Stimulation of Cholesterol Release from Rabbit Foam Cells by the Action of a New Inhibitor for Acyl CoA:Cholesterol Acyltransferase (ACAT), HL-004

ITSUKO ISHII, NORIKO YOKOYAMA, Mamoru Yanagimachi, Naoya Ashikawa, Masayuki Hata, Shigeru Murakami,1 Yumiko Asami,1 Nobuhiro Morisaki,2 Yasushi Saito,2 Shigeru Ohmori3 and Mitsukazu Kitada3

Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba 263-8522, Japan
Accepted for publication May 8, 1998 This paper is available online at http://www.jpet.org

ABSTRACT
A new inhibitor of acyl CoA:cholesterol acyltransferase (ACAT), HL-004 [N-(2, 6-diisopropylphenyl)tetradecylthioacetamide], suppressed the synthesis of cholesterol [14C]oleate at 10−9 – 10−7 M in a concentration-dependent manner in both THP-1 cell-derived macrophages and foam cells prepared from aortic intima of rabbits fed a high cholesterol diet. Incorporation of [3H]cholesterol oleate-β very low density lipoproteins was not inhibited by HL-004 at 10−9 – 10−7 M. Release of radioactivity from the cells loaded with [3H]cholesterol oleate-β very low density lipoproteins was increased by the inhibition of ACAT activity with HL-004. HL-004 did not affect on acid and neutral cholesterol esterases. As a result, cholesterol ester content in foam cells decreased. These data suggested that HL-004 decreases cholesterol ester in foam cells by increasing the release of cholesterol and therefore might suppress atherosclerotic lesions.

The development of atherosclerotic diseases is associated with the accumulation of cholesterol ester in arteries. Large numbers of foam cells derived from macrophages and smooth muscle cells and containing a large amount of cholesterol ester are found in atherosclerotic lesions (Rosenfeld et al., 1987; Lewis et al., 1985; Stary et al., 1990). These foam cells have a high activity of ACAT, which catalyzes the esterification of free cholesterol (Suckling et al., 1985). The increase of lipid deposition in foam cells is due to this excessive cholesterol esterification by the ACAT action (Goldstein et al., 1980). Thus, it might be expected that atherosclerosis is reduced by the inhibition of ACAT activity.

It is known that intracellular cholesterol metabolism is regulated by some enzymes such as acid cholesterol esterase and neutral cholesterol esterase besides ACAT (Brown et al., 1979, 1980; Khoo et al., 1981, 1984; Goldberg et al., 1990). It is reported that an imbalance between cholesterol esterase and ACAT activities might induce either foam cell formation (Ishii et al., 1992, 1994, 1995b) or cell toxicity (Warner et al., 1995). For example, when ACAT activity was induced and neutral cholesterol esterase activity was low, cells accumulated excess cholesterol ester and were converted into foam cells (Ishii et al., 1992, 1994, 1995a). There are several reports on the effects of ACAT inhibitors on intracellular cholesterol content and metabolism. An ACAT inhibitor, Sandoz 58–035, reduced intracellular cholesterol ester content in J774 cells, mouse peritoneal macrophages, Fu5AH hepatoma cells (Bernard et al., 1990) and monkey arterial smooth muscle cells under conditions of hyperlipidemia (Ross et al., 1984). Other ACAT inhibitors, CL 277,082 and CI-976, also inhibited ACAT activity and cholesterol absorption (Krause et al., 1993; Largis et al., 1989). On the other hand, when free cholesterol accumulated by the inhibition of ACAT activity with Sandoz 58–035 and Pfizer 113,818, cell toxicity was induced (Warner et al., 1995). For the safe use of ACAT inhibitors, this problem must be solved. We hypothesized that an ideal condition for reducing cholesterol in foam cells by the use of ACAT inhibitors is to increase the release of intracellular free cholesterol produced by acid and neutral cholesterol esterase. Some kinds of ACAT inhibitors might indirectly stimulate this process. In this study, we investigated this possibility using a new ACAT inhibitor, HL-004 [N-(2, 6-diisopropylphenyl)tetradecylthioacetamide], com-

