Use of $^3$H-Cholesteryl Linoleyl Ether as a Quantitative Marker for Loss of Cholesteryl Ester during Regression of Cholesterol-induced Aortic Atheromas in Rabbits

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In this study, use was made of $^3$H-cholesterol linoleyl ether ($^3$H-CLE) to follow regression of aortic atheromatosis induced by feeding cholesterol to rabbits. After a 3-month induction period, the rabbits were divided into two groups with an attempt to match them by plasma cholesterol levels. They were injected with rabbit plasma labeled with $^3$H-CLE, and the baseline group rabbits were killed 10 to 12 days after injection. The experimental (regression) group rabbits were given rabbit chow containing 3% cholestyramine and were killed up to 330 days thereafter. Aortic $^3$H-CLE of both the baseline and the regression groups correlated highly with the plasma cholesterol levels at the time of injection of label. The radioactivity recovered in the aortas of the baseline and regression groups was not significantly different, indicating retention of label between day 12 and 330 days after injection. During that time, the mean aortic cholesteryl ester content decreased from 7.6±1.3 mg to 3.1±0.7 mg (p<0.01). The specific activity of $^3$H-CLE/cholesteryl ester determined in the aortic arch and the thoracic and abdominal aorta was significantly increased in all three regions examined in the regression group as compared to the baseline group. The present data show that $^3$H-CLE is retained in the atheromatous aorta for at least 330 days and that its use may add another dimension to the quantitative evaluation of regression of atherosclerotic lesions.

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During the past two decades, several studies have provided evidence that regression of aortic atherosclerosis induced by prolonged cholesterol feeding may occur in primates and rabbits. Since in the experimental animals the development and extent of atherosclerotic involvement is quite variable, a quantitative evaluation of regression is rather difficult. In our previous study on cholesterol-fed rabbits, we showed a good correlation between the extent of aortic atherosclerotic lesions, as measured by the cholesteryl ester content, and the influx of $^3$H-cholesterol linoleyl ether ($^3$H-CLE), a nondegradable analogue of cholesteryl ester. $^3$H-CLE was shown to be retained by nonparenchymal liver cells for up to 30 days after injection. Therefore, it seemed that $^3$H-CLE could be used as a marker of atherosclerotic involvement at the end of an induction period and would be useful in a study of regression. Since regression of cholesterol-induced atherosclerosis is a slow process and may commence only some months after removal of cholesterol from the diet, it was important to establish whether $^3$H-CLE taken up by the aorta would remain there throughout an experimental period extending up to 330 days.

In the present study, we induced atherosclerosis by feeding cholesterol to rabbits, labeled the aortic cholesteryl ester by injection of $^3$H-CLE, and then fed the animals a regular diet fortified with a hypcholesterolemic agent to enhance regression. The study was designed to provide information on whether $^3$H-CLE would be retained in the aorta for prolonged periods of time during which regression might occur. If retention of the label was found, regression could be better defined in terms of an increase in specific activity of $^3$H-CLE/cholesteryl ester in the aorta. The presence of $^3$H-CLE might permit determination of whether disappearance of cholesteryl ester occurs preferentially in certain regions of the aorta, that is, would areas with widely divergent specific activity of $^3$H-CLE/cholesteryl ester be encountered in the same aorta?

Methods

Rabbits

Male rabbits were obtained from a local breeder, and they weighed about 2 kg each at the beginning of the cholesterol-feeding period. The animals were kept in individual cages in temperature-controlled rooms. One percent cholesterol (wt/wt) was added to the diet by coating the rabbit chow pellets with a solution of cholesterol in diethyl
ether followed by evaporation of the solvent. This diet was fed daily for the first month and then on alternate weeks for the next 2 months. Plasma cholesterol was monitored by blood samples removed from the ear vein; the last determination was 2 days before injection of $^3$H-CLE.

