Delayed loss of cholesterol from a localized lipoprotein depot in apolipoprotein A-I-deficient mice
(reverse cholesterol transport/atherosclerosis/atheroma/cationized low density lipoprotein/cholesteryl ester)

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ABSTRACT The anti-atherogenic role of high density lipoprotein is well known even though the mechanism has not been established. In this study, we have used a novel model system to test whether removal of lipoprotein cholesterol from a localized depot will be affected by apolipoprotein A-I (apo A-I) deficiency. We compared the egress of cholesterol injected in the form of cationized low density lipoprotein into the rectus femoris muscle of apo A-I K-O and control mice. When the injected lipoprotein had been labeled with [3H]cholesterol, the t1/2 of labeled cholesterol loss from the muscle was about 4 days in controls and more than 7 days in apo A-I K-O mice. The loss of cholesterol mass had an initial slow (about 4 days) and a later more rapid component; after day 4, the disappearance curves for apo A-I K-O and controls began to diverge, and by day 7, the loss of injected cholesterol was significantly slower in apo A-I K-O than in controls. The injected lipoprotein cholesterol is about 70% in esterified form and undergoes hydrolysis, which by day 4 was similar in control and apo A-I K-O mice. The efflux potential of serum from control and apo A-I K-O mice was studied using media containing 2% native or delipidated serum. A significantly lower efflux of [3H]cholesterol from macrophages was found with native and delipidated serum from apo A-I K-O mice. In conclusion, these findings show that lack of apo A-I results in a delay in cholesterol loss from a localized depot in vivo and from macrophages in culture. These results provide support for the thesis that anti-atherogenicity of high density lipoprotein is related in part to its role in cholesterol removal.

The protective role of high density lipoprotein (HDL) in the pathogenesis of coronary heart disease is well known (1), even though the exact mechanism has thus far not been ascertained. HDL and its apolipoproteins, complexed to phospholipid, promote cholesterol efflux from a variety of cells in culture (2–5), and such a mechanism could explain its anti-atherogenic properties. The missing link is the lack of quantitative evidence for enhancement of cholesterol removal from atheromatous vessels by HDL in vivo. Recently, we have developed a novel approach that permits quantitation of cholesterol efflux from a localized depot of lipoprotein in vivo (6). Briefly, this method consists of injection of cationized low density lipoprotein (LDL) into the rectus femoris muscle of small rodents and quantitative determination of cholesterol removal as a function of time. This model system is suitable to study the role of HDL in the efflux of deposited cholesterol.

Recently, genetically modified mice were produced that lack apolipoprotein A-I (apo A-I) (7, 8). In such animals, there is both a reduction in total cholesterol and in HDL cholesterol (7–9), but there was no increased susceptibility to atherosclerosis (9, 10), probably because of low plasma cholesterol levels. In a recent study, several additional parameters were determined that focused on the role of HDL in cholesterol transport (9). With the help of nonmetabolizable tracers such as cholesteryl ether, it was shown that there is a marked reduction in the delivery of cholesterol to peripheral (9) as well as to steroidogenic tissues (11). Because the cholesterol content of peripheral tissues and cholesterol synthesis were unchanged, it was assumed that, in view of lower cholesterol delivery, cholesterol efflux should also be low (9). The aim of this study was to determine whether in vivo cholesterol removal from a well-defined depot of lipoprotein cholesterol is affected by the lack of apo A-I.

MATERIALS AND METHODS

Animals. The animals used were apo A-I-deficient (A-I K-O) mice, created by gene targeting in embryonic stem cells as described by Plump et al. (9). CS7BL/6 mice, obtained from The Jackson Laboratory, were used as controls. All animals were housed in constant temperature rooms with a 12-hr light/12-hr dark cycle; they were weaned at 3–4 weeks of age and fed a pelleted chow diet (No. 19510, Koffolk, Petah Tikva, Israel). The experiments were performed on mice more than 2 months of age.

Lipoproteins. LDL were isolated from human plasma according to Havel et al. (12). Cationization of LDL was carried out according to Basu et al. (13). The cationized LDL was dialyzed extensively for 48 hr with frequent changes of dialysis buffer. Cationized LDL was labeled with [3H]cholesterol (14) and labeling of LDL with [3H]cholesterol oleate was carried out as described (15) and was followed by cationization. All preparations used for injection were concentrated to 20 mg cholesterol/ml, by placing in a dialysis bag and covering with Sephadex, and were sterile filtered.

