximately 400 to 500 mg/dl.3,5,6 In 1975, Thompson et al.7 first used plasma exchange in the successful management of the plasma cholesterol level of homozygotes. Treatment by this procedure at 2-week intervals maintained plasma cholesterol levels between 400 and 150 mg/dl. Although highly successful, this treatment has some practical problems, such as the high cost, the unnecessary depletion of whole plasma components, and the need for a large supply of supplemental replacement plasma protein. To palliate these disadvantages, Lupien et al.8 and Graisely et al.9 developed techniques for removing LDL from the plasma using LDL-specific sorbents such as heparin-agarose beads. Other researchers developed techniques using an anti-LDL antibody coupled to Sepharose10 and double-membrane filtration.11 These LDL-sorbent systems gave excellent specificity but still had the problems of supply, cost, and gel handling (sterilization and mechanical stability). The double-membrane filtration technique is now the best from a practical point of view, but is less specific for LDL than the sorbents.
In this report, we describe experiments with a new material, LA01, that adsorbs LDL selectively and is readily prepared industrially. Our in vitro and in vivo experiments make possible a selective, efficient plasmapheretic system that uses this material to remove LDL from patients with familial hypercholesterolemia.

Methods

Lipoprotein fractions were isolated from citrated human plasma by sequential ultracentrifugation in a NaBr solution at densities less than 1.006 g/ml for very low density lipoprotein (VLDL), from 1.006 to 1.063 g/ml for LDL, and from 1.063 to 1.21 for high density lipoprotein (HDL). Each lipoprotein fraction was dialyzed after separation against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl.

Sorbent beads, LA01 (Kanegafuchi Chemical Industrial Company, Ltd., Osaka, Japan), were prepared using dextran sulfate (Mr 5000) obtained from Sigma Chemical Company, St. Louis, Missouri. The dextran sulfate was covalently linked to cellulose that responded to the high cholesterol diet and homozgyous WHHL rabbits were used for in vivo plasmapheretic experiments.

Cholesterol concentration was determined by an enzymatic assay (Determiner TC5, Kyowa Medics, Tokyo, Japan). HDL cholesterol in the whole plasma was also measured by the same enzymatic method after precipitation of apolipoprotein B-containing lipoproteins with heparin in the presence of Mg2+ (HDL cholesterol test Wako, Wako Pure Chemical Industries, Osaka, Japan). Protein concentration was measured by the method of Lowry et al. Apolipoprotein A-I (apo A-I) concentration was measured by the single radial immunodiffusion technique using an 1% agarose plate containing an anti-apo A-I antibody provided by Dai-ichi Pure Chemical Company Ltd., Tokyo, Japan.

Varying amounts of individual lipoproteins were incubated with a constant amount of the LA01 gel at room temperature with gentle stirring. After a given period of time, the incubation mixtures were centrifuged at 2000 g for 10 minutes. The cholesterol concentration in the supernatant was measured, and its decrease due to the presence of the sorbent beads was considered as cholesterol bound to the sorbent. For typical experiments with VLDL and LDL, the sorbent beads were first equilibrated with a 0.01 M phosphate buffer containing 0.15 M NaCl (pH 7.4). Ten ml wet-packed volume of the beads was suspended in 30 ml of the same buffer. The incubation mixtures contained 200 µl of the beads in suspension and lipoprotein in the same buffer, giving a final volume of 3 ml. In control incubation mixtures, 200 µl of buffer substituted for the sorbent beads suspension. For HDL, 900 µl of the beads in suspension, prepared by suspending 10 ml wet-packed sorbent in 20 ml buffer, was added to the HDL solution, giving a final incubation volume of 2 ml. The solid volume of the sorbent matrix was estimated by dye dilution to be 10% of the wet-packed volume; this number was used for the correction of cholesterol concentrations in the binding study. Binding of lipoproteins reached a maximum within 20 minutes. Therefore, an incubation time of 40 minutes was chosen for all binding experiments.

