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# EFFECT OF SIMVASTATIN (MK-733) ON THE REGULATION OF CHOLESTEROL SYNTHESIS IN Hep G2 CELLS

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**Abstract**—A new antihypercholesterolemic drug, simvastatin (MK-733), which is a prodrug of a potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, inhibited cholesterol synthesis from [$^{14}$C]acetate concentration dependently without inhibiting it from [$^{3}$H]mevalonate in Hep G2 cells. Therefore, MK-733 is thought to be converted to L-654,969, the active $\beta$-hydroxy acid form of MK-733 in the cells and/or medium. MK-733 inhibited cholesterol ester synthesis, but did not affect phospholipid, free fatty acid and triacylglycerol synthesis. This compound increased HMG-CoA reductase activity concentration dependently and raised the specific binding, internalization and degradation of $^{125}$I-labeled low density lipoprotein by Hep G2 cells. Another HMG-CoA reductase inhibitor, pravastatin (CS-514), also behaved like MK-733. However, its potency was far less than that of MK-733.

Simvastatin (MK-733) [1], a chemical derivative of lovastatin (MK-803) [2], is a prodrug of a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34). HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway. The active $\beta$-hydroxy acid form of MK-733, L-654,969 [1], inhibits HMG-CoA reductase with an $IC_{50}$ of $0.94 \times 10^{-9}$ M. MK-733 inhibits *in vitro* cholesterol synthesis from [$^{14}$C]acetate in different cell lines, and reduces the serum cholesterol levels in several animal species and in humans [3–5, †]. Recently, Tsujita *et al.* [6] reported that pravastatin (CS-514), the urinary metabolite of compactin (ML-236B) [7], inhibits HMG-CoA reductase competitively with a $K_i$ value of $2.3 \times 10^{-9}$ M.

Animal cells in tissue culture require cholesterol for the synthesis of plasma membranes. Although the cells can synthesize cholesterol from acetyl CoA, they do so only at a low rate. They derive cholesterol in the form of lipoprotein from the serum in culture medium. To obtain cholesterol in the form of lipoprotein, the cells bind and internalize the lipoprotein in a series of steps. Brown and Goldstein [8] proposed a low density lipoprotein (LDL) receptor theory from the study of familial hypercholesterolemia (FH). Genetic defects in LDL receptors have been reported to induce FH [8]. Bilheimer *et al.* [9] reported that the HMG-CoA reductase inhibitor lovastatin [2] could increase the fractional catabolic rate of LDL in patients with heterozygous FH, which may indicate an increase in the number of LDL receptors [7]. LDL receptors are thought to play an important role in the regulation of serum cholesterol level. However, it is difficult to examine LDL receptor activity in humans. Therefore, LDL receptor activity has been examined using cultured cells. Much information about the metabolism of lipid has been gained from studies on cultured hepatocytes derived from a variety of animals [10]. Recently, a human hepatoma cell line Hep G2 was used to investigate lipid and lipoprotein metabolism [11–13]. Havekes *et al.* [12] reported that Hep G2 cells bind, internalize and degrade human LDL with a high-affinity saturable component and with a low-affinity non-saturable component, and concluded that the Hep G2 possesses specific LDL receptors similar to the LDL receptor demonstrated on extrahepatic tissue cells. Cohen *et al.* [13] demonstrated that compactin decreases cholesterol synthesis in intact Hep G2 cells, and increases the LDL receptor activity. The Hep G2 cell line is thought to be a suitable model to investigate the effect of cholesterol lowering agents on lipid metabolism in human liver.

In the present study, the effects of MK-733 on lipid synthesis, HMG-CoA reductase activity and LDL receptor activity were examined using Hep G2 cells. Pravastatin (CS-514) [6], 25-hydroxycholesterol [14] and progesterone [15] were used as the reference drugs for another HMG-CoA reductase inhibitor, oxysterol and acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitor respectively.

## MATERIALS AND METHODS

*Materials.* MK-733, L-654,969 and CS-514 were prepared in the Merck Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). 25-Hydroxycholesterol and progesterone were purchased from Sigma (St. Louis, MO, U.S.A.). All compounds tested were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.5% (v/v). Under these conditions, DMSO had no

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† Alberts AW, Chen J, Huff J, Hunt V and Kuron G, Comparative studies on the hydroxymethylglutaryl coenzyme A reductase inhibitors mevinolin, MK-733, and CS-514. In: *Ninth International Symposium of Drugs Affecting Lipid Metabolism*, Florence, 22–25 October 1986, p. 8.

significant effect on lipid synthesis and LDL receptor activity. [1-$^{14}$C]Acetic acid sodium salt (54 mCi/mmol), [9,10(n)-$^{3}$H]oleic acid (5 mCi/mmol) and 3-hydroxy-3-methyl[3-$^{14}$C]glutaryl coenzyme A (HMG-CoA, 52 mCi/mmol) were obtained from Amersham International plc (Buckinghamshire, U.K.). *RS*-[5-$^{3}$H(N)]Mevalonolactone (38.8 Ci/mmol) and Na[$^{125}$I] (carrier free, over 17 Ci/mg) were purchased from New England Nuclear (Boston, MA, U.S.A.). Human lipoprotein-deficient serum (LPDS) was prepared as described by Brown *et al.* [16]. All other chemicals used were standard commercial high purity materials.

