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Apoptotic injury in cultured human hepatocytes induced by HMG-CoA reductase inhibitors

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Abstract

Hepatotoxicity is the major complaint during therapy with lipid-lowering agents such as statins, although the cellular mechanisms underlying the statin-induced liver injury are not fully understood. Using cultured human hepatocytes, we investigated the effects of lipophilic as well as hydrophilic statins on the cell viability. Lipophilic statins, including simvastatin, lovastatin, cerivastatin, fluvastatin and atorvastatin, reduced the viability of hepatocytes as assessed by the mitochondrial enzyme activity to reduce WST-8, however, a hydrophilic pravastatin did not cause cell injury. The simvastatin-induced loss of cell viability was attenuated by mevalonate or geranylgeranyl pyrophosphate. Simvastatin-induced DNA fragmentation and increased the number of cells stained with annexin V and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling, both of which were reversed by caspase inhibitors such as zDEVD-fmk, zLEHD-fmk and zIETD-fmk. Consistent with these data, the activities of caspase-3, caspase-9 and caspase-8 were elevated by simvastatin. Simvastatin reduced the protein content and mRNA expression for bcl-2 without affecting bax mRNA expression. On the other hand, both lipophilic and hydrophilic statins significantly reduced the content of endogenous cholesterol. These findings suggest that lipophilic statins cause an apoptotic injury in human hepatocytes by stimulating caspase-3 subsequent to the activation of caspase-9 and caspase-8, in which the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase may be involved.

Keywords: Hepatocytes; HMG-CoA reductase inhibitor; Apoptosis; Caspases; Bcl-2; Bax

1. Introduction

3-Hydroxy-3-methyglutaryl coenzyme A (HMG-CoA) reductase inhibitors such as statins are the most widely used cholesterol-lowering agents for prevention of obstructive cardiovascular events [1–3]. However, severe adverse events including myopathy, rhabdomyolysis and hepatotoxicity associated with lipophilic statins sometimes limit the lipid-lowering therapy with these agents [4,5]. Among them, hepatotoxicity characterized by the elevation of plasma transaminases is most common. In particular, the incidence is heightened, when statin is used in combination with fibrate compounds [6,7], although such combination therapy is necessary for the severe refractory hyperlipidemic patients [8]. It has been reported that the frequency of the elevation of transaminases is 0.5–2.0% during the monotherapy with statin [9,10] but the rate is markedly elevated to 1.3–10% by the combination therapy with statin and fibrate [6,7,11]. However, the precise mechanisms underlying the statin-induced hepatotoxicity remain to be clarified.

It has been reported that a number of lipophilic statins cause apoptosis in a variety of cells, including striated muscle cells [12], cardiac myocytes [13], vascular smooth muscle cells [14–16], and endothelial cells [17,18], by decreasing the amounts of farnesyl pyrophosphate and geranylgeranyl pyrophosphate due to the inhibition of

Abbreviations: HMG-CoA, 3-hydroxy-3-methyglutaryl coenzyme A; MVA, mevalonate; GGPP, geranylgeranyl pyrophosphate; WST-8, 2-(2-methoxy-4-nitorophenyl)-3-(4-nitorophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; AMC, 7-amino-4methyl coumarin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcription-polymerase chain reaction

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HMG-CoA reductase. However, the hydrophilic pravastatin does not possess the toxic action on these cells, since unlike lipophilic statin, the hydrophilic statin can hardly penetrate into cells other than hepatocytes where the hydrophilic statin is transported through the tissue-specific organic anion transporter such as a human liver-specific transporter [19].

The aim of the present study was to compare the toxic action in cultured human hepatocytes among lipophilic and hydrophilic statins and to determine the cellular mechanisms underlying the statin-induced hepatotoxicity. For this purpose, the effects of various statins with different lipophilicity on the viability and nuclear morphology were examined in cultured human hepatocytes. The role for caspases in statin-induced hepatocyte injury was subsequently determined.

2. Materials and methods

2.1. Chemicals

Atorvastatin, cerivastatin and pravastatin were gifts from Sankyo Co. Ltd. The following chemicals and reagents were obtained from commercial sources: fluvastatin, lovastatin, simvastatin, (±)mevalonolacton, and caspase inhibitors such as zLHED-fmk, zDEVD-fmk and zIETD-fmk were obtained from Calbiochem. Fenofibrate and geranylgeraniol were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was from JRH Biosciences. Caspase substrates such as Ac-DEVD-AMC for caspase-3, Ac-IETD-AMC for caspase-8 and Ac-LEHD-AMC for caspase-9 were from Alexis Biochemicals. Cell lysis buffer and dithiothreitol (DTT) were from Bio Vision. 7-Amino-4-methyl coumarin (AMC) was from ICN Pharmaceuticals Inc. Other chemicals and reagents were all of specific grade. Mevalonolacton was converted to mevalonate (MVA) by dissolving in 0.1 M NaOH and incubation at 50 °C for 1 h, followed by neutralizing with 0.1 M HCl. Statins, fenofibrate, caspase inhibitors, and caspase specific substrates were dissolved in dimethyl sulfoxide.

