Acetylated Low-Density Lipoprotein–Encapsulated Cholesteryl 1,3-Diiopanoate Glyceryl Ether for the Detection of Atherosclerosis in Rabbits

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A cholesteryl ester analog, cholesteryl 1,3-diiopanoate glyceryl ether (C2I), was synthesized and investigated for its potential use for the detection of atherosclerotic lesions in rabbits. **Methods:** ^{125}I-labeled C2I was incorporated into acetylated low-density lipoprotein (AcLDL). The resultant complex, ^{125}I-C2I–AcLDL, was injected intravenously into 2 groups of rabbits, fed cholesterol and normal chow, at a dose of 555 kBq/kg. Tissue samples were taken 24 h after injection for the biodistribution study. Atherosclerotic lesions and C2I deposition in aortic samples were examined by Sudan IV staining and autoradiography, respectively. **Results:** The levels of C2I in blood and aortic samples in cholesterol-fed animals were 2- to 3-fold higher than those in the control group ($P < 0.05$). The autoradiography results correlated well with the Sudan IV staining results, indicating sites of C2I deposition superimposed on lesion sites. **Conclusion:** C2I was preferentially taken up and retained at atherosclerotic lesion sites, suggesting its potential use for the detection of early atherosclerosis.

Key Words: cholesteryl 1,3-diiopanoate glyceryl ether; atherosclerosis; plaques; acetylated low-density lipoprotein

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Atherosclerotic lesions are initiated by the accumulation of macrophages and the subsequent formation of foam cells. It is known that scavenger receptors are associated with macrophages and that these receptors are responsible for the uptake of chemically modified human low-density lipoprotein (LDL), such as acetylated LDL (AcLDL) (*1*). On the basis of this information, radiolabeled LDL preparations, such as ^{123}I-LDL, ^{125}I-LDL, ^{111}In-LDL, and ^{99m}Tc-LDL, were explored for their potential use for the detection of atherosclerotic lesions (*2*). A drawback of this strategy, however, is that the labeling is performed on the apolipoprotein B-100 protein moiety of LDL, so that the radioactivity associated with this protein leaves the lesion sites when the protein is degraded, making long-term and accurate estimation of lesion sites difficult (*2*).

In a previous study from our laboratory, in an attempt to overcome the problem associated with the apoprotein labeling of LDL, we studied the potential of incorporating a degradation-resistant lipid analog, cholesteryl iopanoate (CI), into AcLDL for the detection of early atherosclerotic lesions in rabbits (*2*). This approach is superior to the former in that a longer retention of compound-associated radioactivity may be obtained at lesion sites and, because the predominant lipids of atherosclerotic lesions are cholesterol and cholesteryl esters (*1*), the use of cholesteryl ester analogs instead of apoprotein will more accurately reveal the plaque formation process.

In this study, we sought to further explore our approach by synthesizing a new cholesteryl ester analog, cholesteryl 1,3-diiopanoate glyceryl ether (C2I), for the detection of atherosclerotic lesions in rabbits. Bearing 6 iodines on its structure, C2I could have twice the specific radioactivity of CI, which has 3 iodines. The biodistribution profile of C2I was found to be similar to that of CI, and atherosclerotic lesions revealed by Sudan IV staining correlated well with the accumulation of radioactivity from C2I shown by autoradiography results, suggesting the potential application of C2I as a radioimaging or contrast agent for the diagnosis of atherosclerosis.

MATERIALS AND METHODS

C2I was synthesized as illustrated in Figure 1. Compounds a and b were prepared according to a published method (*3*). To synthesize cholesteryl chloride (compound c), cholesterol (5 g,