*Cell cultures.* The established Hep G2 cell line, derived from human hepatoma, was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cell stocks were grown in 80 cm$^2$ flasks containing medium A [Eagle's modified minimum essential medium (MEM, Flow Laboratories, McLean, VA, U.S.A.) supplemented with penicillin G (100 units/mL) and streptomycin (100 μg/mL)] with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and incubated in a humidified incubator (5% $CO_2$) at 37°.

*Lipid synthesis.* Lipid synthesis from [$^{14}$C]acetate or [$^{3}$H]mevalonate was examined according to the method of Brown *et al.* [16] with some modifications. On day 0, $3 \times 10^5$ cells were seeded in 10 cm$^2$ petri dishes containing medium A with 10% FBS. On day 3 or 4, the medium was exchanged for fresh medium. On day 6, the medium was replaced with medium A with 10% LPDS. On day 7, the cells were preincubated with each inhibitor in fresh medium A containing 10% LPDS for the indicated time and then labeled with 1 mM [$^{14}$C]acetate or 50 μM [$^{3}$H]mevalonolactone for 2 hr. At the end of the incubation, petri dishes were cooled in a cold bath, and the medium was aspirated. The cells were washed with cold phosphate-buffered saline (PBS) three times. For the determination of cholesterol synthesis, the cells were dissolved in aqueous 15% KOH. After taking an aliquot for protein determination, lipids in the cell lysate were saponified with 15% KOH in 59% ethanol for 1 hr at 75°. After addition of water, the non-saponifiable lipids were extracted with petroleum ether twice, and the solvent was evaporated under a nitrogen stream. [$^{14}$C] or [$^{3}$H]Cholesterol was isolated on a silica gel G plate (Art 5583, E. Merck, Darmstadt, F.R.G.) using hexane/diethyl ether/acetic acid (85:15:4, by vol.) as a solvent system. The band corresponding to authentic cholesterol was scraped into toluene based liquid scintillation fluid. The radioactivity was counted by a liquid scintillation counter (TRI-CARB 2000CA, Packard Instrument Co., IL, U.S.A.). For the determination of various lipid classes, the cells were harvested using a rubber policeman, and disrupted by ultrasonication (Sonifier 185, Branson Sonic Power Co., Danbury, CT, U.S.A.). Lipids in the homogenate were extracted by the method of Folch *et al.* [17]. Phospholipid, free cholesterol, free fatty acid, triacylglycerol and esterified cholesterol were separated on a silica gel G plate using hexane/diethyl ether/acetic acid (85:15:1, by vol.) as a solvent system. The plate was developed twice to give complete separation of lipids. Lipids on thin-layer chromatography (TLC) were visualized by exposure to $I_2$ vapor. The distribution of radioactivity on TLC was also detected using a radiochromatoscanner (LB-282, Berthold, Wildbad, F.R.G.), and the radioactivity in the area corresponding to authentic standard was counted in a liquid scintillation counter.

*Esterification of cholesterol from* [$^{3}$H]*oleate.* Incorporation of [$^{3}$H]oleate into cholesteryl [$^{3}$H]oleate was examined according to the method of Brown *et al.* [16]. Hep G2 cells were incubated at 37° in medium A containing 10% LPDS. After a 5-hr preincubation with or without inhibitor, the cells were labeled with 0.1 mM [$^{3}$H]oleate bound to albumin [18] for 1 hr. The cells were then washed with cold PBS and harvested. Lipid in the cells was extracted with chloroform/methanol (2:1, v/v) [17]. The cholesteryl [$^{3}$H]oleate was isolated on a silica gel G plate using hexane/diethyl ether/acetic acid (85:15:4, by vol.) as a solvent system, and the radioactivity in the spot corresponding to authentic cholesteryl oleate was counted [18]. Esterification activity is expressed as picomoles of cholesteryl [$^{3}$H]oleate formed per milligram of cell protein per hour.

