Removal of Cholesterol from Blood by Affinity Binding to Heparin-Agarose: Evaluation on Treatment in Homozygous Familial Hypercholesterolemia

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Summary

Extracorporeal removal of cholesterol from blood with heparin-agarose conjugates was studied as a means of reducing the plasma cholesterol concentration in two patients with homozygous familial hypercholesterolemia. Both patients were treated in an outpatient clinic. Patient 1 underwent two separate courses of treatment; during the first course of eight treatments, the plasma cholesterol concentration decreased by a maximum of 54% (from 811-370 mg/dl). The six treatments in the second course resulted in a 35% reduction of plasma cholesterol (from 939-632 mg/dl). In patient two, 12 consecutive treatments resulted in a 56% decrease in the concentration of plasma cholesterol (from 768-298 mg/dl). Repetitive treatments in both patients resulted in a new "steady state" with circulating cholesterol levels lower than the pretreatment baseline value. The decrease in plasma cholesterol is mainly due to the removal of low density lipoproteins. The circulating level of high density lipoproteins is unaffected by the treatment. The treatment has no effect on the chemical composition of both low and high density lipoproteins. The extracorporeal treatment of blood with heparin-agarose was well tolerated by both patients and there were no undesirable effects. The effectiveness and simplicity of the present approach makes it attractive as a possible mode of therapy for homozygous familial hypercholesterolemia.

Speculation

Reduction of plasma cholesterol by the removal of low density lipoproteins in an extracorporeal system is described as a possible mode of therapy for homozygous familial hypercholesterolemia. The effective control of plasma low density lipoprotein level in these patients on long term basis, may permit to evaluate its effect on tissue deposits of cholesterol and atherosclerotic disease.

Homozygous familial hypercholesterolemia (HFH) is characterized by a 4- to 6-fold increase in plasma total and low density lipoprotein (LDL) cholesterol (9). The genetic implications that lead to HFH have been reviewed by Goldstein and Brown (10); several sites and mechanisms appear to be involved. The deficiency of cell surface receptors for LDL as well as their defectiveness in binding LDL have been reported in cultured fibroblasts from patients with HFH (11). This defect in the binding of LDL is associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), a rate-limiting enzyme in the biosynthesis of cholesterol and decreased catabolism of LDL (10). Despite usual therapeutic measures, tendon and tuberos xanthomas develop in these patients in childhood and death from severe coronary atherosclerotic disease usually occurs before age 30 (9, 13). The authors have recently described a new approach

in the management of familial hypercholesterolemia, based on the extracorporeal removal of lipoproteins by their affinity binding to heparin-agarose (17). In this communication, the repeated use of this treatment procedure in lowering plasma total and LDL cholesterol levels in two patients with HFH is reported. The preliminary findings reported here suggest that this form of therapy may find usefulness in the management of HFH, due to its lack of toxicity and significant lowering of both plasma and LDL cholesterol.

MATERIALS AND METHODS

PATIENTS

Two patients were studied after a written informed consent was obtained from the parents of both the patients. The presumptive diagnosis of HFH in these patients was based on the following criteria (9): (a) LDL cholesterol concentration was more than twice that of heterozygotes in the same kindred; (b) elevated LDL cholesterol in both parents or in the family line of an unavailable parent; (c) plasma LDL cholesterol concentration about 500 mg/dl; and (d) juvenile xanthomatosis.

Patient 1 was 3-yr-old at the time of treatment with heparin-agarose and was first referred to the clinic when he was 6 months of age. Total plasma, very low density lipoprotein (VLDL), LDL, and high density lipoprotein (HDL) cholesterol levels were 960, 15, 935, and 10 mg/dl, respectively. Agarose-gel electrophoresis showed a typical type IIa lipoprotein pattern. Planar xanthomas were noted on both Achilles tendons; other clinical and laboratory examinations were unremarkable. Screening of family members revealed parental consanguinity and increased LDL cholesterol in both parents (345 and 188 mg/dl in father and mother aged 28 and 25 yr, respectively); the only brother, aged 5 years, had LDL cholesterol level of 221 mg/dl. At follow-up visits, the patient showed progressive development of xanthomas despite diet and drug therapy with cholestyramine (8 g/day). At 3 yr of age, multiple tuberos xanthomas were observed on both knees, elbows, buttocks, dorsum of the hands and finger folds. Tendon xanthomas were present on the Achilles tendons and planar xanthomas were seen bilaterally in the popliteal fossae. Tubero-eruptive xanthomas were noted on the gluteal folds. A systolic murmur (2/6) of vibratory quality was heard in the left parasternal area. Cholestyramine was discontinued 8 wk before treatment with heparin-agarose.