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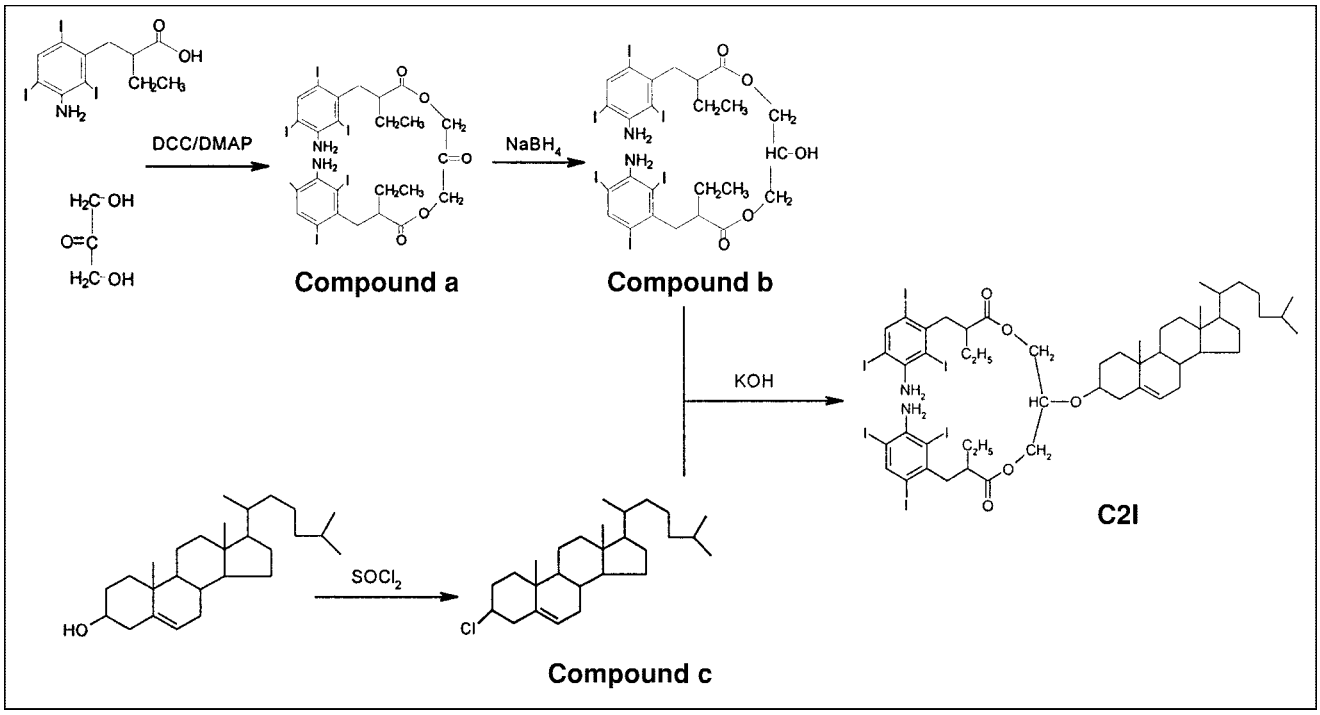

FIGURE 1. Schematic diagram of route of synthesis of C2I.

0.0129 mol) was added to thionyl chloride (5.5 g, 0.046 mol, 3.5 mL). The mixture was kept at 20°C overnight and then poured into 0.4N NaOH (about 25 mL). The product was extracted 3 times with ether (1:1), and the extracts were combined and dried by use of anhydrous $MgSO_4$. Ether was evaporated to afford a semipure whitish solid, which was further purified by silica gel column chromatography with hexane as the eluent. A white cholesteryl chloride solid (3.9 g) was obtained at a yield of 76%. C2I (compound d) was synthesized by mixing cholesteryl chloride (55 mg, 0.14 mmol) in 2 mL of benzene, compound b (150 mg, 0.125 mmol) in 2 mL of dimethyl formamide, and KOH (17 mg, 0.29 mmol) in 2 mL of ethanol:water (50:1, v/v). The mixture was refluxed overnight, concentrated in a vacuum at 50°C, and cooled to room temperature. The white crystals obtained were purified by column separation ($CHCl_3$, silica gel). The yield was about 12.5% (about 25 mg). Infrared details were as follows (per cm): 3,500 and 3,380 (-NH_2), 2,950 and 2,840 (-CH_3), 1,480 (C═C), 1,740 (-C═O), 1,380 [$(CH_3)_2$-CH-], and 1,600 and 920 (Ar). ^{1}H nuclear magnetic resonance details were as follows (parts per million): 8.1 (s, 2H, Ar-H), 5.4 (s, 1H, C═C-H), 4.8 (s, 4H, NH_2), 4.1 (m, 4H, CH_2), 3.7 (m, 2H, -O-CH), 3.4–3.2 (m, 4H, CH_2), 2.8 (m, 2H, $CHCO_2$), 2.2–1.6 (m, 22H, CH_2, CH), 1.1–1.3 (m, 10H, CH_2), 0.9 (d, 15H, CH_3), and 0.7 (s, 6H, CH_3).

The partition coefficient of C2I in 1-octanol:phosphate-buffered saline (PBS) was measured according to a published method (*4*). C2I was labeled with ^{125}I via an iodine-exchange reaction (*2*). ^{125}I-C2I was incorporated into AcLDL to form an ^{125}I-C2I–AcLDL complex by use of a microemulsion transfer technique. The loading efficiency was determined as described previously (*2*).