*Determination of HMG-CoA reductase activity.* Hep G2 cells were incubated with each inhibitor for 18 hr in medium A containing 10% LPDS. After the incubation, the cells were washed twice with medium A and incubated in medium A for another 15 min at 37° to remove intracellular inhibitor. The cells were washed three times with cold PBS and scraped with a rubber policeman. After centrifugation (1000 *g*, 5 min at 4°), the supernatant fraction was removed by aspiration. The cell pellet was frozen and kept at −80° until used. Cell extract was prepared by suspending the thawed pellet in 200 μL of buffer containing 0.1 M potassium phosphate (pH 7.5)/5 mM EDTA/0.2 M KCl/0.25% Brij 96. After sonication for 2 sec at 0°, the cell homogenate was incubated for 10 min at 37°, and then centrifuged at 12,000 *g* for 15 min at 4°. An aliquot of the supernatant fraction was used for the determination of enzyme activity. HMG-CoA reductase activity was determined according to the method described by Brown *et al.* [19] with some modifications. The reductase activity was determined as follows: Aliquots of cell extract (1–50 μg protein) were incubated for 1 hr at 37° in a final volume of 100 μL containing 0.1 M potassium phosphate (pH 7.4)/20 mM glucose-6-phosphate/2.5 mM $NADP^+$/1.0 unit of glucose-6-phosphate dehydrogenase/4 mM dithiothreitol/30 μM [$^{14}$C]HMG-CoA (0.023 μCi). The enzyme reaction was terminated by addition of 20 μL of 2 M HCl. After standing for 15 min at 37° for lactonization of mevalonic acid, [$^{14}$C]mevalonolactone was separated from [$^{14}$C]HMG-CoA on a small column packed with ion exchange resin (AG 1-X8, formate form, Bio-Rad) [20]. Enzyme activity is expressed in picomoles of mevalonic acid formed per milligram of cell protein per minute. The enzyme activity was determined under conditions in which the inhibitory effect of L-654,969 (and CS-514) was overcome by dilution.

*Preparation of* $^{125}$*I-LDL.* Human LDL (d: 1.020–1.063) was isolated from freshly prepared plasma by preparative ultracentrifugation [21]. The LDL fraction was used immediately for $^{125}$I iodination by

the iodine monochloride method as described by Fielding *et al.* [22].

*Binding, internalization and degradation of* $^{125}$I-*LDL.* The LDL receptor assay was performed according to the method of Havekes *et al.* [12] with some modifications. At 18 hr before the experiment, the medium of the cell culture (200–300 μg of cell protein/dish) grown in multiwell dishes (22 mm diameter, Corning, NY, U.S.A.) was replaced with medium A containing 10% LPDS, and each drug was added. The experiment was started by the addition of 10 μg/mL $^{125}$I-LDL (110 cpm/ng) in the presence or absence of 300 μg/mL of unlabeled LDL. After a 3-hr incubation at 37°, the medium was removed for the determination of LDL degradation. The LDL degradation was determined as non-iodine trichloroacetic acid-soluble radioactivity in the medium. The radiolabeled free iodine was removed from a portion of the 10% (w/v) trichloroacetic acid-soluble fraction by extraction with chloroform after addition of an excess of unlabeled KI and $H_2O_2$ as described by Dashti *et al.* [23]. After removal of the incubation medium, each well was washed exactly as described by Goldstein *et al.* [24] and incubated with dextran sulfate releasable buffer [4 mg/mL dextran sulfate/50 mM NaCl/10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.4)] for 1 hr at 4° [25]. The radioactivity released into the buffer reflects the amount of $^{125}$I-LDL bound to the LDL receptor. Internalized LDL was then determined by dissolving the cells in 1 M NaOH. The specific binding, internalization and degradation of $^{125}$I-LDL were calculated by subtracting the amount of $^{125}$I-LDL in the presence of unlabeled LDL from the amount of $^{125}$I-LDL in the absence of unlabeled LDL.

*Determination of protein.* The protein concentrations were determined according to the method of Lowry *et al.* [26] using bovine serum albumin as a standard.

## RESULTS

*Cholesterol synthesis.* Cholesterol synthesis from [$^{14}$C]acetate and [$^{3}$H]mevalonate was linear up to at least 6 hr of incubation in Hep G2 cells (data not shown). As shown in Fig. 1, MK-733 and L-654,969 inhibited cholesterol synthesis from [$^{14}$C]acetate concentration dependently. These results show that MK-733 is converted to L-654,969 in the cells and/or medium. Another HMG-CoA reductase inhibitor, CS-514, inhibited cholesterol synthesis 40 times more weakly than MK-733 and 100 times than L-654,969. 25-Hydroxycholesterol, which is thought to reduce HMG-CoA reductase activity [14], and progesterone also inhibited cholesterol synthesis from [$^{14}$C]acetate (Table 1). Prolonged preincubation (18 hr) decreased the inhibitory activity of HMG-CoA reductase inhibitors and slightly increased that of 25-hydroxycholesterol (Table 1). The $IC_{50}$ values of L-654,969, MK-733, CS-514, and 25-hydroxycholesterol in cholesterol synthesis were $6.6 \times 10^{-9}$, $1.7 \times 10^{-8}$, $6.8 \times 10^{-7}$, and $1.6 \times 10^{-6}$ M respectively (Table 1). Furthermore, progesterone inhibited cholesterol synthesis with an $IC_{50}$ of $1.5 \times 10^{-5}$ M.