Patient 2 was 18-yr-old at the time of study. He was first examined in our clinic at the age of 15 and was known to have xanthomas since early childhood. The total plasma, VLDL, LDL, and HDL cholesterol levels were 647, 19, 599, and 29 mg/dl, respectively. On examination, gigantic tuberos xanthomas were seen on the knees, Achilles tendons, elbows, and dorsum of the

hands. Tendon xanthomas were visible on the Achilles tendons and the extensors of all the fingers of both hands. Tubero-eruptive xanthomas were present on the buttocks. The patient's mother (41-yr-old) had total plasma, VLDL, LDL, and HDL cholesterol levels of 282, 9, 248, and 26, respectively. No data were available on the patient's father who died from heart disease at 46 yr of age. The patient's three sisters aged 15, 19, and 22 yr have total plasma cholesterol concentration of 156, 294, and 267 mg/dl, respectively. Cardiovascular evaluation revealed the presence of a systolic murmur (2/6) in the aortic area of the heart. Bruit were heard (3/6) on both carotid arteries. The treadmill exercise test was normal. The patient was treated with nicotinic acid (0.9–1.5 g/day) and cholestyramine (24 g/day) along with a diet low in cholesterol and saturated fatty acids for 3 yr before undergoing extracorporeal treatment with heparin-agarose during which both diet and drug therapy was continued.

EXTRACORPOREAL TREATMENT OF BLOOD WITH HEPARIN-AGAROSE

Batch chromatography was used for the extracorporeal delipidation of blood with heparin-agarose as described elsewhere (2, 17, 19). The preparation of heparin-agarose and its specificity for binding LDL and VLDL has been previously described (19). All treatments were carried out in the outpatient clinic. Each treatment lasted on an average from 2–3 hr after which the patient was released. In patient 1, cholestyramine was discontinued 2 months before treatment where as patient 2 was taking nicotinic acid and cholestyramine throughout treatment. Blood and plasma volume was measured in both patients by radioiodine specific activity (1250 and 800 ml in patient 1, 4211 and 2665 ml in patient 2). A catheter (Medicut, Sherwood Medical Industries, St. Louis, MO) was introduced in the antecubital vein and its tip connected to a four-way stopcock (Travenol Laboratories, Deerfield, IL). A transfer pack (Travenol Laboratories, Deerfield, IL) containing heparin (Harris Laboratories, Brantford, Ontario), heparin-agarose beads and calcium chloride was connected to the stopcock and 100 or 300 ml of blood was drawn at a time, by gravity, in patients 1 and 2, respectively. The contents of the blood pack were gently mixed and the blood was then returned to the circulation through a filter (Johnson and Johnson, New Brunswick, NJ) as in any standard transfusion. The lipoprotein-heparin-agarose complexes formed in the presence of calcium ions are retained by the transfusion filter. Approximately 13–25 g of heparin-agarose beads were reacted with every 100 ml of blood withdrawn in the presence of calcium at a concentration of 0.02 M. In patient 1, 600 units of heparin were used as anticoagulant and 1500 units in patient 2. The heparin was added only in the first three transfusion packs; 60, 30, and 10% of the total dose in first, second, and third transfusion packs, respectively.

BLOOD SAMPLES AND LABORATORY PROCEDURES

Blood samples were withdrawn under appropriate conditions for various hematologic, lipid, and other clinical chemistry tests before and 10 min after the end of treatment. All blood analysis, except for the lipids, were performed by conventional methods as described previously (17).