**Preparation of Serum Labeled with $^3$H-Cholesteryl Linoleyl Ether**

Sixty milliliters of serum was obtained from cholesterol-fed rabbits. It was centrifuged for 24 hours at $d=1.006$ g/ml in an SW 41 rotor at 100 000 g. The top 2 ml were cut, and the subsoptic that contained 392 mg/dl cholesterol was used for labeling. $^3$H-CLE (about $10^6$ dpm dissolved in chloroform) was placed in a heavy glass-wall test tube, the solvent was evaporated, and the last traces were removed by lyophilization. An aliquot (2 ml) of the $d>1.006$ g/ml of the serum and 2 ml of saline were added to the tube and were sonicated twice for 30 seconds with a 9-mm probe immersed deep in the fluid to prevent foaming by using a Braun-Sonic sonicator at 50% output. The sonicate was removed with the aid of a Pasteur pipette, and sonication was repeated under the same conditions. The pooled sonicates were then added to 50 ml of the $d>1.006$ g/ml serum, which was incubated under N₂ at 37°C with mild agitation in the presence of penicillin and streptomycin for 18 hours. During that time, the labeled cholesteryl linoleyl ether redistributed among the serum lipoproteins by the endogenous cholesteryl ester transfer protein. The labeled serum was used for injection into the rabbits.

**Experimental Design**

On the day of the injection, the rabbits were weighed, and their plasma volume was derived from the hematocrit, assuming a blood volume of 7.2% of body weight. An aliquot of 2 ml $d>1.006$ g/ml fraction of serum (containing $4 \times 10^6$ dpm) per 100 ml estimated plasma volume was injected into each rabbit's ear vein. The $d>1.006$ g/ml fraction was used to remove large triglyceride-rich lipoproteins, which would have been cleared from the circulation at a more rapid rate. The amount injected was determined by weighing the syringe before and after injection. Ten minutes after the injection, about 1 ml of blood was drawn from the marginal vein of the second ear and served for the estimation of initial plasma radioactivity. After the injection, the rabbits were returned to their cages and were fed regular Purina rabbit chow. They were divided into two groups with an attempt to match them by plasma cholesterol levels. Group 1 rabbits were sacrificed 10 to 12 days after injection of $^3$H-CLE-labeled serum and were designated the baseline group; 12 days after injection, the second group of rabbits was transferred to a regression diet and was kept on this diet for up to 330 days. The regression diet was composed of ground chow mixed with cholesteryamine at a final concentration of 3% (w/t/w). The mixture was then repelled and was fed to the rabbits ad libitum.

**Autopsy and Work-up of Tissues**

The rabbits were anesthetized with Ketaset (Bristol Lab, Syracuse, NY), were bled from the carotid or aorta, and were killed with pentobarbital. The organs were removed and weighed, and 1-g aliquots were taken for homogenization. The aortas were removed and cleaned of adventitial tissues under a magnifying glass. They were then opened, and scale drawings were made of the luminal surfaces. The aortas were then subdivided into three regions: ascending aorta and arch, thoracic aorta, and abdominal aorta. Homogenization was carried out in 10 volumes of methanol followed by 10 volumes of chloroform in a Kontes (Vineland, NJ) whole-glass conical homogenizer.

**Chemical and Radiochemical Determination**

$^3$H-CLE was synthesized using a modification of the method of Stoll. The purity of the compound was ascertained by thin-layer silicic acid chromatography and was found to be more than 98.5% pure. $7a$-$n$-$^3$H-cholesterol (specific activity 95 Ci/mmol) was purchased from Amersham International, Amersham, United Kingdom. Lipo-ids were extracted and purified according to the method of Folch et al., and aliquots were taken for radioactivity and cholesterol determination. Radioactivity was measured in a Packard scintillation spectrometer. Aortic free and total cholesterol were determined by gas liquid chromatography by using campesterol as the internal standard. Plasma cholesterol was determined by the cholesterol oxidase procedure by using Boehringer kits.