ABBRVIATIONS: ACAT, acyl CoA:cholesterol acyltransferase; VLDL, very low density lipoproteins; FBS, fetal bovine serum; HDL, high density lipoprotein; HL-004, N-(2, 6-diisopropylphenyl) tetradecylthioacetamide; LPDS, lipoprotein-deficient serum; TPA, 12-tetra-decanoylphorbol-13-acetate.
pared with CI-976 (2,2-dimethyl-N1-(2,4,6-trimethylphenyl)dodecanamide).

Materials and Methods

Materials. HL-004 was synthesized by Taisho Pharmaceutical (Ohmiya, Japan). HL-004 was synthesized as reported (Japanese patent No. JP-316129, 1995), and the purity was >99% by HPLC. CI-976 was obtained from Warner-Lambert (Ann Arbor, MI). HL-004 and CI-976 were first dissolved in DMSO, and the final concentration was 10 μM. [3H]Cholesterol oleate (3.0 GBq/mmol) and [14C]oleic acid (1.5 GBq/mmol) were purchased from New England Nuclear Corporation (Boston, MA); [8-3H]Adenine (666 GBq/mmol) was purchased from Amersham Corp. (Buckinghamshire, UK). TPA was obtained from Sigma (St. Louis, MO). THP-1 cells (human monocye leukemia cell line) were obtained from Japan Cell Culture System (Tokyo, Japan). DMEM and RPMI 1640 were obtained from Nissui Pharmaceutical (Tokyo, Japan).

Preparation of cells. THP-1 cells (5 × 10⁶ cells/ml) were treated with 1 × 10⁻⁸ M TPA overnight in RPMI 1640 containing 10% FBS (10% FBS/RPMI), and the adherent cells were used as macrophages (Hayashi et al., 1991).

Rabbit atherosclerotic lesion cells were obtained as reported previously (Jaakkola et al., 1988; Pitas et al., 1990). Rabbis were fed a high cholesterol diet containing 1% cholesterol in a normal chow diet for 16 weeks. The aorta was removed and the adventitia was carefully separated. Then, the media and intima were cut into pieces (1 × 1 mm) and digested with collagenase (Sigma, Type III, 500 units/ml) and elastase (Sigma, Type III, 100 units/ml) for 30 min at 37°C. The tissue digest was centrifuged at 1000 × g for 5 min and the pellet was suspended with DMEM. This process was repeated 3 times. The resultant cell suspension was seeded onto plates and incubated in DMEM containing 10% FBS (10% FBS/RPMI). After 4 hr, unattached cells were washed off with DMEM. We called these adherent cells atherosclerotic lesion cells. Esterase staining demonstrated that 75% ~ 82% of the attached cells were able to hydrolizze α-naphthylacetate (data not shown), indicating that these cells were mainly macrophages and the remaining cells were smooth muscle cells.

Measurement of ACAT inhibitor-induced cell toxicity. ACAT inhibitor-induced cell toxicity was measured by the modified (Warner et al., 1995; Reid et al., 1992) method of Shirhatti and Krishna (Shirhatti et al., 1985). Briefly, after 18-hr incubation of cells with DMMEM containing 0.2% BSA, 37 kBq of [3H]adenine was added to each well and cells were incubated for 2 hr. Then, wells were washed 3 times with 1 ml of DMEM containing 0.2% BSA (0.2% BSA/DMMEM) and incubated for an additional 10 min with 0.2% BSA/DMMEM. Media were removed, and fresh 0.2% BSA/DMMEM was added with ACAT inhibitor. At indicated times, 200 μl of the media was removed from triplicate cultures and their radioactivities were counted. [3H]Adenine release from macrophages was expressed as a percentage of release compared with control treatment (0.2% BSA): (cpm in medium of treatment – cpm in medium of control) /[3H]Adenine content at time zero) × 100, or as a percent release of total [3H]adenine content at time zero.