In order to study the composition of various lipoprotein fractions, VLDL, LDL, and HDL were prepared by sequential ultracentrifugation in a L2-65B ultracentrifuge with 40.3 rotor (Beckman Instruments, Palo Alto, CA) according to the method described by Havel *et al.* (12). If only cholesterol was determined in the various lipoproteins, then the plasma fraction with a density of more than 1.006 g/ml was analyzed, before and after precipitation with heparin and manganese (5) at a final concentration of 412 USP units/ml and 95 mM, respectively. LDL cholesterol was obtained by difference. Total cholesterol and triglycerides were measured after extraction with isopropanol using Technicon Auto-Analyzer II (22). Total and unesterified cholesterol were also measured enzymatically (21) using manual reagent kit (CHOD/PAP, Boehringer Mannheim) and esterified cholesterol was cal-

culated from the difference. Phospholipid was determined by the method of Zilversmit and Davis (26) and protein by the dye-binding method of Bradford (4) using crystalline Bovine serum albumin as a standard. Agarose gel electrophoresis of plasma lipoproteins was carried out by a modification of the procedure of Noble (20) as described previously (19). Plasma levels of apoprotein-B were determined by the rocket immunoelectrophoretic method of Laurell (14) using commercially prepared anti- β -lipoprotein serum (Behring diagnostics, Montreal, Québec). The immunoelectrophoresis was run at 2V/cm for 18 hr.

RESULTS

Patient 1 underwent two separate courses of extracorporeal treatments with heparin-agarose during which no lipid-lowering drugs were taken. Figure 1 summarizes the effects of repetitive treatments, on plasma cholesterol levels during the first course of eight treatments which lasted 20 days. Initially, 700 ml of blood were delipidated with heparin-agarose (100 ml at a time) which resulted in the removal of 1.9 g of cholesterol and the plasma concentration of cholesterol was reduced by 30%. With cessation of treatment, plasma cholesterol levels increased gradually and attained the baseline level after 7 days. A similar effect was observed during the next treatment except that the return to pretreatment levels occurred after 3 days. The subsequent five treatments were given at 1- to 2-day intervals and 300–500 ml of blood were delipidated at each treatment. These successive treatments resulted in a decrease in plasma cholesterol concentration from 768–370 mg/dl. The maximum decrease in the plasma cholesterol concentration during the first course of eight treatments was 54% (from 811–370 mg/dl); a total of 3150 ml of blood was treated which resulted in a cumulative removal of 9.04 g of cholesterol.

Several months later, patient 1 underwent a second course of six treatments with heparin-agarose but this time at 2-wk intervals for 10 wk (Fig. 2). It will be noted that during the first three treatments, plasma cholesterol levels between treatments always returned back to pretreatment baseline values. However, during the last three treatments, plasma cholesterol levels did not regain their initial values but appeared to have reached a new threshold of steady-state which was approximately 200 mg/dl lower than observed in the earlier phases of treatment. A total of 3800 ml of blood was treated which resulted in a cumulative removal of 6.4 g of cholesterol. Maximum decrease in plasma cholesterol was 35%. No lipid lowering drug was given throughout the treatment period.

Patient 2 was treated with diet and drug therapy (cholestyramine

Fig. 1. Changes in plasma cholesterol concentration during the first course of extracorporeal removal of cholesterol from blood with heparin-agarose in patient 1. Numbers in parenthesis at the top of the figure, cumulative blood volume treated in ml at each treatment. Solid lines with an arrow, plasma cholesterol values before and after the treatment. ---, show the increase in cholesterol between treatment periods.

Fig. 2. Changes in plasma cholesterol concentration during the second course of extracorporeal removal of cholesterol from blood with heparin-agarose in patient 1. For other details, see legend to Figure 1.