**Statistical Analysis**

Statistical correlations were determined by least-squares linear regression analysis and by Student’s $t$-test.

**Results**

Twenty rabbits were fed 1% cholesterol in their diet for 1 month and on alternate weeks for an additional 2 months. At the end of the feeding period, when their mean plasma cholesterol was $1296 \pm 125$ mg/dl (mean $\pm$ SE), rabbit plasma labeled with $^3$H-CLE was injected into each rabbit's ear vein. In 12 rabbits, blood samples were drawn from the second ear about 10 minutes after injection. The recovery of the label in the plasma compartment ranged from 85% to 93% of the injected dose. In the baseline group, which was sacrificed 10 to 12 days after injection, plasma radioactivity declined to low values ($0.0065\%$ of injected label/ml plasma). The experimental group of rabbits consisted of 10 animals that were kept on the regression regimen diet for up to 330 days; one animal died and was not included in the study. During this time, the rabbits gained weight and did not display any untoward effects of the drug. The plasma levels of cholesterol were less than 200 mg/dl at 4 weeks after the initiation of the regression regimen, and the radioactivity in 0.5-ml plasma aliquots was not above background levels. Two rabbits were killed after 8 and 9 months because of signs of infection.

**Distribution of Radioactivity in Organs**

Autopsies of the baseline group showed that the livers were yellowish to white, indicating a marked increase in lipid content. No gross changes were seen in the other organs. The distribution of radioactivity in the organs of the baseline group is shown in Table 1. Data on eight rabbits are given, because the organs of two rabbits were
Table 1. Uptake of \(^{3}\)H-CLE by Rabbit Organs after Injection of Labeled Plasma Lipoproteins

| Rabbit | Liver | Spleen | Heart | Lung | Kidney | Adrenal |
|--------|-------|--------|-------|------|--------|---------|
| 1      | 55.4  | 0.35   | 0.01  | 0.07 |        |         |
| 2      | 61.6  | 0.25   | 0.04  | 0.15 | 0.2    | 0.20    |
| 4      | 58.2  | 0.47   | 0.02  | 0.15 |        |         |
| 5      | 67.7  | 0.8    | 0.03  | 0.3  | 0.2    | 0.13    |
| 7      | 64.6  | 0.3    | 0.03  | 0.2  | 0.3    | 0.34    |
| 8      | 64.4  | 1.0    | 0.02  | 0.2  | 0.2    | 0.20    |
| 9      | 60.2  | 0.4    | 0.03  | 0.3  | 0.5    | 0.12    |
| 10     | 62.2  | 1.7    | 0.03  | 0.3  | 0.3    | 0.20    |

The rabbits were injected with plasma labeled with \(^{3}\)H-cholesterol linoleyl ether (\(^{3}\)H-CLE) and were killed on days 10 to 12 after injection.

Table 2. Distribution of \(^{3}\)H-CLE In Organs of Rabbits Kept on a Regression Regimen for up to 330 Days

| Rabbit | Liver | Spleen | Heart | Lung | Kidney | Adrenal |
|--------|-------|--------|-------|------|--------|---------|
| 11     | 20.9  | 0.01   | 0.01  | 0.03 | 0.10   | 0.05    |
| 12     | 24.8  | 0.04   | 0.01  | —    | —      | 0.16    |
| 13     | 25.9  | 0.01   | 0.01  | 0.04 | 0.10   | 0.17    |
| 14     | 28.7  | 0.02   | 0.01  | 0.05 | 0.10   | 0.12    |
| 15     | 19.9  | 0.02   | 0.01  | 0.07 | 0.10   | 0.06    |
| 16     | 20.4  | 0.03   | 0.01  | 0.06 | 0.08   | 0.21    |
| 17     | 28.1  | 0.03   | 0.04  | 0.10 | 0.18   | 0.21    |
| 18     | 21.4  | 0.03   | 0.01  | 0.06 | 0.22   | 0.26    |
| 20     | 24.4  | 0.04   | 0.02  | 0.01 | 0.09   | 0.13    |

The rabbits were injected with plasma labeled with \(^{3}\)H-cholesterol linoleyl ether (\(^{3}\)H-CLE) and were fed 3% cholestyramine during the regression period. Rabbits 20 and 12 were killed 8 and 9 months after injection of \(^{3}\)H-CLE, the other rabbits, after 330 days on the regression regimen.