Preparation of lipoproteins. HDL (d = 1.063 – 1.21 g/ml) and LPDS (d > 1.25 g/ml) were isolated from human plasma by sequential ultracentrifugation and were dialyzed against 20 mM Tris HCl (pH 7.4), 2 mM EDTA, 0.15 M NaCl and 0.02% NaN₃ for 24 hr (Havel et al., 1955).

Preparation of reconstituted [3H]cholesterol oleate into βVLDL. βVLDL (density < 1.006) was isolated from cholesterol-fed rabbit serum by ultracentrifugation for 16 hr (Brown et al., 1975). Incorporation of [3H]cholesterol oleate into βVLDL was done essentially by the method of Brown et al. (1975). Then, 37 MBq of [3H]cholesterol oleate was added with 1 ml of DMSO. The mixture was sonicated for 5 sec. Then 2 ml of plasma density buffer (0.154 M NaCl, 1 mM EDTA, 10 mM Tris HCl, pH 7.4, 0.01% NaN₃) was added, and the mixture was sonicated for 30 sec. It was then added dropwise to 6 ml of βVLDL (10 mg total cholesterol/ml) in 3 min. The solution was incubated for 8 hr at 37°C and then was dialyzed against 3 liters of plasma density buffer for 10 hr. After dialysis, the solution was centrifuged for 16 hr at 105,000 × g. The top layer was used as [3H]cholesterol oleate-incorporated βVLDL. The specific activity was about 3.4 × 10⁵ dpm/mg total cholesterol and 5.4 × 10⁵ dpm/mg cholesterol ester.

[3H]Cholesterol oleate-βVLDL incorporation into cells. TPA-treated THP-1 cells (1 × 10⁶ cells) and atherosclerotic lesion cells (5 × 10⁶ cells) were plated in 12-well plates and incubated for a certain time periods in 0.75 ml of DME containing 5% LPDS (5% LPDS/DMEM) and 200 μg of [3H]cholesterol oleate-βVLDL (1.6 × 10⁶ dpm). Then, after 8-hr incubation, the cells were washed 3 times with 10% FBS/DMEM, and their radioactivity was measured with a scintillation counter. Furthermore, to determine the free [3H]cholesterol released from the cells during incubation, organic solvent (chloroform/methanol = 2:1) was added to the medium, and lipids were extracted from the chloroform layer. The lipids were applied to thin layer chromatography (Gillies et al., 1986). The radioactivity in the free cholesterol fraction was then counted. The total uptake amounts were the sums of intracellular radioactivity and free [3H]cholesterol radioactivity in the medium.

Release of radioactivity from [3H]cholesterol oleate-βVLDL-loaded cells. TPA-treated THP-1 cells (2 × 10⁶ cells) and atherosclerotic lesion cells (2 × 10⁶ cells) were plated in 12-well plates and incubated for 24 hr in 1 ml of 5% LPDS/DMEM containing 200 μg of [3H]cholesterol oleate-βVLDL. Then the cells were washed 3 times with 5% LPDS/DMEM. These [3H]cholesterol oleate-βVLDL-loaded cells were incubated further in 2 ml of 5% LPDS/DMEM containing LDL (0.3 mg protein/ml). At the times indicated in figure 3, 0.4 ml of the medium was removed, and its radioactivity was measured.

Synthesis of cholesterol [14C]oleate from [14C]oleic acid in intact cells. Cells (2 × 10⁶ cells/well) were treated with βVLDL (100 μg cholesterol/ml) and albumin-[14C]oleic acid complex (0.1 μCi/ml) (Goldstein et al., 1974). After incubation for 8 hr, the cells were washed with medium three times and with PBS twice. The intracellular lipids were extracted by chloroform/methanol (2:1) and separated by thin-layer chromatography (Merck). After incubation with hexane diethyl ether acidic acid (146:50:4). Nonlabel free cholesterol and cholesterol ester were spotted as marker in the plate, and the spot was detected by iodide. The radioactivity of the cholesterol ester fraction was estimated as ACAT activity in intact cells.