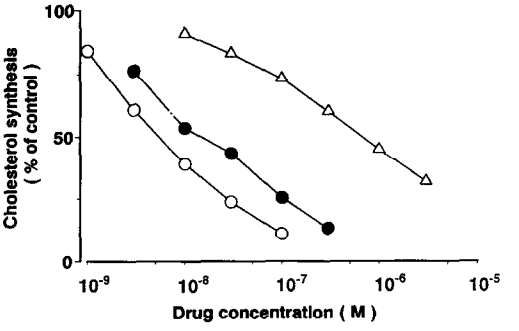


Fig. 1. Effects of HMG-CoA reductase inhibitors on [$^{14}$C]cholesterol synthesis from [$^{14}$C]acetate in Hep G2 cells. Hep G2 cells (on day 7) were preincubated with the indicated concentration of MK-733 (●), L-654,969 (○), and CS-514 (△) for 1 hr, and then 1 μCi/mL of [$^{14}$C]acetate was added (final concentration: 1 mM). After 2 hr of incubation, [$^{14}$C]cholesterol was extracted, separated and quantified as described in Materials and Methods. The control value (100%) was 7400 dpm/mg cell protein/hr. Each value is the mean of duplicate determinations.

Cholesterol synthesis from [$^{3}$H]mevalonate was examined in Hep G2 cells (Table 2). MK-733 and CS-514 did not inhibit cholesterol synthesis from [$^{3}$H]mevalonate except at an extremely high concentration. 25-Hydroxycholesterol also did not inhibit it. On the other hand, progesterone inhibited

Table 1. Effects of HMG-CoA reductase inhibitors, oxysterol and progesterone on [$^{14}$C]cholesterol synthesis from [$^{14}$C]acetate in Hep G2 cells

| Compounds | $IC_{50}$ (M) | |
|---|---|---|
| | 1-hr Preincubation | 18-hr Preincubation |
| MK-733 | $1.7 \times 10^{-8}$ | $1.5 \times 10^{-7}$ |
| L-654,969 | $6.6 \times 10^{-9}$ | $8.0 \times 10^{-8}$ |
| CS-514 | $6.8 \times 10^{-7}$ | $>10^{-5}$ |
| 25-Hydroxycholesterol | $1.6 \times 10^{-6}$ | $7.0 \times 10^{-7}$ |
| Progesterone | $1.5 \times 10^{-5}$ | * |

Hep G2 cells (on day 7) were preincubated with inhibitor for 1 or 18 hr, and then 1 μCi/mL of [$^{14}$C]acetate was added (final concentration: 1 mM). After a 2-hr incubation, [$^{14}$C]cholesterol was extracted, separated and quantified as described in Materials and Methods.

* Not tested.

Table 2. Effects of HMG-CoA reductase inhibitors, oxysterol and progesterone on [$^3$H]cholesterol synthesis from [$^3$H]mevalonate in Hep G2 cells

| Concn (M) | [$^3$H]Mevalonolactone → [$^3$H]Cholesterol (dpm/mg/hr) | | | | |
|---|---|---|---|---|---|
| | MK-733 | L-654,969 | CS-514 | 25-Hydroxycholesterol | Progesterone |
| Control | 3210 (100%) | 3210 | 3210 | 3210 | 3210 |
| $3 \times 10^{-8}$ | 3530 (110%) | 3380 (105%) | 3700 (115%) | * | * |
| $3 \times 10^{-7}$ | 2900 (90%) | 2880 (90%) | 3740 (117%) | 3270 (102%) | * |
| $3 \times 10^{-6}$ | 2560 (80%) | 2260 (70%) | 3480 (108%) | 2970 (93%) | 3200 (100%) |
| $3 \times 10^{-5}$ | * | * | 2810 (87%) | 2820 (88%) | 928 (29%) |

Hep G2 cells (on day 7) were preincubated with inhibitor for 1 hr, and then 2 $\mu$Ci/mL of [$^3$H]mevalonolactone was added (final concentration: 50 $\mu$M). After a 2-hr incubation, [$^3$H]cholesterol was extracted, separated and quantified as described in Materials and Methods. Each value is the mean of duplicate determinations. Numbers in parentheses indicate percent of control value.

* Not tested.