Lost. The liver was the major site of uptake of \(^{3}\)H-CLE, and 61.8±1.4% (mean±SE) of the injected label was recovered in the liver (Table 1). In seven of eight rabbits, the radioactivity in the spleen was 1% or less of the injected dose (0.65±0.17%). Low radioactivity was found in the heart (0.02±0.003%), while the lungs and kidneys took up 0.21±0.03%, respectively, and 0.28±0.05% (mean±SE)% of the injected dose. The adrenals showed a high uptake relative to their weight, which amounted to 0.2±0.04% of the injected dose.

In the rabbits that were killed after being fed the regression diet, the most prominent finding was the normal appearance of the liver. The distribution of radioactivity among the organs is shown in Table 2. The radioactivity in the liver was reduced to 23.6±1% of the injected dose. The label in spleen, heart, lung, and kidney decreased to 0.03±0.004%, 0.014±0.003%, 0.08±0.011%, and 0.12±0.02% of injected dose, respectively. When compared with corresponding values in the baseline group (Table 1), these differences were significant (p<0.001 for liver, spleen, and lung, and p<0.02 for heart and kidney). The radioactivity in the adrenals did not change significantly and amounted to 0.15±0.02% of the injected dose (Table 2).

Analysis of Aortas

The aortas were removed and were examined under a magnifying glass. In the baseline group, the most severe

Table 3. Uptake and Retention of \(^{3}\)H-CLE In Atherosclerotic Aorta of Rabbits

|                         | Baseline | Regression |
|-------------------------|----------|------------|
| Initial plasma cholesterol (mg/dl) | 1344±195 | 1270±166  |
| \(^{3}\)H-CLE, dpm/aorta | 9066±1419 | 8661±1532 |
| Cholesterol (mg/aorta) |          |
| Total                   | 13.2±2.1 | 9.5±1.9   |
| Esterified              | 7.6±1.3  | 3.1±0.7   |

Baseline: Rabbits were injected with plasma labeled with \(^{3}\)H-cholesterol linoleyl ether (\(^{3}\)H-CLE) and were killed 10 to 12 days later. Regression: Rabbits were injected with plasma labeled with \(^{3}\)H-CLE and were fed 3% cholestyramine during the regression period. Rabbits 20 and 12 were killed 240 and 270 days after injection of \(^{3}\)H-CLE, the other rabbits, after 330 days on the regression regimen.

Values are means±SE of 10 rabbits in the baseline group and nine rabbits in the regression group. *p<0.01.

Atherosclerotic involvement was seen in the arch and in the thoracic aorta. The data in Table 3 show that in all 10 aortas, the esterified cholesterol was markedly increased and accounted for 38% to 68% of total cholesterol. The total cholesterol content of the entire aorta correlated highly (r=0.84) with the levels of plasma cholesterol at the time of injection of \(^{3}\)H-CLE. The radioactivity recovered in each aorta was highly correlated (r=0.877) with the plasma cholesterol levels at the time of injection. A high correlation (r=0.875) was also found between the \(^{3}\)H-CLE
and the abdominal aorta, the values of $^3$H-CLE/mg cholesteryl ester were determined and are presented in Figure 3. The mean±SE specific activity in the arch was $1574±216$ dpm/mg cholesteryl ester in the baseline group and $3679±460$ in the regression group. These values were $1137±145$ for the thoracic aorta in the baseline group and $2609±413$ in the regression group. A similar difference was also seen between the abdominal aorta of the two groups, that is, $1239±213$ versus $262±251$. Each of the differences between the baseline and the regression groups were statistically significant ($p<0.005$) as determined by Student's $t$ test.