2.2. Cell culture

Human adult hepatocytes Chang liver cells were obtained form American Type Culture Collection (Walkersville, MD, USA). Cells were grown in MEM eagle medium (Dainippon Pharmaceuticals, Co. Ltd., Osaka, Japan) supplemented with 2 mM L-glutamine and 10% FBS on 75 cm^2 tissue culture flasks (Corning Costar, Corning, NY, USA) at 37 °C in an air supplemented with 5% CO_2 under humidified condition.

2.3. Evaluation of cell viability

The cell viability was estimated from the mitochondrial activity to reduce 2-(2-methoxy-4-nitorophenyl)-3-(4-nitorophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8) to the water-soluble formazan [20]. Briefly, cells (2×10^4 cells per well) were seeded on 24-well plates (Falcon, Bectom Dickinson Labware, Lincoln Park, NJ, USA) and incubated for 48 h, then exposed to various statins (1–30 μM) in combination with or without fenofibrates (10 μM) for 24 h. After washing twice with phosphate-buffered saline, cells were incubated with 200 μl of serum-free medium containing 20 μl of WST-8 assay solution (5 mM) and incubated at 37 °C in humidified air supplemented with 5% CO_2 for 1 h. The incubation medium (100 μl) was transferred to 96-well flat bottom plastic plates (Corning Costar). The amount of the formazan formed was measured at OD of 450 nm wavelength using a microplate reader (Immuno-mini NJ-2300, Inter Med.).

2.4. Detection of phosphatidylserine on membrane surface

The extrusion of phosphatidylserine on membrane surface was detected by annexin V stain by using commercial assay kit (MEBCYTO-apoptosis kit, Medical & Biological Lab. CO., Ltd.). Briefly, cells were cultured on collagen-coated chamber slide glass (Iwaki) at 2.0×10^4 cells per chamber. At 48 h after seeding, cells were incubated for 24 h with various statins. In a set of experiments where the effects of caspase inhibitors were examined, subtype-specific caspase inhibitors were added 1 h before addition of the statin. After exposure to the statin, cells were washed twice with phosphate-buffered saline, and incubated with 100 μl binding buffer containing fluorescent isothiocyanate (FITC)-labeled annexin V (10 μl) for 30 min in the dark. The stained cells were visualized using a fluorescence microscope (100× magnification).

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) stain

The nuclear damage was also determined by TUNEL stain. Briefly, the simvastatin-treated cells were washed with phosphate-buffered saline and fixed for 30 min at room temperature with 4% (w/v) paraformaldehyde in phosphate-buffered saline. Cells were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate on ice. TUNEL stain was carried out using a commercial assay kit (Cell Death Detection kit, Roche Applied Science, Tokyo), according to the manufacturer's instructions. The stained cells were visualized by a fluorescence microscope (BX51, Olympus) with 100× magnification.

2.6. Quantitative measurement of simvastatin-induced apoptosis

Apoptosis was quantitatively determined using APO-Percentage™ apoptosis assay kit (Biocolor Ltd., Belfast,

Northern Ireland). The assay uses a dye that is selectively incorporated into apoptotic but not necrotic cells. The incorporation of dye is considered to be due to the membrane ''flip-flop'' event when phosphatidylserine is translocated from the inner membranes to the outer site [21]. The simvastatin-treated cells on 24-well plates (Falcon) were washed with PBS, cells were incubated with 200 μl of serum-free medium containing 10 μl of APOPercentage dye and incubated at 37 °C in humidified air supplemented with 5% CO_2 for 1 h. After washing twice with PBS, cell bound dye was extracted by lysis solution and measured at OD of 590 nm wavelength using a microplate reader (Inter Med).

2.7. DNA electrophoresis

Cells (1.0×10^6) were seeded in 25 cm^2 flask, and incubated for 48 h, then exposed to 30 μM simvastatin for 24 h. After incubation of cells with simvastatin in the absence or presence of caspase inhibitors, cells were collected, and washed twice with phosphate-buffered saline (PBS). Then, DNA was extracted by using ApopLadder EX TM reagent (Takara Biomedicals). The extracted DNA was applied to 2% agarose gel electrophoresis, and visualized with ethidium bromide.