Experimental Procedures. The animals were anesthetized with peroxide-free ether, the inguinal area was shaved, and the animal was strapped in a supine position, with adhesive tape holding the extremities stretched parallel to the long axis of the body. The bulging anterior muscles of the thigh were palpated and a longitudinal incision of 3–4 mm was made in the skin, and 10 μl of lipoproteins were injected with a 1/2-inch 27-gauge needle using a 25-μl Hamilton syringe. The needle was introduced near the distal end of the rectus femoris muscle parallel to its long axis, up to one-half the length of the needle, and the contents injected. The needle was kept in place for 60 sec and slowly withdrawn; if a droplet of liquid appeared, it was blotted and the radioactivity was counted. The incision was closed using three discontinuous sutures (5–0 Surgilene Cu-

Abbreviations: FC, free cholesterol; CE, cholesteryl ester; HDL, high density lipoprotein; LDL, low density lipoprotein; apo A-I, apolipoprotein A-I; SMC, smooth muscle cells.
The lipoproteins were injected into the rectus femoris of the right leg, whereas the left muscle was removed at death for estimation of recirculated radioactivity and endogenous cholesterol content. At different time intervals after injection, ranging from minutes to 3 weeks, the animals were anesthetized with ether and blood was drawn from the aorta. The rectus femoris muscle on the injected and contralateral side was removed, weighed immediately, and the tissue was minced and homogenized in a dual glass conical homogenizer (Kontes, No. 21) in 2 x 1 ml of methanol followed with 2 x 1 ml of chloroform. In some experiments, additional animals were used for morphological studies as described previously (6).

Analytical Procedures. For cholesterol determination, stigmasterol was added as internal standard and the homogenate was left overnight at room temperature to allow lipid extraction. After centrifugation at 3,000 rpm for 10 min, the lipid extract was brought to chloroform/methanol (2:1 vol/vol) and purified according to Folch et al. (16). Aliquots of chloroform extracts of muscle were taken for determination of radioactivity, analysis of lipids by thin-layer chromatography, and determination of total and free cholesterol (FC) by HPLC (17). Separation of \[^{3}H\]cholesterol from \[^{3}H\]cholesterol ester (CE) by thin-layer chromatography was performed using chloroform/ethyl acetate (95:5 vol/vol). For determination of cholesterol by HPLC, the chloroform was evaporated, the dry extracts were dissolved in 2-propanol, and the reagent (Boehringer Mannheim) for determination of either free or total cholesterol was added. After incubation at 37°C for 30 min, methanol was added and the product extracted with petroleum ether, which was evaporated, and the residue dissolved in 2-propanol was analyzed by HPLC [Kontron 400 equipped with a spectrophotometric detector 430 (Zurich)], using the Altech (Deerfield, IL) reversed-phase Econosphere C18 5-μm column. The product was eluted with acetonitrile:2-propanol (82:18), at a flow rate of 1 ml/min, and the cholesterol and stigmasterol oxidation products were detected at 242 nm.

Estimation of endogenous and exogenous cholesterol in muscle was as follows: Total and free cholesterol was determined on the noninjected contralateral muscle of each animal and expressed per 100 mg wet weight. The mean of all determinations (25–30 samples in each experiment) was designated endogenous cholesterol. The value of exogenous total and free cholesterol in the injected muscle was obtained after subtraction of the endogenous cholesterol. Values of exogenous CE were obtained by subtraction of free from total cholesterol.

Radioactivity was also determined on chloroform extracts of liver. Serum was separated by centrifugation and total and HDL cholesterol were determined by an enzymatic procedure using a Boehringer Mannheim kit. HDL cholesterol was measured on the supernatant, after dextran sulfate precipitation of whole plasma or serum, using a TLA-100 Beckman ultracentrifuge rotor.

Cell Cultures. Peritoneal macrophages were obtained from C57BL mice 4 days after intraperitoneal injection of thioglycollate (18). Macrophages (3.5 x 10⁶) were seeded in 12-well multi-well clusters in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). The nonadherent cells were removed after 2 hr and the adherent macrophages were cultured for 24 hr in MEM containing 10% FBS. Bovine smooth muscle cells (SMC) were grown from explants of aorta and subcultured in Dulbecco–Vogt medium supplemented with 10% FBS (19). For experiments of 10³ cells, were seeded in 12-well multi-wells (Costar) and grown for 6–10 days. To label cells with \[^{3}H\]cholesterol, the latter was added to serum containing medium (1 μCi/ml; 1 Ci = 37 GBq) (14). The labeled medium was added to macrophages after removal of nonadherent cells; SMC were grown in the labeled medium for 8 days from the time of seeding.