**Discussion**

In the present study, we used $^3$H-CLE to serve as a stable marker of the extent of aortic atheromatosis induced by cholesterol feeding in rabbits. In agreement with our previous findings, there was a high correlation between the amount of total cholesterol, as well as of esterified cholesterol and radioactivity recovered in the aorta 10 to 12 days after injection of $^3$H-CLE. The main objective of this study was to investigate the potential usefulness of labeled CLE in the evaluation of regression of atheromatoses, and to that end we kept the rabbits for almost a year on a hypolipidemic drug. This experimental design was based on the results of studies summarized in recent reviews, which pointed out that atherosclerotic lesions of rabbits fed cholesterol for 2 months or more regress very slowly, however, enhancement of regression was reported when the animals were given a hypolipidemic drug, such as cholestyramine or probucol. The reason for the slow regression and even temporary progression of the lesions was attributed to Daugherty et al. to the huge cholesterol stores in the liver, continued overproduction of cholesteryl ester-rich very low density lipoprotein, and its persistent delivery to the arterial wall. The slow rate of regression could also be ascribed to the accumulation in the lesions of cholesterol crystals derived from hydrolysis of esterified cholesterol, which are resistant to removal from the aorta. In addition, we might have underestimated the extent of regression because the

**Figure 1.** Linear regression analysis of correlation between plasma cholesterol at time of injection and $^3$H-cholesterol linoleyl ether ($^3$H-CLE) in aorta of the regression group up to 330 days after injection, $r=0.9470$, $p<0.001$.

**Figure 2.** Linear regression analysis of correlation between aortic cholesteryl ester and $^3$H-cholesterol linoleyl ether ($^3$H-CLE) of the regression group up to 330 days after injection, $r=0.9741$, $p<0.001$.

and the cholesteryl ester content of the aorta (data not shown). The aortas of rabbits kept on the regression regimen for up to 330 days were examined in a similar manner. In many instances, the lesioned areas, which were seen mainly in the arch and in the thoracic aorta, looked whiter than in the baseline group. In these regions, numerous small aneurysma-like dilations were seen. The radioactivity recovered in these aortas, up to 330 days after injection of $^3$H-CLE, correlated highly ($r=0.947$) with the plasma cholesterol levels at the time of injection (Figure 1) and also with the cholesteryl ester in the aorta ($r=0.974$, Figure 2). The mean radioactivity in nine aortas of the regression group was not significantly different from that found in the baseline group and was about 9000 dpm/aorta (Table 3). The mean cholesteryl ester content was $3.1±0.7$ mg/aorta, which was significantly different ($p<0.01$) from that found in the baseline group, $7.6±1.3$ mg/aorta. On the other hand, the amount of free cholesterol was $5.54±0.98$ and $6.41±1.33$ mg/aorta in the baseline and regression groups, respectively. These values were not statistically significant.

**Relation of $^3$H-Cholesterol Linoleyl Ether to Aortic Cholesteryl Ester**

To compare the extent of regression in the three aortic regions, the ascending aorta and arch, the thoracic aorta,
results were given per whole aorta and were not normalized to aortic weight or surface area, which could have increased during the 330 days of the regression period.