Measurement of acid and neutral cholesterol esterase activity. Macrophages (2 × 10⁷ cells) were washed 3 times with PBS and suspended in 1 ml of 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose. Then the cells were sonicated twice for 15 sec and used as the enzyme solution. The reaction mixture (Ishii et al., 1992; Ishii et al., 1994, 1995) contained 0.5 mM cholesterol oleate, 0.37 MBq of [14C]oleate, 0.5 mM phosphatidic acid and 100 mM Tris-HCl (pH 7.4) for neutral cholesterol esterase, or 0.5 mM phosphatidylcholine and sodium acetate buffer (pH 4.0) for acid cholesterol esterase, certain concentration of ACAT inhibitors, and the enzyme solution, in a total volume of 200 μl. The incubation was carried out at 37°C for 1 hr. The [14C]oleate released was extracted by modification of the method of Belfrage et al. (1969). Briefly, the reaction was stopped with 3.25 ml of chloroform/methanol/heptane (1:2.1:2.5:1.00), and then 1 ml of 0.1 N NaOH was added. The radioactivity in the water phase was measured.

Determination of cholesterol ester content. The cholesterol ester content was measured as the difference between the total and free cholesterol contents (Ishii et al., 1995). The lipids of the washed cells in each well were extracted with 1 ml of hexane/isopropanol (2:1). The organic solvent was evaporated and the pellet was dissolved in 100 μl of methanol, and the total and free cholesterol contents in the methanol solution were assayed enzymatically with...
Determination protein concentration. Protein concentration was determined with a kit using Bradford’s method (BioRad, Protein Assay, Richmond, CA).

Statistics treatment. The significance of differences in a treatment series was determined by a one-way analysis of variance. Individual treatments were compared with the control by Dunnett’s test.

Results

Effect of HL-004 on synthesis of cholesterol [14C]oleate in TPA-treated THP-1 cells. To determine the effect of the ACAT inhibitor, HL-004 on cholesterol esterification in macrophages compared with the effect of CI-976, we examined the inhibition of cholesterol [14C]oleate synthesis in TPA-treated THP-1 cells. Figure 1A shows that HL-004 decreased the synthesis of cholesterol [14C]oleate in a concentration-dependent manner and the effect of HL-004 was stronger than that of CI-976, the inhibition being ~90% at 10^{-7} M and ~100% at 10^{-6} M. Then, 50% inhibition (IC_{50}) of HL-004 and CI-976 were 1.1 \times 10^{-9} M and 1.2 \times 10^{-7} M, respectively.

In preliminary studies, we observed that the release of radiolabeled adenine correlated with the release of lactate dehydrogenase, a well accepted marker of cell toxicity in cholesterol-loaded macrophages. Figure 1B shows [3H]adenine release from TPA-treated THP-1 cells incubated in the presence of HL-004 and CI-976. The release of [3H]adenine from CI-976-treated cells increased in a concentration-dependent manner, and the amount of the released radioactivities from HL-004-treated cells was nearly equal in all groups. There was a statistically significant difference between HL-004- and CI-976-treated cells. These data suggested that HL-004 did not induce cell toxicity when it inhibited ACAT activity compared with CI-976.

Figure 2 shows the time course of the effect of the ACAT inhibitors. HL-004 (10^{-7} M) inhibited 80% ~ 90% of the synthesis of cholesterol [14C]oleate during the first 24-hr incubation, but the activity then returned to 70% of control at 48 hr. CI-976 (10^{-7} M) inhibited ~60% during 12-hr incubation but ~20% after 24-hr incubation. These data suggested that the duration of HL-004 effect was longer that that of CI-976. In the following experiments, the HL-004 effect was determined within 24 hr.