Efflux of \[^{3}H\]Cholesterol from Cultured Cells. To study \[^{3}H\]cholesterol efflux from labeled cells, the medium was removed, and the cells were washed with PBS and incubated with 3 ml MEM containing 1% BSA and with 3 ml serum-free medium at 37°C for 15 min each. Thereafter, 0.5 ml of acceptor medium was added and incubation was carried out for 4 or 24 hr. The acceptor medium consisted of serum-free MEM and serum of apo A-I-deficient (apo A-I K-O) or control mice. Serum was used as such or delipidated with ethanol and ether (20), and the dried powder redissolved in the culture medium. In some experiments, liposomes of dioleoyl phosphatidylcholine, prepared as described previously (5), were used.

Materials. Culture medium and FBS were obtained from Gibco. \[^{7}al(n)-^{3}H\]Cholesterol and \[^{3}H\]cholesteryl oleate were obtained from Amersham. All reagents were of analytical grade and were obtained from Sigma.

Statistical Evaluation. The results were presented as means ± SE. The difference between groups was evaluated with the Student’s t test.

RESULTS

Studies in Vivo. Plasma levels of total cholesterol were determined at the end of each experiment and as seen in Table 1; both total and HDL cholesterol were markedly reduced in the apo A-I K-O mice. In the first three experiments, we compared the disappearance of label from the muscle injected with \[^{3}H\]FC-cationized LDL. As seen in Fig. 1, the loss of the labeled cholesterol was monoeponential; however, whereas the t½ in the control was about 4 days, it was more than 7 days in the apo A-I K-O mice. The greater retention of \[^{3}H\]cholesterol at the site of injection in apo A-I K-O mice was reflected by a lower recovery of \[^{3}H\]label in the liver (as compared with controls), 1 and 7 days after injection (Table 2). Evidence for the metabolism of the injected lipoprotein was derived from the finding of \[^{3}H\]CE in the muscle, which after 7 and 14 days amounted to 27 and 50% of \[^{3}H\]label recovered in muscle in both apo A-I K-O and control mice (data not shown).

In subsequent experiments, the cationized LDL had been labeled with trace amounts of \[^{3}H\]cholesteryl oleate, and the loss of label from the site of injection was compared. In contrast to the findings with \[^{3}H\]FC, more than 90% of the injected label was still recovered in the muscle after 2 days, and 79% and 62% was recovered after 4 days in apo A-I K-O and control mice, respectively (Fig. 2). At the later time intervals of 7 and 14 days, the slower rate of loss of \[^{3}H\]cholesterol from the site of injection in apo A-I K-O mice persisted. The percent of label recovered in the muscle in the form of \[^{3}H\]CE decreased to 73% and 48% in A-I K-O animals and 73% and 50% in controls, on days 4 and 7, respectively, indicating that hydrolysis of \[^{3}H\]CE was comparable in both groups.

In view of the large difference in plasma cholesterol levels between the apo A-I K-O and control mice (Table 1), which could have affected cholesterol exchange, it was necessary to validate these results by determination of the egress of exogenous (inject- ed) cholesterol mass as a function of time. This necessitated measurement of endogenous cholesterol mass in the noninjected, contralateral muscle, and no difference was found between apo

| Table 1. Plasma levels of total and HDL cholesterol in apo A-I K-O and control mice |
|------------------|------------------|------------------|
|                  | cholesterol, mg/dl | A-I K-O          | Control         |
| Plasma           | 26 ± 2 (44)       | 96 ± 7 (46)      |
| HDL              | 15 ± 1 (44)       | 71 ± 5 (46)      |

In eight apo A-I K-O and eight control mice the phospholipid content of plasma was examined as well. In the A-I K-O animals it was reduced commensurate with total cholesterol. Cholesterol and phospholipids (mg/dl); 12 ± 1 and 46 ± 2 in apo A-I K-O and 96 ± 4 and 236 ± 8 in controls, respectively. Values are means ± SE. Number in parentheses is the number of animals. A-I K-O, apo A-I-deficient mice.
A-I K-O and control mice (86 ± 4 and 89 ± 8 μg cholesterol per 100 mg wet weight, respectively). Data from five experiments in which cationized LDL was injected into the leg muscle are shown in Fig. 3. In contrast to the monoexponential loss of [3H]FC (Fig. 1), there was an initial slow and a later more rapid component in the disappearance curves of exogenous total cholesterol mass (Fig. 3). The disappearance curves of cholesterol mass of apo A-I K-O and controls started to diverge after the first 4 days and the difference became statistically significant after 7 days. Because CE has to be hydrolyzed prior to removal from the tissue, the above results could have been affected by impairment of CE hydrolysis. This was not the case, since on day 4 the amount of injected CE mass hydrolyzed (calculated by subtraction of the amount of exogenous CE mass recovered from that found at 0 time) was about 40% and increased to about 60% on day 7 in A-I K-O and control mice (Fig. 4). Histological examination of sections of muscle injected with cationized LDL revealed no difference between apo A-I K-O and control mice with respect to infiltration with mononuclear cells between days 1, 4, 7, and 14 and resolution of the infiltrate by day 21.