2.8. Measurement of bcl-2 protein by an enzyme immunoassay

The content of bcl-2 protein was determined by an enzyme immunoassay, according to the method of Beck et al. [22]. Briefly, cells (1.0×10^6) were seeded in 25 cm^2 flask, and incubated for 48 h, then exposed to 30 μM simvastatin in the absence or presence of 30 μM MVA for 12 h. After the cells were collected and washed twice with PBS, the bcl-2 content was determined using a bcl-2 ELISA kit (Oncogene Research Products, San Diego, CA, USA) according to the manufacturer's instructions, and the absorbance at OD of 450 nm wavelength was measured using a microplate reader (Inter Med).

2.9. Reverse transcription-polymerase chain reaction (RT–PCR)

To determine the changes in mRNA expression for bcl-2 and bax after exposure to simvastatin, cells (1.0×10^6) were seeded in 25 cm^2 flask, and incubated for 48 h, then exposed to 30 μM simvastatin in the absence or presence of 30 μM MVA for 24 h. The total RNA was isolated using a TRIzol reagent (Gibco BRL). RT–PCR was carried out by using Ready-To-Go RT–PCR Beads (Amersham Pharmacia Biotech). The oligonucleotide primers for bcl-2, bax and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed based on the sequences described by Lee et al. [23] The sequences of PCR primers were as follows: 5′-TGCACCTGACGCCCTTCAC-3′ (sense) and 5′-AGACAGCCAGGAGAAATCAAACAG-3′ (antisense) for bcl-2, 5′-ACCAAGAAGCTGAGCGAGTGTC-3′ (sense) and 5′-ACAAAGATGGTCACGGTCTGCC-3′ (antisense) for bax, and 5′-GGGAGCCAAAAGGGTCATCATC-3′ (sense) and 5′-GAGTGGGTGTCGCTGTTGAAG-3′ (antisense) for GAPDH. The PCR products were subjected to electrophoresis on 2% agarose gel, and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. Then, the intensities of PCR products were semi-quantified densitometrically by using Alpha Imager 2200 (Alpha Innotech Corporation). The mRNA for GAPDH was used as the standard.

2.10. Assay for caspase activity

The activities of caspase-3, caspase-9 and caspase-8 were estimated from the liberation of fluorescent AMC after degradation of peptide substrates specific for caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC) and caspase-9 (Ac-LEHD-AMC), based on the protocols for the caspase activity assay kit (BioVision, Inc.). The cell mixtures were then centrifuged at 150 × *g* for 10 min, and the resultant pellets were suspended in 1 ml lysis buffer (BioVision, Inc.) and subjected to caspase activity assay. The reaction was started by incubating the enzyme extracts with each caspase substrate (10 μM) for 2 h. To ascertain that the reactions were mediated by the activation of the respective caspase subtypes, subtype-specific caspase inhibitors (3 μM) such as Ac-zDEVD-fmk for caspase-3, Ac-zLEHD-fmk for caspase-9 and Ac-zIETD-fmk for caspase-8 were included in the reaction mixture. After centrifugation at 10,000 × *g* for 10 min, the concentration of AMC in the supernatant was determined at an excitation wavelength of 380 nm and an emission wavelength of 460 nm by using a fluorescent spectrofluorometer (Corona Co., Ltd.). The caspase activity was expressed as nmol of AMC produced per milligram protein.

2.11. Assay for endogenous cholesterol in cells

Cellular cholesterol was extracted, as described previously [24]. In brief, cells were treated with various statin for 12 h, then washed twice with PBS. Cholesterol was extracted with the mixture of hexane and isopropanol (3:2, v/v). One milliliter of 2% Triton X-100/chloroform mixture was added to the extract, and the mixture was dried with nitrogen gas. The extracted cholesterol was reconstituted with 500 μl distilled water, and cholesterol content was determined with Amplex Red Cholesterol Assay Kit (Molecular Probes, Inc.).

2.12. Protein assay

Protein content was determined by the method of Bradford [25]. Bovine serum albumin was used as the standard.