Table 3. Effects of HMG-CoA reductase inhibitors, oxysterol and progesterone on [$^{14}$C]lipid synthesis from [$^{14}$C]acetate in Hep G2 cells

| Compounds | Concn (M) | [$^{14}$C]Acetate → [$^{14}$C]Lipid (dpm/mg/hr) | | | | |
|---|---|---|---|---|---|---|
| | | FC | EC | PL | FFA | TG |
| Control | 0 | 24,700 | 1,590 | 16,800 | 2,780 | 30,300 |
| MK-733 | $2 \times 10^{-5}$ | 423 | 99 | 19,700 | 2,930 | 35,700 |
| CS-514 | $2 \times 10^{-5}$ | 4,120 | 148 | 19,900 | 3,250 | 35,700 |
| | $2 \times 10^{-4}$ | 1,150 | 113 | 21,600 | 3,380 | 37,600 |
| 25-Hydroxycholesterol | $3 \times 10^{-5}$ | 3,870 | 3,120 | 20,100 | 3,140 | 33,300 |
| Progesterone | $5 \times 10^{-5}$ | 1,690 | 65 | 3,350 | 492 | 3,280 |
| | $2 \times 10^{-4}$ | 568 | 58 | 965 | 232 | 941 |

Hep G2 cells (on day 7) were preincubated with inhibitor for 1 hr, and then 1 $\mu$Ci/mL of [$^{14}$C]acetate was added (final concentration: 1 mM). After a 2-hr incubation, free cholesterol (FC), cholesterol ester (EC), phospholipid (PL), free fatty acid (FFA) and triacylglycerol (TG) were extracted, separated and quantified as described in Materials and Methods. Each value is the mean of duplicate determinations.

Table 4. Effects of HMG-CoA reductase inhibitors, oxysterol and progesterone on cholesterol ester synthesis in Hep G2 cells

| Compounds | Concn (M) | [$^3$H]Oleate → Cholesteryl [$^3$H]oleate (pmol/mg/hr) | |
|---|---|---|---|
| | | −LDL | +LDL* |
| Control | 0 | 500 (100%) | 883 (100%) |
| MK-733 | $2 \times 10^{-7}$ | 94 (19%) | 567 (64%) |
| | $2 \times 10^{-5}$ | 71 (14%) | 345 (39%) |
| CS-514 | $2 \times 10^{-7}$ | 340 (68%) | 874 (99%) |
| | $2 \times 10^{-5}$ | 84 (17%) | 516 (58%) |
| 25-Hydroxycholesterol | $3 \times 10^{-5}$ | 987 (197%) | 1434 (162%) |
| Progesterone | $5 \times 10^{-5}$ | 62 (12%) | 111 (13%) |

Hep G2 cells (on day 7) were preincubated with inhibitor in the presence or absence of LDL for 5 hr. Then 2 $\mu$Ci/mL of [$^3$H]oleate-albumin (final concentration: 0.1 mM) was added. After a 1-hr incubation, cholesteryl [$^3$H]oleate was extracted, separated and quantified as described in Materials and Methods. Each value is the mean of duplicate determinations. Numbers in parentheses indicate percent of control value.

* +LDL: 30 $\mu$g protein/mL of LDL was added.

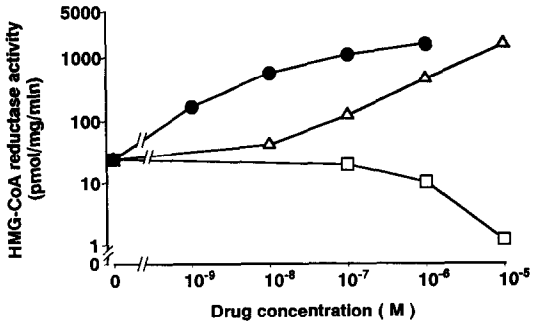


Fig. 2. Effects of HMG-CoA reductase inhibitors and oxysterol on HMG-CoA reductase activity in Hep G2 cells. Hep G2 cells (1 to 1.5 mg cell protein) were incubated for 18 hr in medium A containing 10% lipoprotein-deficient serum (LPDS) with the indicated concentration of MK-733 (●), CS-514 (△) and 25-hydroxycholesterol (□). HMG-CoA reductase activity in the cell extract was determined as described in Materials and Methods. Each value is the mean of duplicate determinations.

cholesterol synthesis potently. Progesterone has been reported not to inhibit cholesterol synthesis at the site of HMG-CoA reductase [27].

*Lipid synthesis.* As shown in Table 3, MK-733 strikingly reduced cholesterol and cholesterol ester synthesis, but did not show any suppressive effect on the synthesis of phospholipid, free fatty acid and triacylglycerol. CS-514 also reduced cholesterol and cholesterol ester synthesis without affecting other parameters. 25-Hydroxycholesterol reduced cholesterol synthesis but increased cholesterol ester synthesis. It did not affect other lipid synthesis. Progesterone strongly inhibited all of them. Incubation for 3 hr with progesterone ($2 \times 10^{-4}$ M) did not affect cell viability (data not shown).