Adsorption of lipoprotein by LA01 from human plasma was also studied using sorbent columns of 2 to 3 ml volume. The column was first equilibrated with the same buffer as the incubation buffer. Heparinized human plasma (40 to 50 ml) was passed through the column with a flow rate of 0.5 ml/min; 0.4 ml fractions were collected. The concentrations of cholesterol, HDL-cholesterol, apo A-I, and protein were measured in each fraction. Lipoproteins were pre-stained with Sudan Black B and analyzed by electrophoresis in 3.75% polyacrylamide gels. The quantity of lipoprotein in each band was estimated using a densitometric scanner (Fujiriken FD A-IV, Tokyo). Proteins in the eluate fractions were identified by immunoelectrophoresis using antihuman plasma rabbit serum.

For plasmapheretic treatment of the rabbits, an extracorporeal circuit including LA01 was constructed using elements of a plasmapheretic system (APEX-II, Kawasumi Laboratories, Inc., Tokyo). The plasma separator consisted of polysulfone hollow fibers (Kanegafuchi Chemical Industrial Company Ltd.) with an average pore diameter of 0.2 µm in a 5 ml chamber (the intrafibrous volume was 1.8 ml). The plasma was first separated from the blood cells by passing through the hollow fibers; plasma was then passed through a LA01 column (25 ml; inclusion volume was about 18 ml). After passing through this column, plasma was mixed continuously with heparin (5 unit/min) and then recombined with the blood-cell-rich portion retained by the hollow fibers; this was infused into the rabbit. Polyvinylchloride tubes were used for the circuit. The whole extracorporeal circulation volume was about 40 ml (Figure 1). Animals (3 to 3.5 kg in weight) were anesthetized with 2 ml of Nembutal (Abbot Laboratories, North Chicago, Illinois) injected intravenously before operation. The extracorporeal circulation device was first filled with physiological saline containing heparin (10 unit/ml). Blood was withdrawn from an ear artery at a rate of 6 ml/min and plasma was pumped from the separator at a rate of 2 ml/min by using peristaltic pumps. The arterial blood and the filtered plasma were monitored before and after passing through the sorbent for total and HDL cholesterol concentrations and protein concentration. The platelet count was
Figure 1. An experimental extracorporeal circuit for plasmapheretic treatment of rabbits using the specific sorbent (LA01) of LDL. Polysulfone hollow fibers with an average pore diameter of 0.2 μm were used in a 5 ml chamber (intrafibrous volume was 1.8 ml). The volume of the LA01 column was 25 ml; the total extracorporeal circulation volume was 40 ml. P₁ = a pump for blood withdrawal; P₂ = a pump for plasma filtration.

Figure 2. Typical binding profiles of lipoproteins to LA01. Each incubation mixture contained 50 μl of the wet-packed sorbent for LDL and VLDL. For HDL, each 2 ml incubation mixture contained 300 μl of the wet sorbent. ● = LDL; ○ = VLDL; □ = HDL. Lines represent calculated binding curves using the parameters listed in Table 1.

Results

Binding of Human Lipoproteins to the Sorbent In Vitro

The typical profiles of lipoproteins bound to the sorbent beads are shown in Figure 2. The amount of cholesterol bound to a constant volume of the sorbent is displayed as a function of the concentration of added cholesterol. The amount of lipoprotein bound to the sorbent increased as lipoprotein concentration increased until saturation (maximum) was reached. When tested for individual lipoprotein, the saturating level for HDL was much lower than for LDL and VLDL. The binding data were analyzed according to the Langmuir adsorption isotherm using the equation:

\[(S - LP_b)/LP = K_d\] (1)

where S is the molarity of the lipoprotein bound at saturation level, LP_b and LP are concentrations of bound and free lipoprotein, and K_d is the dissociation constant.

This analysis was based on the assumption that lipoproteins were adsorbed reversibly and noncooperatively to the surface of the sorbent. The molarity of the lipoprotein was calculated using the factors of 1 g of cholesterol/liter = 3.62 x 10^{-7} M for VLDL; 1.45 x 10^{-6} M for LDL; and 2.80 x 10^{-5} M for HDL.