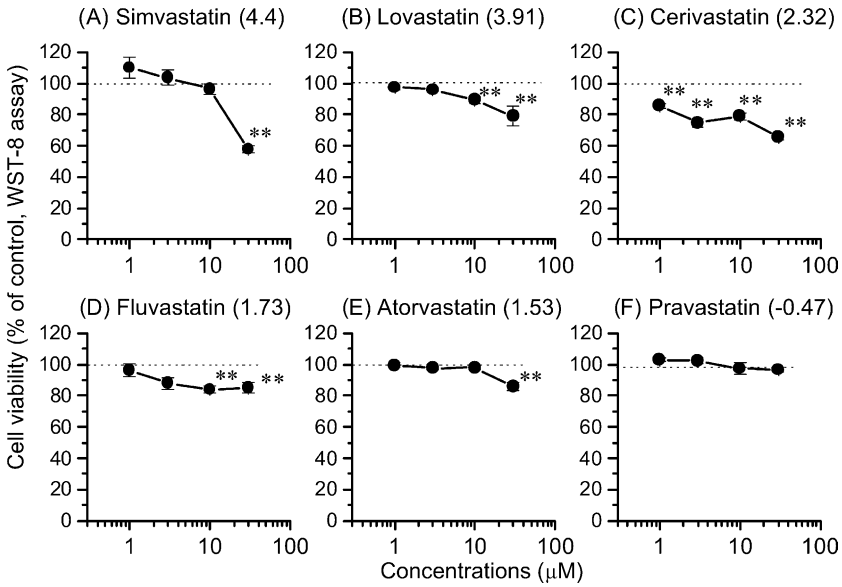

Fig. 1. Comparative effects of various statins on the viability of cultured human hepatocytes. Cells were exposed to various concentrations of statins including simvastatin (A), lovastatin (B), cerivastatin (C), fluvastatin (D), atorvastatin (E) and pravastatin (F) for 24 h, and the viability was determined by WST-8 assay. Figures in parentheses show the log *P* (partition coefficient between octanol and water) values. Each point represents the mean ± S.E.M. of four to eight experiments. For control, cells were exposed to vehicle (0.1% DMSO). $^{**}P < 0.01$ as compared with the respective control group (Dunnett's test).

2.13. Statistical analyses

Data were expressed as the mean ± S.E.M. and statistically analyzed by one-way analysis of variance followed by Dunnett's test for multiple comparisons, or by Student's *t*-test for comparison between two groups (Stat View; Abacus Concepts). Statistical significance was defined as $P < 0.05$.

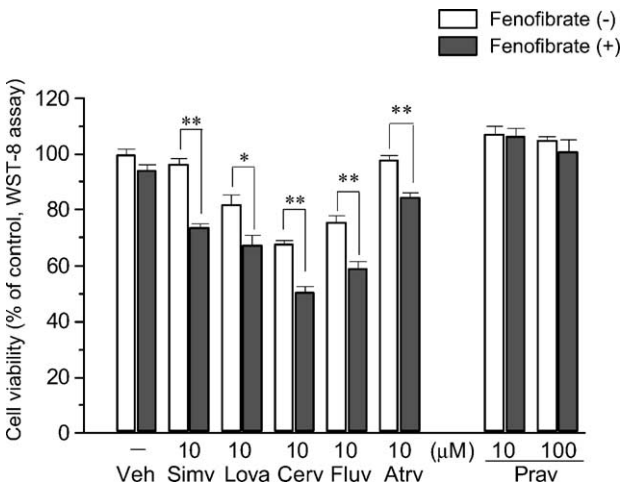

Fig. 2. Augmentation by fenofibrate of hepatotoxocity induced by various lipophilic statins. Cells were incubated with various statins (10 μM for lipophilic statins, 10 and 100 μM for pravastatin) in the absence or presence of 10 μM fenofibrate for 24 h. Cell viability was assessed by WST-8 assay. Each column represents the mean ± S.E.M. of six experiments. Abbreviations: Veh, vehicle; Simv, simvastatin; Lova, lovastatin; Cerv, cerivastatin; Fluv, fluvastatin; Atrv, atorvastatin; Prav, pravastatin. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

3. Results

3.1. Effect of various statins on the viability of Chang liver cells

Lipophilic statins, including simvastatin (log $P = 4.4$), lovastatin (log $P = 3.91$), cerivastatin (log $P = 2.32$), fluvastatin (log $P = 1.73$) and atorvastatin (log $P = 1.59$), decreased the viability of cultured human hepatocytes, whereas a hydrophilic statin pravastatin (log $P = -0.47$) had no influence on the cell viability (Fig. 1). The hepatotoxic effect of lipophilic statins was augmented by a low