Fig. 3. Changes in plasma cholesterol concentration during extra-corporeal removal of cholesterol from blood with heparin-agarose in patient 2. Numbers at top, treatments; 1800 ml of blood was delipidated at each treatment except treatments 1, 4, and 6 during which 3000 and 1200 ml of blood were treated, respectively. For other details, see legend to Figure 1.

and nicotinic acid) for 3 yr before he underwent extracorporeal treatment of blood with heparin-agarose; both drugs were continued during the study and the patient received 12 extracorporeal treatments. The results on plasma cholesterol levels are shown in Figure 3. Initially, 3000 ml of blood were delipidated with heparin-agarose (300 ml at a time) which resulted in the removal of 3.6 g of cholesterol and the plasma concentration of cholesterol was reduced by 21%. One wk after treatment, plasma cholesterol concentrations had decreased instead of rising to baseline values as had been noticed in patient 1. During subsequent treatments, the plasma cholesterol concentrations decreased further and the levels between treatments never reached pretreatment baseline values but rather remained approximately 250 mg lower than the initial value. A total of 23,400 ml of blood was delipidated in patient 2 during 12 treatments which resulted in the removal of 23.6 g of cholesterol; maximum decrease in plasma cholesterol was 56% (from 678–298 mg%).

The effect of various treatments on the plasma concentration of cholesterol in both patients is summarized in Table 1. During the

first and second course of treatment with heparin-agarose in patient 1, a mean decrease of 28 and 11% is noticed as compared to average cholesterol concentration during 2-yr treatment with diet and cholestyramine. Similarly, in patient 2, the mean cholesterol level during combined diet, drug and heparin-agarose treatment is 25% lower than the average cholesterol concentration observed during diet and drug therapy for a period of 3 yr.

A mean decrease of 18%, per treatment, in the concentration of plasma cholesterol is similar to that of phospholipid as shown in Table 2. The lowering of plasma cholesterol level is mostly due to the decrease of LDL-cholesterol, because the concentration of HDL-cholesterol is virtually unaffected by the treatment. Although VLDL-cholesterol is greatly reduced during the treatment, its contribution to the overall reduction of plasma cholesterol is not appreciable due to low cholesterol content of this fraction. In fact, the decrease in VLDL-cholesterol as well as plasma triglycerides (Table 2) is more likely due to hydrolysis of triglycerides and conversion of VLDL into intermediary lipoprotein particles, by lipoprotein lipase which is released in the circulation by heparin

Table 1. Plasma cholesterol concentration during various treatments in patients 1 and 2¹

Treatment	N	Cholesterol (mg/dl) ¹	P
Patient 1			
Rx	13	853 ± 93 (744–1,088)	
Heparin-agarose 1st treatment	15	612 ± 120 (310–788)	< 0.001
2nd treatment	11	757 ± 104 (632–929)	< 0.05
Patient 2			
Rx	16	525 ± 68 (450–678)	
Rx+heparin-agarose	25	393 ± 69 (298–537)	< 0.001

¹ Values are mean ± 1 SD for 2-yr period during Rx and for 3- and 10-wk period during 1st and 2nd course of treatment with heparin-agarose in patient 1. In patient 2, the values are for 3-yr treatment period during Rx and for 12 wk during Rx + heparin-agarose. Values in parenthesis indicate range. N is number of observations. The statistical significance of differences between cholesterol values was analyzed by Student's *t* test. For Rx, see *Materials and Methods*.

Table 2. Plasma lipid and lipoprotein levels before and after treatment with heparin-agarose in patient 2

	Initial ¹ (mg/dl)	Final ² (mg/dl)	P
Cholesterol	496 ± 73 (18)	409 ± 57 (18)	< 0.02
Triglycerides	111 ± 38 (65)	39 ± 17 (65)	< 0.001
Phospholipids	344 ± 57 (18)	281 ± 52 (18)	< 0.05
VLDL-cholesterol	13 ± 7 (85)	2 ± 1 (85)	< 0.001
LDL-cholesterol	452 ± 65 (16)	379 ± 55 (16)	< 0.05
HDL-cholesterol	30 ± 3 (3)	29 ± 3 (3)	N.S.
Apoprotein B	206 ± 22 (20)	164 ± 17 (20)	< 0.005

¹ Values are mean ± 1 SD for eight treatments; 1800 ml of blood were delipidated at each treatment.