*Esterification of cholesterol from* [$^3$H]*oleate.* [$^3$H]Oleate was incorporated into cholesteryl [$^3$H]oleate in a linear fashion after up to 3 hr incubation in the absence and presence of LDL (data not shown). As shown in Table 4, LDL increased the incorporation of [$^3$H]oleate into cholesteryl ester by 1.8 times. MK-733 decreased the incorporation of

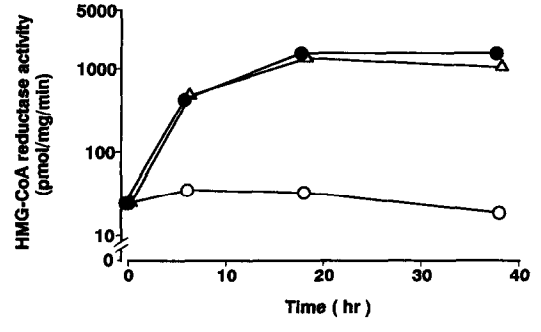


Fig. 3. Increase in HMG-CoA reductase activity in Hep G2 cells incubated with HMG-CoA reductase inhibitors. Hep G2 cells (1 to 1.5 mg cell protein) were incubated for the indicated time in medium A containing 10% LPDS with no drug (○), $10^{-6}$ M MK-733 (●) and $10^{-5}$ M CS-514 (△). HMG-CoA reductase activity in the cell extract was determined as described in Materials and Methods. Each value is the mean of duplicate determinations.

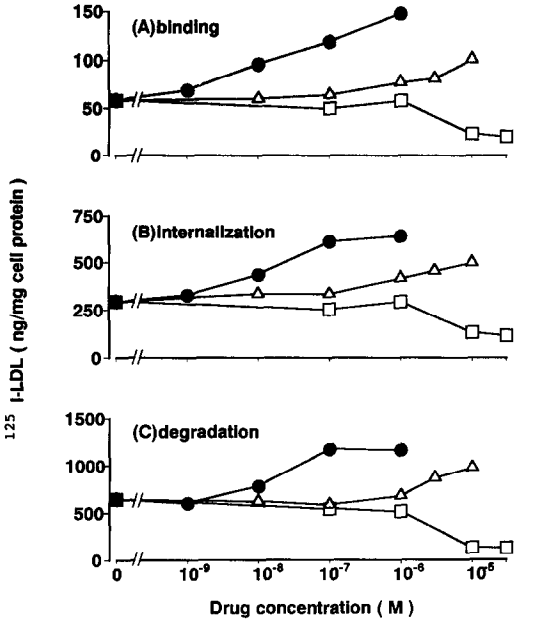


Fig. 4. Effects of HMG-CoA reductase inhibitors and oxysterol on the (A) binding, (B) internalization, and (C) degradation of $^{125}$I-LDL by Hep G2 cells. At 18 hr before the assay, Hep G2 cells (200–300 μg cell protein) were preincubated in medium A containing 10% LPDS with the indicated concentration of MK-733 (●), CS-514 (△) and 25-hydroxycholesterol (□). $^{125}$I-LDL (10 μg/mL) was added to Hep G2 cells in the absence or presence of excess unlabeled LDL (300 μg/mL). After incubation at 37° for 3 hr, the amounts of high-affinity binding, internalization and degradation of $^{125}$I-LDL were determined as described in Materials and Methods. Each value is the mean of duplicate determinations.

[$^3$H]oleate into cholesteryl [$^3$H]oleate remarkably, due to the inhibition of cholesterol synthesis. The inhibitory effect of MK-733 was less potent in the presence of LDL than in its absence. CS-514 inhibited cholesteryl ester synthesis more weakly than MK-733, reflecting its lower potency in inhibiting cholesterol synthesis. On the other hand, progesterone, which is known to inhibit ACAT activity [15], suppressed cholesteryl ester synthesis in both the presence and absence of LDL. 25-Hydroxycholesterol stimulated cholesteryl ester synthesis in the presence and absence of LDL.

*Induction of HMG-CoA reductase.* Hep G2 cells were incubated in the presence of different concentration of inhibitors for 18 hr. As shown in Fig. 2, MK-733 and CS-514 increased HMG-CoA reductase activity in Hep G2 cells concentration dependently. On the other hand, 25-hydroxycholesterol decreased HMG-CoA reductase activity. 25-Hydroxycholesterol ($1 \times 10^{-5}$ M) decreased the activity to a negligible level (Fig. 2). HMG-CoA reductase inhibitors increased the reductase activity time dependently (Fig. 3).