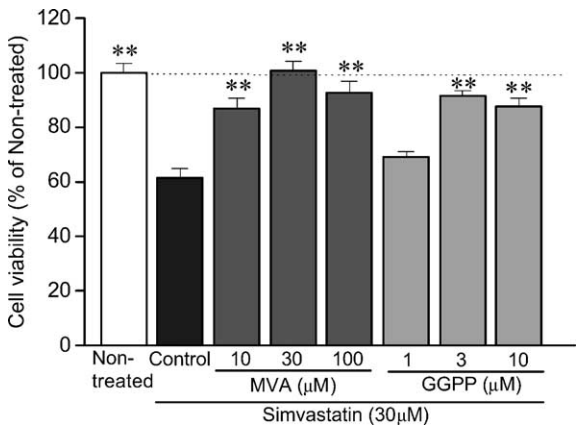

Fig. 3. Reversal by mevalonate (MVA) and geranylgeranyl pyrophosphate (GGPP) of the simvastatin-induced decrease in cell viability in cultured human hepatocytes. Cells were incubated with 30 μM simvastatin in the absence or presence of 10–100 μM MVA or 1–10 μM GGPP for 24 h, and the viability was assessed by WST-8 method. Data represent the mean ± S.E.M. of five to six experiments. $^{**}P < 0.01$ vs. control (Dunnett's test).

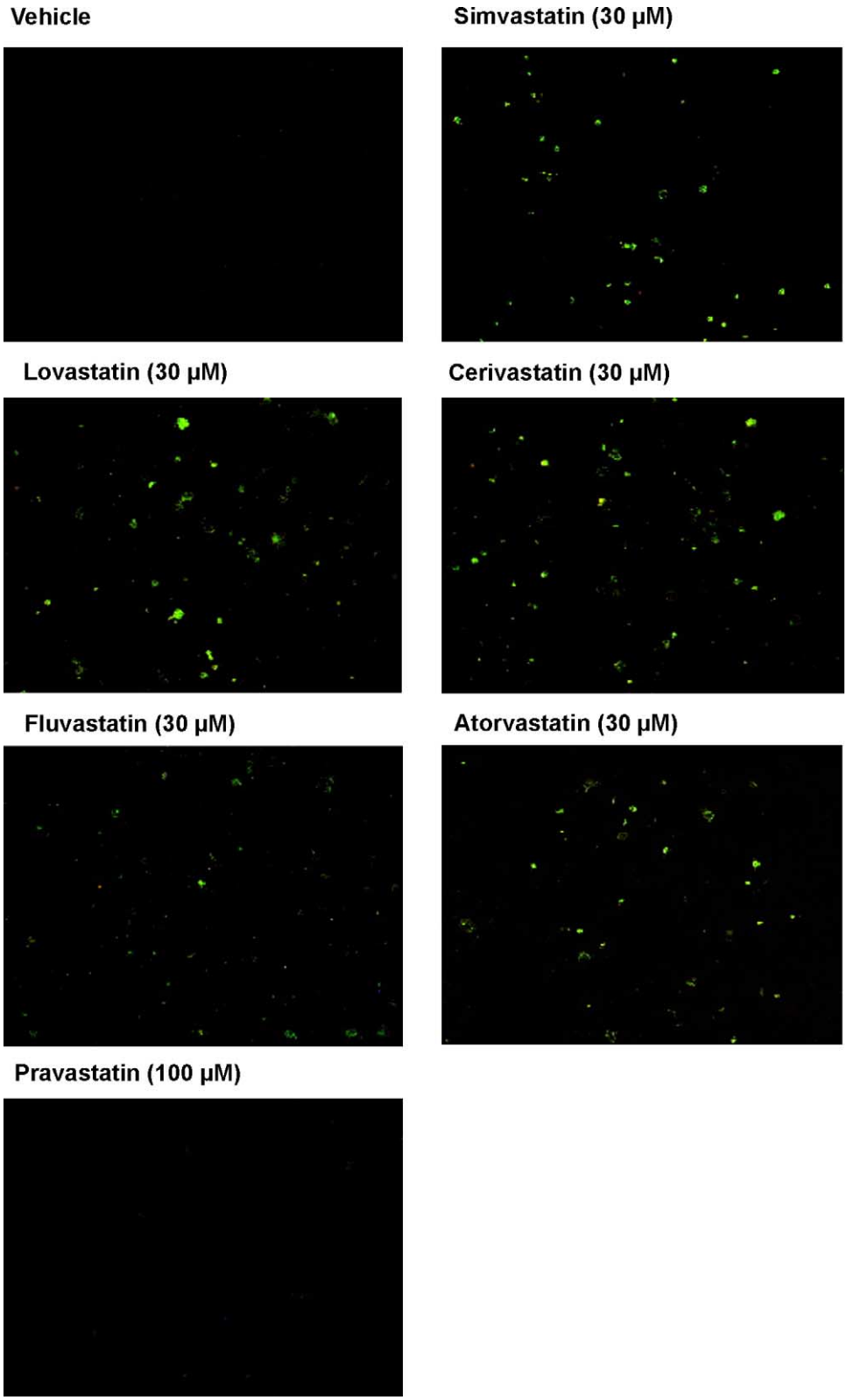

Fig. 4. Increase in the number of annexin V-positive hepatocytes after exposure to lipophilic but not hydrophilic statins. Cells were exposed to vehicle (0.1% DMSO) or various statins, including 30 μM each of simvastatin, lovastatin, cerivastatin, fluvastatin and atorvastatas lipophilic statins and a hydrophilic statin pravastatin for 24 h. Magnification, 100×.