Effect of HL-004 on cholesterol metabolism in TPA-treated THP-1 cells. Cholesterol metabolism of macrophages is as follows; macrophages take up lipoproteins such as βVLDL and denatured LDL. First, cholesterol ester in lipoproteins is hydrolyzed by acid cholesterol esterase in lysosomes (Brown et al., 1979; Brown et al., 1980). Then the product, free cholesterol, is reesterified by ACAT and stored as cholesterol ester in intracellular lipid droplets. After that, the reesterified cholesterol is hydrolyzed by neutral cholesterol esterase (Kho et al., 1981, 1984; Goldberg et al., 1990), and finally, free cholesterol is released from the cells. Thus, the effects of HL-004 on the incorporation of βVLDL, release of cholesterol, acid cholesterol esterase activity and neutral cholesterol esterase activity were measured compared with CI-976.

HL-004 and CI-976 did not change the incorporation of [3H]cholesterol oleate-βVLDL (fig. 3A). The released radioactivity from cells treated with both ACAT inhibitors increased in a dose-dependent manner. However, the release from HL-
Fig. 3. Effect of concentration of HL-004 and CI-976 on incorporation of \[^{3}H\]cholesterol oleate-βVLDL and release of radioactivity from TPA-treated THP-1 cells loaded with \[^{3}H\]cholesterol oleate-βVLDL. A, Incorporation of \[^{3}H\]cholesterol oleate-βVLDL into TPA-treated THP-1 cells. TPA-treated THP-1 cells (1 × 10^6 cells/well) were incubated with ACAT inhibitors at indicated concentrations for 12 hr. The medium was changed and the cells were treated with \[^{3}H\]cholesterol oleate-βVLDL, and again with ACAT inhibitors for 8 hr. Incorporation of \[^{3}H\]cholesterol oleate-βVLDL into the cells was measured as described in Materials and Methods. B, Release of radioactivity from TPA-treated THP-1 cells loaded with \[^{3}H\]cholesterol oleate-βVLDL. TPA-treated THP-1 cells (1 × 10^6 cells/well) were incubated with \[^{3}H\]cholesterol oleate-βVLDL for 24 hr. After that, cells were incubated with 5% LPDS/DMEM containing HDL (0.3 mg protein/ml) and ACAT inhibitors for 12 hr. Total incorporated and released radioactivity were measured as described in Materials and Methods. •, HL-004-treated cells; ○, CI-976-treated cells. Triplicate determination was measured at each experiment. Values are mean ± S.D. for three experiments. *P < .05, **P < .01.

004 was about twice that from CI-976 at the concentration of 10^{-7} M (fig. 3B). Chemical form of radioactivity in media with HDL (fig. 3B) was free cholesterol (~80%) and cholesterol ester (~20%). Cellular free cholesterol mass was measured but there is no significant difference at each concentration of each ACAT inhibitor. However, the ratio of free \[^{3}H\]cholesterol to total \[^{3}H\]cholesterol in THP-1 cells treated with HL-004 and CI-976 was 44% and 32%, respectively. Figure 4 shows that HL-004 did not change the acid and neutral cholesterol esterase activities at the concentration of 10^{-9} ~ 10^{-7} M. CI-976 decreased both enzyme activities at 10^{-7} M. Then, we suggested that HL-004 is more efficient for cholesterol transport.

Effect of HL-004 on cholesterol metabolism of foam cells. Next, we examined the effects of HL-004 on the cholesterol metabolism of foam cells. Figure 5A shows the effect of HL-004 and CI-976 on the synthesis of cholesterol \[^{14}C\]oleate in foam cells. Synthesis of cholesterol \[^{14}C\]oleate of control cells was in the range of 3.7 ~ 5.7 nmol/mg protein. HL-004 decreased the synthesis in a concentration-dependent manner, the inhibition being 83% at 10^{-7} M. Although CI-976 decreased the synthesis in a concentration-dependent manner, the inhibition was much lower than that of HL-004, being 21% at 10^{-7} M. Figure 5B shows the effect of HL-004 and CI-976 on the release of \[^{3}H\]adenine. The release of HL-004-treated groups was almost the same as CI-976-treated groups and was not different from that of nontreated group. These data on HL-004 were quite similar to those in Materials and Methods. •, HL-004-treated cells; ○, CI-976-treated cells. Values are mean ± S.D. for three experiments. Triplicate determination was measured at each experiment. Data are expressed as mean ± SD values obtained from 3 animals. *P < .05, **P < .01.