*LDL receptor activity.* Hep G2 cells bound, internalized and degraded LDL. Pretreatment of the cells with MK-733 for 18 hr increased the specific surface binding, internalization and degradation of $^{125}$I-LDL (Fig. 4). CS-514 also increased LDL receptor

Table 5. Effects of LDL, oxysterol and protein inhibitors on the increase of $^{125}$I-LDL binding to Hep G2 cells by L-654,969

| Drug | $^{125}$I-LDL specific binding (ng LDL/mg cell protein) | |
|---|---|---|
| | Control | L-654,969 (1 μM) |
| No addition | 36.3 | 53.2 |
| LDL (200 μg/mL) | 16.6 | 9.0 |
| 25-Hydroxycholesterol (10 μM) | 6.9 | 3.6 |
| Cycloheximide (100 μM) | 30.3 | 26.6 |
| Actinomycin D (15 μg/mL) | 20.4 | 13.1 |

Hep G2 cells were incubated with each drug in the presence or absence of L-654,969 for 18 hr. After the cells were washed with PBS, LDL receptor binding activity was determined as described in Materials and Methods. Each value is the mean of duplicate determinations.

activity. However, the effect of CS-514 on receptor activity was found to be much less potent than that of MK-733. These inhibitors increased maximum binding, but did not affect the apparent dissociation constant ($K_d = 2 \times 10^{-8}$ M). 25-Hydroxycholesterol decreased the binding, internalization and degradation of $^{125}$I-LDL. LDL and 25-hydroxycholesterol inhibited the increase in LDL receptor activity by HMG-CoA reductase inhibitor (Table 5). Treatment with cycloheximide and actinomycin D also suppressed the induction of LDL receptors (Table 5). These results suggest that induction of LDL receptor activity by HMG-CoA reductase inhibitor requires RNA and protein synthesis.

## DISCUSSION

Hepatocytes synthesize lipids such as sterol, triacylglycerol and phospholipid. The synthesis and secretion of hepatic lipids and lipoproteins are regulated exquisitely by a considerable number of factors (hormones, nutrients, metabolites, etc.). Hepatocytes also take up the lipids carried by lipoproteins. However, there have been few studies to investigate lipid synthesis and metabolism using human cells. Cohen *et al.* [13] reported that a human hepatoma cell line, Hep G2, was suitable to examine lipid metabolism. Therefore, the effects of HMG-CoA reductase inhibitors on cholesterol metabolism were examined using Hep G2 cells.

MK-733 and L-654,969 inhibited cholesterol synthesis from [$^{14}$C]acetate in Hep G2 cells concentration dependently without inhibiting cholesterol synthesis from [$^{3}$H]mevalonate. These results demonstrated that MK-733 and L-654,969 act as specific HMG-CoA reductase inhibitors in Hep G2 cells. MK-733 is a prodrug for the HMG-CoA reductase inhibitor L-654,969 [1]. MK-733 is thought to be converted easily to L-654,969, in the cells and/or medium, because MK-733 inhibited cholesterol synthesis as potently as L-654,969. CS-514 has been reported to inhibit HMG-CoA reductase activity with a potency about 1/9 that of L-654,969.* However, CS-514 inhibited cholesterol synthesis from [$^{14}$C]acetate about 1/40 to 1/100 as potently as MK-733 and L-654,969 in Hep G2 cells (Table 1). CS-514 has been reported to inhibit cholesterol synthesis in liver cells more potently than in extrahepatic tissue cells [6]. Tsujita *et al.* [6] examined cholesterol synthesis in suspended rat hepatocytes isolated by collagenase treatment. The use of collagenase for cell dispersion has made it possible to obtain functional isolated cells. However, it has been shown recently that freshly isolated hepatocytes have greatly impaired functions and that the cells recover their functions during primary culture [28]. Collagenase treatment may damage cells, increase membrane permeability to drugs, and potentiate the inhibitory activity of CS-514 in suspended liver cells.

25-Hydroxycholesterol did not inhibit cholesterol synthesis from [$^{3}$H]mevalonate. These results suggest that 25-hydroxycholesterol inhibits cholesterol synthesis at the level of HMG-CoA reductase. Compactin at a concentration of 0.5 μg/mL, which strongly inhibits the incorporation of [$^{14}$C]acetate into cholesterol, has been reported to inhibit cholesterol synthesis from radiolabeled mevalonate by 50% in L-cells [29]. MK-733, L-654,969 and CS-514, however, did not inhibit cholesterol synthesis from [$^{3}$H]mevalonate in Hep G2 cells at concentrations which strongly inhibited the incorporation of [$^{14}$C]acetate into cholesterol. These differences may be caused by the different kind of cell line used.