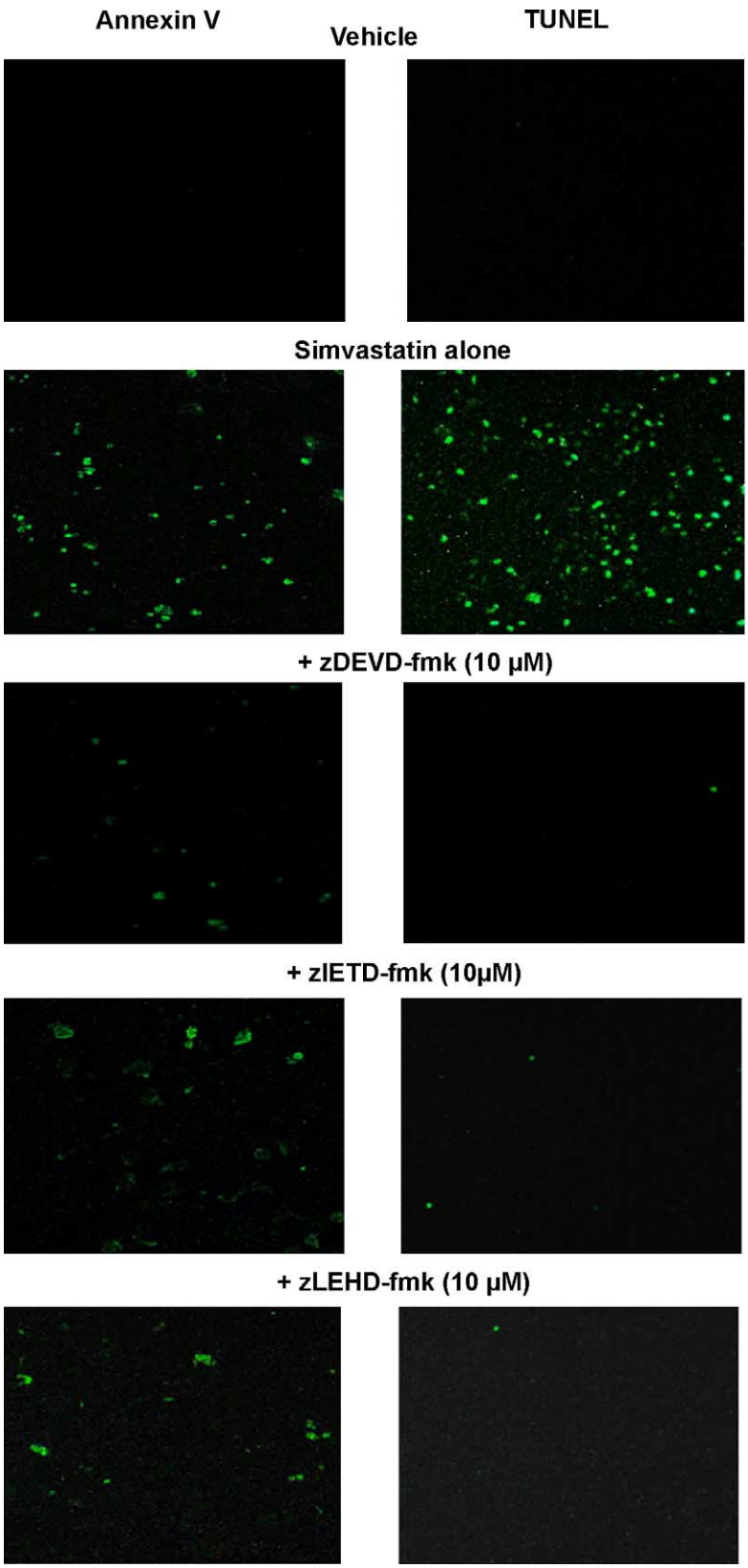

Fig. 5. Reversal by several caspase inhibitors of simvastatin-induced apoptosis as assessed by annexin V stain (left) and TUNEL stain (right) in cultured human hepatocytes. Cells were exposed to 30 μM simvastatin for 24 h in the absence or presence of caspase inhibitors (10 μM), including zDEVD-fmk, zIETD-fmk and zLEHD-fmk. Magnification, 100×.

concentration (10 μM) of fenofibrate (Fig. 2), although fenofibrate alone reduced concentration-dependently the cell viability, in which the significant effect was observed at ≥100 μM (data not shown). However, pravastatin even at 100 μM did not reduce the viability, when it was applied in combination with fenofibrate.