The linear plot of the data according to this equation is shown in Figure 3. The data for each lipoprotein subclass fit to a straight line, which indicates that binding of lipoprotein to the sorbent is, indeed, a simple Langmuir-type adsorptive binding. Dissociation constants (K_d) and the maximum capacity (S) for each lipoprotein subclass are obtained from these plots as the ordinate intercept and the slope, respectively. The values of K_d and S are listed in Table 1. The values of S were also converted to the area occupied by lipoprotein, assuming a monomolecular layer of spherical lipoproteins. The area of the lipoprotein monomolecular layer was calculated using

Table 1. Binding Parameters of Lipoproteins to LA01

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>K_d x 10^9 M</th>
<th>g/liter</th>
<th>m^2/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>20</td>
<td>1.8</td>
<td>927</td>
</tr>
<tr>
<td>LDL</td>
<td>29.7</td>
<td>7.1</td>
<td>1394</td>
</tr>
<tr>
<td>HDL</td>
<td>53.8</td>
<td>0.03</td>
<td>29</td>
</tr>
</tbody>
</table>

K_d is the dissociation constant, expressed as molarity of lipoproteins. S is the saturating level of lipoprotein binding in terms of lipoprotein concentration, expressed as grams of cholesterol per liter of wet-packed sorbent as well as the area for the binding per liter of the sorbent.
the factors of 1.9 mg cholesterol/m² for VLDL, 3.7 mg/m² for LDL, and 1.2 mg/m² for HDL. The K values (inverse of affinity) were similar for LDL and HDL, while VLDL had a much higher affinity to the sorbent. The capacity of the sorbent, however, was much smaller for HDL than for VLDL or LDL. The presence of Ca²⁺ (10 mM) in the incubation mixture did not have any significant effect on the binding parameters. Thus, the binding of HDL to the sorbent was limited not because of the low affinity, but because the surface to which HDL can bind is limited. Therefore, one would expect selective adsorption of LDL and VLDL even from human plasma, which normally contains 5 to 10 times more HDL particles than LDL.

**Lipoprotein Adsorption from Human Plasma**

Human plasma from a normal subject and from a patient with familial hypercholesterolemia was passed through the sorbent column. Total and HDL cholesterol and protein were measured for each eluate fraction. As shown in Figure 4, both plasmas gave essentially the same results. Protein was eluted without any retention and the protein concentration in the eluate was the same as in the original plasma. The HDL cholesterol level showed exactly the same elution profile as the total protein level did, and apo A-I also eluted as a similar profile to HDL cholesterol, reaching the same concentration as in the original plasma (125 mg/dl for a normal subject) (data not included in Figure 4). On the other hand, the elution profiles of total cholesterol levels corresponded to HDL cholesterol for the first several fractions, indicating that all the unbound cholesterol was
due to HDL alone and that only a negligible amount of HDL was adsorbed. Then the total cholesterol level gradually increased until it reached the total cholesterol concentration in the original plasma after 30 ml plasma volume. The results of electrophoretic analysis also showed a selective removal of LDL (Figure 5).

These results demonstrate that independently measured binding parameters are valid for lipoproteins present in plasma and account for the selective removal of LDL (and VLDL) from plasma. Immuno-electrophoresis of the original and the eluted plasma against antihuman plasma rabbit serum showed that the major plasma protein components were recovered from the column. From the amount of lipoprotein bound in the experiment, the capacity of the sorbent was estimated at about 9 g/liter of cholesterol for normal plasma, and 12 g/liter cholesterol for hypercholesterolemic plasma. These values are in good agreement with the S value obtained from the binding experiments. Scaling up the experiment did not change the capacity of the sorbent; when 2 liters of human plasma were passed through the 200 ml column with a flow rate of 50 ml/min, the capacity of the sorbent was 10 to 12 g/liter of cholesterol.

LA01 Plasmapheresis of Rabbits

The sorbent for apolipoprotein B-containing lipoprotein (LA01) was used for plasmapheretic treatment of rabbits. Plasmapheresis was performed with the device described in the Methods section. Control experiments were done with the same device except for the replacement of the LA01 column by a blank column with the same free saline volume. In control experiments, a WHHL rabbit plasma cholesterol level decreased by 5% to 7% after 100 ml of plasma was treated, probably because of nonspecific lipoprotein adsorption of the polymers used in the components of the instrument and also because of the dilution of the plasma by the initial extracorporeal circulation volume. When the LDL adsorptive device was used for normal rabbits, the plasma cholesterol level of 35 mg/dl, most of which was in HDL, decreased by only a small percentage after treatment with 100 ml of plasma. The HDL cholesterol level of 30 mg/dl to 32 mg/dl did not change throughout the treatment.