None of the HMG-CoA reductase inhibitors tested affected phospholipid, free fatty acid and triacylglycerol synthesis. 25-Hydroxycholesterol did not affect these parameters, but progesterone inhibited phospholipid, free fatty acid and triacylglycerol synthesis. Progesterone may inhibit the fatty acid synthetic pathway.

HMG-CoA reductase inhibitors strongly inhibited cholesterol ester synthesis from [$^{3}$H]oleate in the absence of LDL (Table 4). But in the presence of LDL, inhibitory activity of these compounds was diminished. In the presence of LDL, cells synthesize cholesterol ester from exogenously supplied cholesterol. Cholesterol obtained from medium is supposed to direct to the ACAT substrate pool [30]. MK-733 was found to inhibit directly microsomal ACAT in rabbit intestinal mucosa in our previous experiment [31]. The $IC_{50}$ value was $2 \times 10^{-5}$ M. We also found that MK-733 inhibited Hep G2-microsomal ACAT activity with an $IC_{50}$ of $1.4 \times 10^{-5}$ M (data not shown). In the absence of LDL, MK-733 ($2 \times 10^{-7}$ M) inhibited the incorporation of [$^{3}$H]oleate into cholesteryl [$^{3}$H]oleate by 81% in Hep G2 cells (Table 4). This value was much lower than the $IC_{50}$ value of ACAT inhibition by MK-733. Therefore, we concluded that the inhibitory effect of MK-733 on cholesterol ester synthesis in Hep G2 cells was due to the inhibition of cholesterol synthesis. 25-

* Alberts AW, Chen J, Huff J, Hunt V and Kuron G, Comparative studies on the hydroxymethylglutaryl coenzyme A reductase inhibitors mevinolin, MK-733, and CS-514. In: *Ninth International Symposium of Drugs Affecting Lipid Metabolism*, Florence, 22–25 October 1986, p. 8.

Hydroxycholesterol increased cholesterol ester synthesis in the presence or absence of LDL because it increases ACAT activity [32]. Progesterone inhibited cholesterol ester synthesis in the presence of LDL as potently as in the absence of LDL, since it inhibits ACAT activity directly [15].

HMG-CoA reductase inhibitors induced HMG-CoA reductase activity. The inducibility of reductase activity by MK-733 was more potent than that by CS-514. This fact may reflect the inhibitory potency in cholesterol synthesis. It has been shown that compactin induces this enzyme activity in Hep G2 cells [13]. 25-Hydroxycholesterol (polar sterol) decreased HMG-CoA reductase activity in Hep G2 cells. A squalene-2,3-epoxide cyclase inhibitor, U18666A, decreases HMG-CoA reductase activity at concentrations less than $3 \times 10^{-6}$ M, but increases it at concentrations higher than $3 \times 10^{-6}$ M [33]. Boogaard *et al.* [33] suggested that polar sterols may play a role in suppressing reductase activity.

Hep G2 cells bound, internalized and degraded $^{125}$I-LDL in part by a receptor-mediated mechanism as reported by Havekes *et al.* [12]. MK-733 increased the specific binding, internalization and degradation of $^{125}$I-LDL in Hep G2 cells. CS-514 also behaved like MK-733, but its potency was much weaker than that of MK-733. In skin fibroblasts from normal humans, compactin has only a minor effect on LDL receptor activity [16, 34]. In Hep G2 cells, however, HMG-CoA reductase inhibitors increased LDL receptor activity by about two times. Liscum and Faust [35] recently reported that U18666A blocks LDL-mediated stimulation of cholesterol esterification and LDL-mediated suppression of LDL receptor activity. In our study, down-regulation of LDL receptor and activation of ACAT by LDL were observed in the presence of MK-733 or L-654,969 (Tables 4 and 5). Therefore, MK-733, different from U18666A, did not alter the action of LDL on LDL receptor and ACAT activities.

Fung and Khachadrian [36] reported that 25-hydroxycholesterol decreased LDL receptor activity in cultured human fibroblasts. 25-Hydroxycholesterol was found to decrease LDL receptor activity in Hep G2 cells. LDL receptor [37], HMG-CoA reductase [38] and HMG-CoA synthase [39] genes have been reported to be controlled by an octanucleotide consensus sequence in the 5′ flanking region of these genes. As mentioned above, 25-hydroxycholesterol decreased HMG-CoA reductase activity. Therefore, oxysterol was thought to regulate the expression of these genes.

The reduction of *de novo* cholesterol synthesis in cells regulates several events, including the induction of LDL receptors, induction of HMG-CoA reductase activity, and the decrease in cholesterol ester synthesis [40]. From our studies, MK-733 was found to increase LDL receptor and HMG-CoA reductase activity, and decrease cholesterol ester synthesis. These results suggest that MK-733 would decrease total and LDL-cholesterol levels in humans.

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