As shown in Fig. 3, the simvastatin-induced reduction in cell viability was reversed by 10–100 μM MVA, in which the reversal by 30 μM MVA was complete. Moreover, the simvastatin-induced hepatotoxicity was reversed by 1–10 μM geranylgeranyl pyrophosphate.

3.2. Positive stains with annexin V and TUNEL after exposure to statins

It has been shown that phosphatidylserine is translocated from inner site to the outer membrane during the early stage of apoptosis, which can be detected by staining with annexin V, a phospholipid-binding protein with high affinity for phosphatidylserine [26]. Therefore, to determine whether lipophilic statins induce apoptosis, cells were stained with FITC-labeled annexin V after exposure to 30 μM of various lipophilic statins. As shown in Fig. 4, a number of annexin V-positive cells were observed after exposure to lipophilic statins, including simvastatin, lovastatin, cerivastatin, fluvastatin and atorvastatin. In contrast, few cells were stained with annexin V after exposure to pravastatin (100 μM).

Moreover, the apoptosis was confirmed by TUNEL stain. As shown in Fig. 5, a number of TUNEL-positive cells were observed after exposure to the lipophilic statin simvastatin.

3.3. Effects of caspase inhibitors on simvastatin-induced cell damage

Caspases are known to be implicated in apoptotic death in a variety of cells. To determine whether caspases contribute to the lipophilic statin-induced apoptosis, the effects of several inhibitors specific for caspase-3, caspase-9 and caspase-8 on the simvastatin-induced increase in the number of cells positively stained with annexin V and TUNEL were investigated. As shown in Fig. 5, the number of annexin V- or TUNEL-positive cells was remarkably reduced by a caspase-3 inhibitor zDEVD-fmk [27] and moderately suppressed by a caspase-9 inhibitor zLEHD-fmk [27] or a caspase-8 inhibitor zIETD-fmk [28]. In addition, quantitative measurement for apoptosis using APOPercentage dye showed that the increase in the amount of the dye induced by 30 μM simvastatin was completely reversed by zDEVD and partially inhibited by zIETD or zLEHD (Fig. 6A).