**Studies in Cell Culture.** The aim of these experiments was to compare the cholesterol removal capacity of serum derived from apo A-I K-O with that from control mice. However, the difference in plasma total cholesterol levels between the two groups could have made such a comparison difficult, if the rate of efflux is affected by cholesterol levels in the medium. Therefore, in the next set of experiments we compared the cholesterol removal capacity of serum in its native state or after delipidation. A significantly lower efflux of [3H]cholesterol was found when macrophages were incubated with medium containing 2% native or delipidated serum derived from apo A-I K-O than from control mice (Fig. 5). Similar results were also obtained after exposure of SMC to serum from A-I K-O and control mice (Fig. 6). Another difference between apo A-I K-O and control serum is the phospholipid concentration and, therefore, we also compared the efflux potential of this serum in the presence of phosphatidylcholine liposomes. Results of three experiments are presented in Fig. 7. It can be seen that the addition of only 20 μg of phosphatidylcholine per ml to the delipidated serum of apo A-I K-O or control mice increased the efflux of [3H]cholesterol from

<table>
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<th>Day after injection</th>
<th>[3H] in liver, percent of 0 time</th>
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<tr>
<td></td>
<td>A-I K-O</td>
</tr>
<tr>
<td>1</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>7</td>
<td>2.7 ± 0.4</td>
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<tr>
<td>14</td>
<td>3.0 ± 0.3</td>
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<tr>
<td>21</td>
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Values are means ± SE of eight animals per time point. A-I K-O, apo A-I deficient. The 0 time is as in Fig. 1. *Difference between control and A-I K-O, P < 0.02.
macrophages by 100%. The addition of phospholipid to medium containing 2% native serum from apo A-I K-O mice also increased the efflux by 50%, but when added to control serum the increase was only 13%.

DISCUSSION

In this study we have used a newly developed method that permits quantitative measurement of the removal of cholesterol from a well-defined depot in vivo (6). In analogy to our previous findings, the t1/2 of [3H]cholesterol loss from the rectus femoris muscle injected with [3H]PC-cationized LDL was about 4 days for the control mice but was significantly longer in the apo A-I K-O animals. These findings were also corroborated when the egress of cholesterol mass was compared in control and apo A-I K-O mice. On the other hand, no difference was seen between control and apo A-I K-O mice with respect to the metabolism of the cholesterol moiety of the injected cationized LDL. This was evidenced by the finding of a similar percentage of [3H]CE after injection of cationized LDL labeled with free cholesterol, as well as by determination of CE hydrolysis by thin-layer chromatography and HPLC after injection of cationized LDL labeled with [3H]cholesteryl oleate. Moreover, the cellular reaction to the injected cationized LDL was also similar.

One of the principal differences between apo A-I K-O and control mice is the level of plasma cholesterol. In previous studies (9, 10), a very marked reduction in plasma cholesterol was reported and this was confirmed in this study. Likewise, very low levels of plasma HDL were found. In view of the anti-atherosclerotic role of the overexpression of human apo A-I in transgenic animals (21–23), it was expected that the lack of apo A-I should enhance atherogenesis, but this was not the case even when the mice were kept on high fat diets (9, 10). However, it would be difficult to envisage development of atherosclerosis in the presence of low levels of atherogenic lipoproteins and, therefore, the protective role of apo A-I could not be demonstrated. In this model in which the reverse cholesterol transport system was challenged with a depot of injection of cationized LDL labeled with free cholesterol, as well as by determination of CE hydrolysis by thin-layer chromatography and HPLC after injection of cationized LDL labeled with [3H]cholesteryl oleate. Moreover, the cellular reaction to the injected cationized LDL was also similar.

FIG. 4. Hydrolysis of CE mass in muscle after injection of cationized LDL (200 μg cholesterol) into the rectus femoris muscle of apo A-I K-O and control mice. CE hydrolysis was calculated by subtraction of the amount of CE mass on days 4 or 7 from that found at 0 time. Data were obtained from 4–5 experiments and are the means ± SE of 13–20 animals per time point.