After injection into rabbits, **3H-CLE** was predominantly recovered in the liver, which accounted for more than 60% of the injected dose. The liver radioactivity declined to about 20% of the injected dose in 330 days and was probably eliminated from the liver through the bile. This route was demonstrated in our previous studies in which rats were injected with **3H-CLE**-labeled chylomicrons,**6,7** and after a lag period, radioactivity appeared in the bile and feces. It was also shown that elimination of the **3H-CLE** from the liver was not accompanied by redistribution of the label in the body**17** as determined by measuring radioactivity in peripheral organs and the entire carcass. Moreover, Hough and Zilversmit**8** have shown that, while up to 10 hours after injection the clearance of labeled cholesteryl ester and cholesteryl ester ether from the circulation was identical, the disappearance curves began to diverge 24 hours after injection and differed by a factor of 10 after 11 days. This divergence of disappearance of plasma cholesteryl ester and cholesteryl ester ether was attributed to resorption of cholesterol (derived from hydrolysis of cholesteryl ester) and further supports our findings in rats.**6,7** That cholesteryl ester is not resorbed from the liver into the plasma. In the present study, no measurable radioactivity was found in aliquots of 0.5 ml of plasma of rabbits obtained 4 weeks after institution of cholestyramine treatment, that is, 6 weeks after injection of **3H-CLE**. Thus, it seems that resorption of cholesteryl ester ether from the liver into the plasma and its subsequent deposition in the artery may not have been significant.

In the rabbits killed 330 days after injection of **3H-CLE**, the radioactivity declined in the spleen and lung but was retained in the adrenals. The mean radioactivity recovered in the aortas of the nine rabbits that were killed up to 330 days after injection was similar to that found in the aortas of the baseline group killed 10 to 12 days after injection. The finding that the aortic radioactivity in both groups correlated highly with the initial plasma cholesterol levels may lend further support to the possibility that the **2H** label in the aorta of the regression group represents originally taken-up label, even though it does not rule out possible redistribution. The problem of retention of labeled cholesteryl ether in the aortas of rabbits over a period of 7 or 25 days was investigated by Hough and Zilversmit.**18** In that study, loss of cholesteryl ether was measured as a difference in estimated influx of **2H-** and **14C**-labeled cholesteryl ester ether injected 7 or 25 days apart, and a 50% loss was found irrespective of the length of time interval between injections. These authors discuss this unexpected time-independence of percent "loss" of cholesteryl ester ether and suggest that the loss could have been underestimated because of the assumption that the influx rates were constant over time. In addition, the time-independence of the estimated loss of cholesteryl ether might suggest that, if there is a loss of cholesteryl ether from the artery, it could occur within 7 days after injection of the label from a putative pool accessible to cholesteryl ester transfer protein. The latter was shown to enhance cholesteryl ester efflux from extracellular lipoprotein deposits in the in vitro model systems.**19** The retention of **3H-CLE** in the aorta during the regression period would suggest that, after the initial 10 to 12 days, the **3H-CLE** was not accessible to cholesteryl ester transfer protein. The inaccessibility of the **3H-CLE** could have been due to proposed stratification of sequential lipid deposits**16** in atheroma, which would be expected to continue to enlarge, even during the first months of regression.**14** Cholesteryl ester transfer protein would not have been expected to also remove intracellular cholesteryl ether.**19**

In the present study, the occurrence of regression of aortic atherosclerosis was documented by a 60% reduction in cholesteryl ester content after 330 days on the regression regimen. At the same time, the total aortic cholesterol was not significantly different, indicating an accumulation of some free cholesterol as was shown to occur during the later stages of regression of atherosclerosis in nonhuman primates.**15** The retention of **3H-CLE** in the aorta in spite of loss of cholesteryl ester permitted us to further conclude that the lower cholesteryl ester values in the experimental group were indicative of regression, rather than variability, of atherosclerotic involvement. Moreover, it was possible to compare the specific activities of **3H-CLE**/cholesteryl ester in three aortic regions of the baseline and regression groups. The significant rise in mean specific radioactivity in the regression group, as compared to the baseline group, indicated that loss of cholesteryl ester had occurred from all three regions of the aorta. In the regression group, the mean specific activity of **3H-CLE**/cholesteryl ester in the arch and thoracic aorta was not significantly different. However, in two animals, the specific activity in the arch was much higher than in the thoracic aorta, suggesting that, in some instances, regression might proceed at different rates in these two regions. The present study has shown that **3H-CLE** is retained in the aorta for long periods of time; its use may add another dimension to the quantitative evaluation of regression of the atherosclerotic lesions.

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