Animal models were established as described previously but with modifications (*2*). Eight male New Zealand White rabbits (2.5–4.7 kg) were randomly divided into 2 groups. One group was maintained on normal rabbit chow ($n = 4$, control group) for 12 wk, and the other group was maintained on rabbit chow supplemented with 1% cholesterol ($n = 4$, cholesterol diet group) for 12 wk to induce early atherosclerotic lesions. The ^{125}I-C2I–AcLDL complex was injected via the marginal ear vein into both groups of rabbits at a dose of 555 kBq/kg. Rabbits were killed 24 h after injection, and tissues, including the liver, spleen, lungs, kidneys, adrenal glands, blood, and aorta, were sampled for the determination of radioactivity by use of a γ-counter (*2*). Sudan IV staining and autoradiography of aortic samples were performed as described previously (*2*). The in vivo stability of C2I was determined by measuring its degradation in the liver (*5*).

TABLE 1
Distribution of Radioactivity in Various Tissues of Atherosclerotic and Control Rabbits

Tissue	% Administered dose/g of tissue in each group (mean ± SD)	
	Cholesterol diet	Control
Liver	13.7 ± 2.3	18.5 ± 1.1
Spleen	23.3 ± 3.3	27.4 ± 3.7
Lungs	12.2 ± 2.7	8.5 ± 2.2
Kidneys	2.8 ± 0.3	29.2 ± 0.52
Adrenal glands	8.5 ± 0.3*	22.9 ± 4.4
Blood	7.4 ± 1.1*	2.7 ± 0.5
Aorta	0.26 ± 0.04*	0.12 ± 0.02

*Significantly different from control group ($P < 0.05$).
Radioactivity (kBq) was determined 24 h after intravenous injection of ^{125}I-C2I–AcLDL at 555 kBq/kg (4 rabbits per group).