Effect of HL-004 on cholesterol metabolism of foam cells. Acid and neutral cholesterol esterase activity are measured as described in Materials and Methods. A, Acid cholesterol esterase. B, Neutral cholesterol esterase. •, HL-004-treated cells; ○, CI-976-treated cells. Triplicate determination was measured at each experiment. Values are mean ± S.D. for three experiments. *P < .05, **P < .01. (Asterisks beside circles, compared with nontreated groups.)

Fig. 4. Effect of concentration of HL-004 and CI-976 on acid and neutral cholesterol esterase activities. Acid and neutral cholesterol esterase activities were measured as described in Materials and Methods. A, Acid cholesterol esterase. B, Neutral cholesterol esterase. •, HL-004-treated cells; ○, CI-976-treated cells. Triplicate determination was measured at each experiment. Values are mean ± S.D. for three experiments. *P < .05, **P < .01. (Asterisks beside circles, compared with nontreated groups.)

Fig. 5. Effect of concentration of HL-004 and CI-976 on cholesterol \[^{14}C\]oleate synthesis in atherosclerotic lesion cells and release of \[^{3}H\]adenine. A, Atherosclerotic lesion cells (5 × 10^6 cells/well) were preincubated with HL-004 or CI-976 at indicated concentrations for 24 hr. The medium was changed and the cells were treated with βVLDL (100 μg cholesterol/ml) and \[^{14}C\]oleate (37 MBq/well), and again with HL-004 or CI-976 for 8 hr. Incorporation of \[^{14}C\]oleate into cellular cholesterol \[^{14}C\]oleate was measured as described in Materials and Methods. 100% Value was 118.2 nmol/mg cell protein. B, Release of \[^{3}H\]adenine; Release of \[^{3}H\]adenine from TPA-treated THP-1 cells (5 × 10^6 cells/well) treated with HL-004 or CI-976 was measured as described in Materials and Methods. •, HL-004-treated cells; ○, CI-976-treated cells. Values are mean ± S.D. for three experiments. Triplicate determination was measured at each experiment. Data are expressed as mean ± SD values obtained from 3 animals. *P < .05, **P < .01. (Asterisks beside circles, compared with nontreated groups.)
Results

Groups: Released radioactivity from the HL-004-treated groups was significantly higher than that from the control groups. Released radioactivity from foam cells treated with $10^{-9}$ M HL-004 was most effective. In addition, HL-004 did not influence the total incorporation of [3H]cholesterol oleate-βVLDL into cells in either of the experiments (fig. 6), suggesting that the difference in the release of cholesterol is not due to the different incorporation of βVLDL.

These data suggested that the release of free cholesterol from foam cells could be accelerated by the inhibition of ACAT activity by HL-004 and that there might be two pathways for the release of free cholesterol from the cells: one is dependent on acid cholesterol esterase and the other on neutral cholesterol esterase.

Finally, we measured the cholesterol ester content in foam cells after incubation with βVLDL, and it was observed to increase significantly. However, by preincubation with HL-004 followed by incubation with βVLDL, the content in cells decreased concentration-dependently and that in treated with $10^{-7}$ M HL-004 was almost the same as that in control cells (fig. 9).