With WHHL rabbits, the plasma cholesterol level decreased from around 500 mg/dl to 200 mg/dl after 100 ml of plasma passed through the sorbent column. HDL cholesterol did not show any significant change during the operation (18 mg/dl to 15 mg/dl). The protein concentration did not change either. A typical profile of change in cholesterol, HDL cholesterol, and plasma protein level during the treatment is shown in Figure 6. Electrophoretic analysis of plasma lipoproteins also showed specific reduction of LDL and VLDL after treatment of plasma with LA01 (Figure 7). The estimated total capacity of the LA01 column for cholesterol binding was again about 10 g/liter.
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Figure 7. Electrophoretic profiles of lipoproteins of a WHHL rabbit plasma circulating in the body (top), immediately after the plasma separator (middle), and immediately after the LA01 column (bottom) when treated with 40 ml of plasma.

Figure 8. Typical changes in protein, total cholesterol, and HDL cholesterol concentration in the plasma of a rabbit with hypercholesterolemia induced by feeding a cholesterol-rich diet. --- = total cholesterol concentration in the plasma circulating in the rabbit; •—• = cholesterol after the plasma separator; ○—○ = cholesterol after the LA01 column; △—△ = HDL cholesterol in the plasma; ▲—▲ = HDL cholesterol after the separator; and △—△ = HDL cholesterol after the sorbent column.

Diet-induced hypercholesterolemic rabbits were also treated by the same procedure. By feeding a 2% cholesterol diet, the total plasma cholesterol levels of the rabbit were first elevated to 1500 mg/dl to 1900 mg/dl. A considerable increase of lipoproteins with the sizes of chylomicrons and VLDL was observed. By treating 100 ml of plasma with LA01 plasmapheresis, the reduction of total cholesterol level was again about 300 mg/dl. Neither HDL cholesterol nor protein concentration was affected by the treatment. However, due to the presence of very large lipoprotein particles, the plasma separator did not segregate the lipoproteins effectively from the cells. Even the filtered large lipoproteins were adsorbed by the sorbent less effectively than LDL-size particles (Figures 8 and 9). It appears from our electrophoretic analysis that these large lipoproteins with diameters larger than 0.2 μm did not efficiently pass through the hollow fiber’s pores. We feel that it is the size of the particle that is also responsible for the lack of adsorption to LA01; the pore size of the sorbent does not seem to be large enough to let large lipoproteins diffuse into the beads.

Finally, no hemolytic reaction was ever observed and the platelet count did not change by the plasmapheretic treatment of the rabbits; the count stayed consistently at around 5 × 10^9/mm³.
Discussion

Thus, dextran sulfate-coated cellulose beads (LA01) have been proven a potent specific sorbent of apolipoprotein B-containing lipoproteins for plasmapheresis treatment of familial hypercholesterolemias. Heparin-agarose beads and anti-LDL antibody-agarose beads have also been shown9–10 to be LDL-specific sorbents in the experimental system. We have developed support materials for cellulose beads because of the difficulties encountered in the system mentioned earlier and described in the introductory section. The mechanical properties of microporous cellulose beads are markedly good; they are easily autoclaved and can tolerate flow rates as high as 5 ml/min-cm² without losing their ability to bind LDL. Even extensive dehydration and hydration had no effect on the size and shape of the beads. Furthermore, the absence of organic crosslinks in cellulose promises much less antigenic ability and specific binding of blood components than would be the case for agarose beads. Finally, the lower cost and the ease of reproducing the starting materials warrant the use of cellulose instead of agarose.

Although dextran and dextran sulfate are widely used for intravenous injections, dextran has reportedly sensitized some recipients after intravenous injection.18–21 When we proceeded to clinical trials of LA01, this possibility must be carefully examined and excluded. Since a covalent bond between the ligand and the cellulose bead is very stable,22 the ligand is not likely to be transferred from the bead into the plasma. Therefore, as long as the sorbent is kept apart from the blood cells, sensitization could be avoided. High molecular weight dextran sulfate reportedly enhanced the precipitation of fibrinogen and the aggregation of platelets, resulting in toxicity. However, this did not occur with the low molecular weight dextran sulfate used in this study.23,24 Adsorption of minor components of plasma has not yet been excluded. During additional trials, this must also be intensively examined.

References


Index Terms: hyperlipoproteinemia • plasmapheresis
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