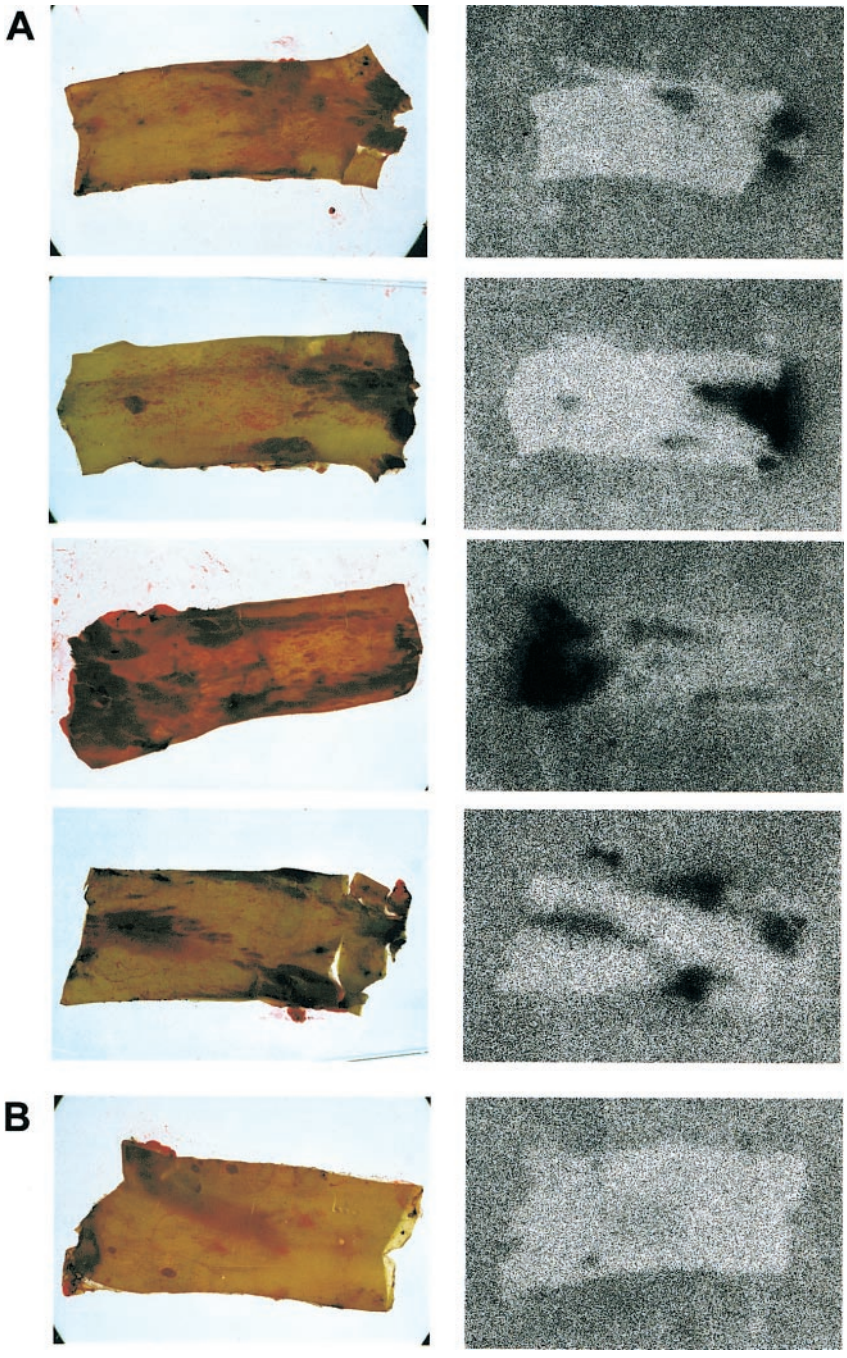

FIGURE 2. Results of Sudan IV staining (red staining, left panels) and autoradiography (black regions, right panels) of aortic samples taken at 24 h. (A) Samples from cholesterol diet group. (B) Sample from control group.

RESULTS

The compound C2I was synthesized (Fig. 1), and its structure was confirmed by infrared and nuclear magnetic resonance data. The partition coefficient of C2I in octanol and PBS defined as log ($C_{octanol}/C_{PBS}$) was 2.529 ± 0.072. The efficiency of loading of C2I into AcLDL, defined as the number of C2I molecules per AcLDL molecule, was determined to be 162.41 ± 23.38. The tissue distribution at 24 h showed that the accumulation of ^{125}I-C2I in the blood and aortic samples from the cholesterol-fed group was significantly higher than that for the control group (2.7 and 2.1 times, respectively) (Table 1). In the adrenal glands, however, less ^{125}I-C2I was found in the cholesterol-fed group than in the control group ($P < 0.05$). No statistically significant difference in ^{125}I-C2I accumulation was found in other tissues for the 2 groups. The in vivo stability test showed that 91.2% of the radioactivity in the liver was associated with C2I, indicating that C2I was chemically stable and that the level of radioactivity reflected the content of the intact C2I compound.

The results of Sudan IV staining and autoradiography of aortic samples from the cholesterol-fed group showed a

clear superimposition of atherosclerotic lesions and radioactivity deposition (Fig. 2A), suggesting that ^{125}I-C2I selectively accumulates at lesion sites in the aorta. The results of both Sudan IV staining and autoradiography were negative for aortic samples from the control group (Fig. 2B).

DISCUSSION

In a previous study, we examined the potential use of ^{125}I-CI–AcLDL for the detection of early atherosclerotic lesions in rabbits (*2*). In this study, we synthesized a new compound, C2I, for the same purpose. Like CI, C2I also has high in vivo stability because of the chemical nature of the ether linkage between cholesterol and 1,3-diiopanoate glycerol. Compared with CI, which has 3 iodine atoms per molecule, each C2I molecule contains 6 iodine atoms, a factor that could result in twice the specific radioactivity of CI. The higher specific radioactivity of C2I is more desirable for scintigraphic or CT studies.

The tissue distribution study showed that there was no difference in C2I deposition for the cholesterol-fed and control groups, except for blood, aortic, and adrenal gland samples. The accumulation of C2I in blood and aortic samples was 2- to 3-fold higher in cholesterol-fed rabbits than in control rabbits, a finding consistent with the results obtained with CI (*2*). Reduced accumulation in the adrenal glands of cholesterol-fed animals was also observed (Table 1). When the ratio of the lesion area to the whole area of aortic samples was considered, the actual specific radioactivity of aortic plaque was found to be at least 10 times higher (Fig. 2A).

Sudan IV staining was used to visualize lipid-laden foam cells, a hallmark of early atherosclerosis (*2*). In this experiment, lesions in cholesterol-fed animals were readily identified by this method (Fig. 2A). The accumulation of ^{125}I-C2I in aortic samples, as visualized by autoradiography (Fig. 2A), was superimposed on lesion sites, indicating that C2I accumulates and is retained specifically in regions of plaque.

CONCLUSION

In this study, a new compound, C2I, was synthesized and radiolabeled with ^{125}I. Results from animal studies demonstrated selective uptake and retention of radiolabeled C2I in rabbit atherosclerotic lesions when iodinated C2I was carried via AcLDL. It is anticipated that when labeled with ^{123}I or ^{131}I, C2I incorporated into AcLDL will permit scintigraphic imaging of lesion sites in the aorta. More studies on these topics are under way.

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