² Values in parentheses are percent decrease from the initial value. The statistical significance of differences between initial and final values was analyzed by Student's *t* test.

(9) used as an anticoagulant. However, some VLDL and intermediary lipoprotein particles may also be removed by heparin-agarose, because it has been shown previously that VLDL also bind to heparin-agarose although it has lower affinity as compared to LDL (19). As shown in Table 2, a decrease of 20% in the plasma level of apoprotein B is higher than the 16% decrease in the concentration of LDL-cholesterol. These results further indicate that some VLDL is also being removed, which may explain a higher decrease in the plasma concentration of apoprotein B as compared to LDL-cholesterol.

The extracorporeal treatment of blood with heparin-agarose has no effect on the chemical composition of both LDL and HDL as shown in Table 3. Although the composition of VLDL is greatly modified (Table 3), it is unlikely that it may be caused by heparin-agarose. The changes in the composition rather reflect the hydrolysis of VLDL by lipoprotein lipase activity as discussed earlier. The effect of extracorporeal treatment of blood with heparin-agarose on serum calcium level and various hematologic determinations is presented in Table 4. It will be noted that in spite of the addition of exogenous calcium for the binding of LDL to heparin-agarose, the circulating concentration of calcium ions is not increased. This is due to the retention of calcium ions by heparin-agarose as shown previously in the *in vitro* studies (19). The heparin-agarose treatment resulted in a marginal decrease in erythrocyte counts, hemoglobin, hematocrit, and platelet counts. This effect is attributed to some loss of blood during repeated transfusions and also due to blood samples taken for various laboratory tests. The leucocyte count showed a small but significant increase during the treatment, which was transitory and always remained in the normal range. The increase in leukocyte count is accompanied by alterations in the distribution of neutrophils and lymphocytes, but the differences observed are small and not significant. These transient changes noticed during extracorporeal treatment of blood with heparin-agarose may be related to repeated withdrawal and reinfusion of blood. No significant alterations were seen in several routine clinical chemistry and immunologic tests as also described previously (2, 17, 19). The increase in prothrombin time is, however, due to partial heparinization of the patient, as judged from mixing studies which did not show coagulation factor deficiency.

DISCUSSION

HFH is characterized by very high levels of plasma and LDL cholesterol and coronary atherosclerotic disease progresses very rapidly in these patients, whose average life expectancy is less than 20 yr (13). Dietary manipulations associated with combination drug therapy are usually without adequate response in the decrease of plasma cholesterol levels except in a few instances (7, 15, 16). The data presented here clearly demonstrate that the plasma cholesterol concentration can be lowered in HFH patients by the extracorporeal removal of cholesterol through treatment with heparin-agarose. The results shown in Table 1 compare the effects of treatment with heparin-agarose to that of conventional

diet and drug therapy. It will be noticed that an additional mean decrease of 11–28% is obtained over diet and drug therapy, during very short periods of treatment with heparin-agarose. The average removal of cholesterol from circulation per 100 ml of blood treated, during the first and second course of treatment in patient 1, was 287 and 176 mg as compared to 100 mg in patient 2. In two other patients (heterozygotes, type IIa) reported previously (17), an average of 176 and 150 mg of cholesterol was removed per 100 ml of blood treated. These differences are probably due to varying proportion of heparin-agarose conjugates used in the treatment of blood in each case, as well as due to variation in the binding capacity of various heparin-agarose batches used in the treatment.

The data in patient 1 indicate that during the initial phase of treatment, there is a transient but substantial decrease in the concentration of plasma cholesterol which rapidly increases to baseline value upon cessation of treatment. However, with sustained treatments, the plasma cholesterol concentration decreased further and attained a new steady state, at a lower circulating level of cholesterol. Similar effects were noted in patient 2 except that the posttreatment cholesterol levels never reached the pretreatment value throughout the study. This difference may be attributed to the additional beneficiary effect of hypolipidemic drugs in this patient as also previously reported by Thompson *et al.* (24, 25).