Discussion

The concentration-dependent effect of HL-004 on rabbit foam cells was almost the same as that on THP-1 derived macrophages (figs. 1 and 3). It was also similar to that on rat macrophages (Murakami et al., 1995b) and smooth muscle cells (Murakami et al., 1995a). These data indicate that the inhibitory effect on ACAT by HL-004 is universal regardless of cell type and origin. From our results, the difference of HL-004 and other ACAT inhibitor (CI-976 in this report) is as follows: (1) the effective concentration of HL-004 was low ($10^{-9} \sim 10^{-7}$ M), (2) the cell toxicity of HL-004 was low and (3) the effect of HL-004 on incorporation of βVLDL, acid cholesterol esterase and neutral cholesterol esterase was very little, whereas CI-976 affected these metabolisms. These data suggested that HL-004 was a unique ACAT inhibitor compared with other ACAT inhibitors.
HL-004 was effective on macrophages and foam cells at a very low concentration (10^{-9} \text{ to } 10^{-7} M), apparently without any cell toxicity (figs. 1B and 5B). Orally administered ACAT inhibitors generally inhibit ACAT activity of various organs such as the liver and intestine, but the side effect of adrenocortical cytotoxicity has been reported (Dominick et al., 1993). Then, the question arises why HL-004 is free from toxicity. HL-004 is a noncompetitive inhibitor against oleoyl-CoA and its specificity for ACAT is very high because it does not influence the synthesis of phospholipids and triglycerides. If this ACAT inhibitor acts on only ACAT and free cholesterol is transported out of cells, cell toxicity would not occur. To find the differences between both HL-004 and CI-976, the stable structures were obtained by a semiempirical molecular orbital method with P3 approximation (Stewart, 1989a, 1989b) in figure 10. For HL-004, the structure of molecule at grand minimized potential energy was a linear conformation. Phenyl ring and peptide bond might be conjugated but area of delocalization might be smaller compared with that of CI-976. When “S” in HL-004 was changed to “C” and the minimum energy was calculated, the energy of a turned conformation was 1.9 kcal/mol low compared with the energy of a linear conformation. Thus, a presence of “S” may be important for a linear conformation. For CI-976, the structure was folded between peptide bond and alkyl chain. Conjugation of phenyl ring, methoxy group and peptide bond would raise up delocalization of electrons, and then the molecule might be stable. To clear this reason, we calculated the potential energy of grand minimum between a linear conformation and a turned conformation of CI-976. The energy of the turned conformation was 2.1 kcal/mol low. Therefore, the turned conformation was stable compared with a linear conformation. These results suggested that the unique structure of HL-004 indicates that this inhibitor specifically acts on ACAT as a noncompetitive inhibitor and does not affect other proteins related to the free cholesterol transport system.

Cholesterol efflux was very low compared with that in figures 7 and 8 without HDL (data not shown). This suggested that HL-004 enhanced the HDL-mediated cholesterol efflux from atherosclerotic lesion cells. It is reported that ACAT inhibitors enhanced HDL-mediated cholesterol efflux from macrophages (Furuchi et al., 1993; Schnitz et al., 1988, 1985). In the experiments of figures 7 and 8, a sufficient level of HDL (0.3 mg protein/ml) was added to the medium. In a similar experiment using 10% FBS/DMEM, the total release of cholesterol was low (the released cholesterol was up to \sim 30% of the total incorporated cholesterol) and there was no statistical difference between control and HL-004-treated foam cells (data not shown). Both cholesterol-releasing pathways from lysosome and cytosol required HDL (figs. 7 and 8). It was recently reported that free cholesterol produced by the addition of ACAT inhibitor induced cell toxicity (Warner et al., 1995). This points out the need for sufficient acceptors for the released cholesterol, such as HDL, to avoid such toxicity by ACAT inhibitors.

Clinically, it is speculated that plaque rupture caused by foam cells containing a lot of cholesterol ester is a trigger of acute coronary syndrome (Davies et al., 1985; Hackett et al., 1988). Our studies showed that HL-004 was effective at low concentrations (10^{-9} \text{ to } 10^{-7}) and might decrease foam cell formation without toxicity. Considering these findings, it seems reasonable to expect that acute coronary syndrome (acute coronary thrombosis, and so on) can be reduced by the use of HL-004 together with a sufficient presence of HDL.

Acknowledgments

The computations were carried out by the DRIA System at the Faculty of Pharmaceutical Sciences, Chiba University.

References


Send reprint requests to: Dr. Itsuko Ishii, Faculty of Pharmaceutical Sciences, Chiba University, Inage-ku 1–33, Yayoi-cho, Chiba, 263-0022, Japan.