FIG. 5. Efflux of [3H]cholesterol from macrophages in the presence of serum derived from apo A-I K-O or control mice. Mouse peritoneal macrophages labeled with [3H]cholesterol were exposed to medium containing 2% serum. Serum was from control (Con) or apo A-I K-O (K-O) mice and was either native (N) or delipidated (D). Values are the means ± SE of eight dishes from four experiments. *, P < 0.05.

FIG. 6. Efflux of [3H]cholesterol from SMC in the presence of serum derived from apo A-I K-O or control mice. Aortic SMC, labeled with [3H]cholesterol, were exposed to medium containing 2% serum. The serum was from control (Con) or apo A-I K-O (K-O) mice and was either native (N) or delipidated (D). Values are the means ± SE of eight dishes from four experiments. *, P < 0.05.

FIG. 7. Effect of dioleyl phosphatidylcholine liposomes on efflux of [3H]cholesterol from macrophages. Mouse peritoneal macrophages labeled with [3H]cholesterol were exposed to medium containing 2% serum. The serum was from control (Con) or apo A-I K-O (K-O) mice and was either native (N) or delipidated (D). In each experiment, phosphatidylcholine liposomes were added to one-half of the dishes to give 20 μg phospholipid/ml. Data are the means from three experiments performed in triplicate and are presented as the increment in the presence of phosphatidylcholine, taking the efflux without phosphatidylcholine as 100%. *, P < 0.05.
cholesterol derived from exogenously delivered LDL, the absence of apo A-I did contribute to the slower rate of cholesterol removal. More recently, the protective role of apo A-I in atherogenesis was reinforced when the apo A-I K-O mice were crossed with mice overexpressing the human apo B transgene (24, 25). Under this experimental condition, which enables development of aortic atherosclerosis, the lack of apo A-I resulted in a marked enhancement of lesion formation. It seems relevant to also point out that in humans, apo A-I deficiency leads to premature atherosclerosis if present in conjunction with additional cardiovascular risk factors, such as high LDL levels or hypertension (26, 27).

The other interesting finding in the studies with apo A-I K-O mice was that the lack of apo A-I markedly diminished CE stores in the adrenal gland, which culminated in a decreased basal corticosteroid synthesis (11). HDL was known to play an important role in the delivery of cholesterol to adrenal glands of rodents (28, 29), but the direct role of apo A-I in vivo had not been demonstrated. Studies with adrenal cells in culture provided evidence for selective uptake of HDL CE by these cells (30, 31), and the putative role of apo A-I in this process (31). The finding that scavenger receptor BI is responsible for selective uptake of HDL–CE (32) and that the lack of apo A-I up-regulates SR-BI in adrenals (33) shed new and convincing light on the role of apo A-I in cholesterol transport.

The results obtained with cultured cells supplement the findings in vivo and support the conclusion that the lesser capacity of apo A-I K-O serum to promote cholesterol efflux resulted in the slower removal of the lipoprotein cholesterol injected into the leg muscle. A reduced cholesterol removal potential from cultured cells of serum derived from apo A-I K-O mice was first described by Atger et al. (34). Our results extend these findings to delipidated serum devoid of cholesterol and deficient in phospholipids. The cooperative action of apolipoproteins and phospholipids in cholesterol efflux has been well established, and the combined effect was shown to be more than additive (3, 4). In a recent study (35), the role of serum phospholipid in cholesterol removal has been reemphasized. The low phospholipid/apo A-I ratio in HDL from mice that were high overexpressors of human apo A-I was blamed for the lack of correlation between apo A-I and cholesterol efflux from cultured cells (35). It seems plausible that in addition to the lack of apo A-I, the low plasma phospholipid content of apo A-I K-O mice also played a role in the slower clearance of deposited cholesterol in vivo. Indeed, when phospholipid liposomes were added to medium containing 2% serum from apo A-I K-O mice, a significant increase in cholesterol efflux from macrophages was noted; this amount of phospholipid was not effective when added to medium containing serum from control mice.

Even though apo A-I is considered to play a prominent role in cholesterol removal from cells, it shares this capacity with other apoproteins such as apo A-IV and apo E (3). It should also be noted that serum albumin, when added to cells at concentrations found in the extracellular fluid, is also effective in cholesterol efflux (36).

**CONCLUSION**

These findings show that the lack of apo A-I results in a delay in cholesterol loss from a localized depot in vivo and from macrophages in culture. These results provide support for the thesis that anti-atherogenicity of HDL is related in part to its role in cholesterol removal.

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