The return of cholesterol to baseline value after cessation of extracorporeal removal of blood cholesterol, may result from increased synthesis of cholesterol or may be due to mobilization of tissue cholesterol. In a similar approach but using the plasma exchange technique, Thompson *et al.* (19, 20) have shown that reduction of plasma cholesterol by replacement of the patients plasma with cholesterol-free purified protein fraction resulted in an influx of tissue cholesterol into the plasma compartment. Plasmapheresis has been shown to lower plasma cholesterol concentrations in rats (21), with a concomitant increase in the plasma cholesterol during the postplasmapheresis period due to an increase in the rate of cholesterol synthesis. However, it was suggested that the increase in plasma concentration of cholesterol was due to an increased rate of protein and lipoprotein synthesis in response to protein depletion caused by plasmapheresis. Such a mechanism is unlikely to operate in the present approach, because plasma protein concentration is virtually unaffected by the extracorporeal removal of cholesterol with heparin-agarose.

The use of plasmapheresis and plasma exchange (1, 3, 8, 25) in the management of HFH patients has been suggested. This approach is different from the conventional plasmapheresis in that it is specific for the removal of LDL; although VLDL may be removed, there is no effect on the circulating levels of HDL. This finding is of particular interest, because it has been suggested that HDL may be involved in the regulation of tissue-cholesterol (18) and reduction in its concentration may accelerate the process of atherosclerosis. The extracorporeal treatment of blood with heparin-agarose was without side effects in both patients, as judged from routine physical examination as well as hematologic and biochemical tests. It has certain advantages over plasmapheresis

Table 3. Chemical composition of plasma lipoproteins before and after delipidation of blood with heparin-agarose in patient 2¹

	Protein	Phospholipid	Triglyceride	Cholesterol esters	Cholesterol (unesterified)
Before treatment					
VLDL	10	26	41	18	6
LDL	17	22	5	46	9
HDL	51	21	5	22	2
After treatment					
VLDL	10	48	33	7	2
LDL	17	23	4	45	10
HDL	48	23	5	23	2

¹ Values are expressed as percent of the sum of all constituents analyzed. Values are mean of two determinations carried out in two separate treatments during which 1800 ml of blood was delipidated. The mean decrease in the concentration was 17, 52, 15, 75, 15, and 3% for cholesterol, triglyceride, phospholipid, VLDL, LDL, and HDL cholesterol, respectively.

Table 4. Serum calcium levels and hematologic indices before and after extracorporeal treatment of blood with heparin-agarose in patient 2¹

	N ²	Initial ³	Final ³	P
Calcium (mg%)	12	9.9 ± 0.4	9.6 ± 0.6	N.S.
Erythrocyte count (× 10 ⁶)	12	4.4 ± 0.3	4.2 ± 0.3	N.S.
Hemoglobin (g%)	12	11.9 ± 0.6	11.5 ± 0.6	N.S.
Hematocrit (%)	12	34.9 ± 1.8	33.7 ± 1.8	N.S.
Platelets (× 10 ³)	6	372 ± 35	360 ± 37	N.S.
Leucocyte count (× 10 ³)	12	6.3 ± 1.0	8.2 ± 2.1	< 0.02
Neutrophils (%)	12	55.6 ± 3.6	53.4 ± 9.5	N.S.
Lymphocytes (%)	12	33.4 ± 2.5	37.2 ± 8.2	N.S.
Prothrombin time (sec)	6	10.6 ± 0.6	14.4 ± 0.9	< 0.01

¹ The following analyses were unaffected by the treatment: glucose, blood urea-nitrogen, total proteins, globulins, phosphorous, magnesium, uric acid, creatinine, sodium, potassium, chloride, alkaline phosphatase, lactic dehydrogenase, glutamic-oxaloacetic, and glutamic-pyruvic transaminases.

² N is number of observations, the statistical significance of differences between initial and final values was analyzed by Student's *t* test.

³ Values are mean ± 1 SD.

and plasma exchange, because it is selective in the removal of plasma LDL and VLDL and, furthermore, no exogenous plasma products are introduced that may lead to various complications (6). It is suggested that the present treatment procedure can be used more frequently and for longer periods than plasmapheresis and may serve as a useful mode of therapy for HFH patients.

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