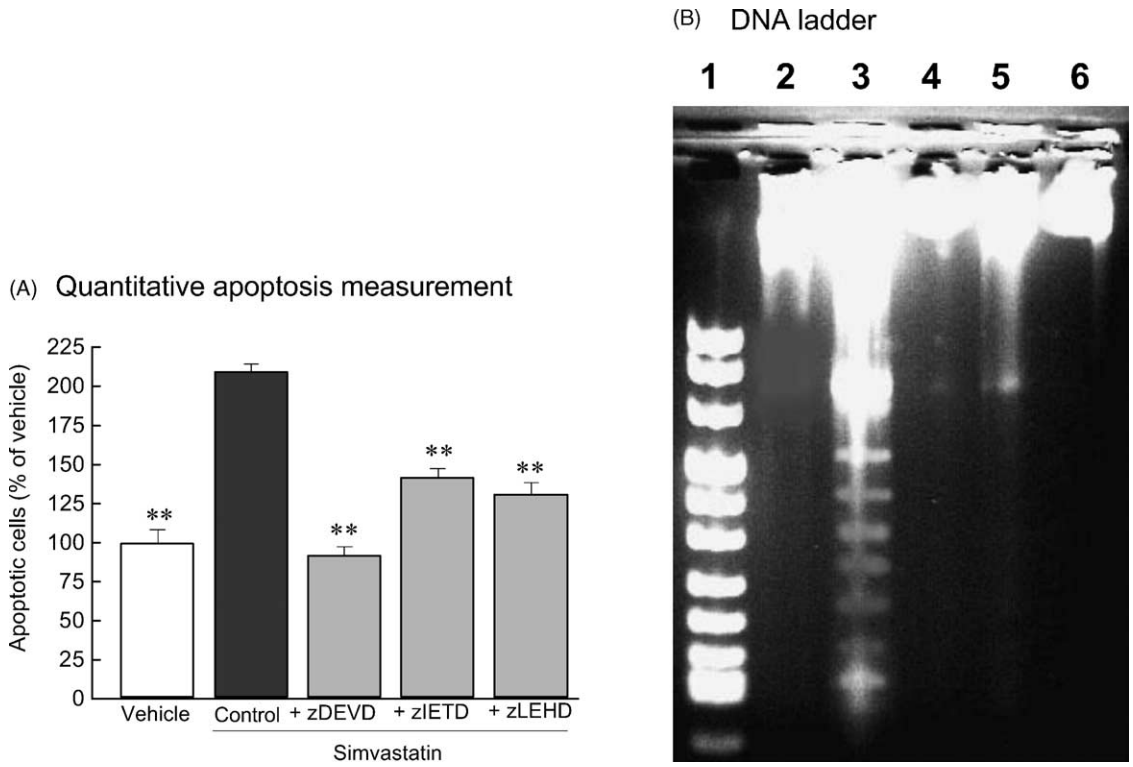

Fig. 6. Involvement of caspases in simvastatin-induced apoptosis (A) and DNA fragmentation (B) in cultured human hepatocytes. In (A), apoptosis was quantitated using APOPercentage™ apoptosis assay kit in cells exposed to 30 μM simvastatin for 24 h in the absence or presence of various caspase inhibitors. Caspase inhibitors were added to the serum-free medium 1 h before simvastatin treatment and included throughout the experiment. In (B), the total DNA was extracted from simvastatin-treated cells. Lane 1: marker; lane 2: 0.1% DMSO; lane 3: simvastatin (30 μM); lane 4: simvastatin (30 μM) + zDEVD-fmk (10 μM); lane 5: simvastatin (30 μM) + zIETD-fmk (10 μM); lane 6: simvastatin (30 μM) + zLEHD-fmk (10 μM). $^{**}P < 0.01$ as compared with control (Dunnett's test).

On the other hand, simvastatin caused DNA fragmentation as assessed by DNA electrophoresis (Fig. 6B). The simvastatin-induced DNA fragmentation was almost completely reversed by zDEVD-fmk and zLEHD-fmk, but partially blocked by zIETD-fmk.

3.4. Enhancement of caspase activity after exposure to simvastatin

To confirm that the activities of several caspase subtypes increase after exposure to simvastatin, the activities of caspase-3, caspase-9 and caspase-8 were determined by the degradation of the fluorogenic peptide substrates for the respective caspase subtypes. As shown in Fig. 7A, simvastatin at 30 μM markedly enhanced the activities of caspase-3, caspase-9 and caspase-8. It was noteworthy that the activation of caspase-3 was partially blocked by z-IETD-fmk and z-LEHD-fmk, but completely reversed by the combination of z-IETD-fmk and z-LEHD-fmk (Fig. 7B). Therefore, it is suggested that simvastatin-induced activation of caspase-3 is mediated through activation of both caspase-9 and caspase-8.

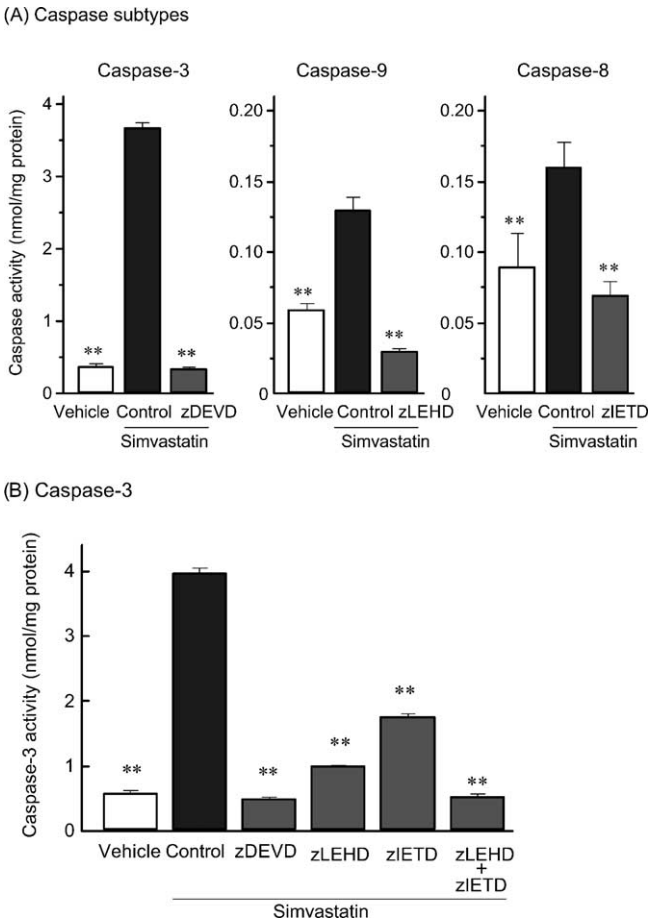

Fig. 7. Enhancement of the activities of caspase-3, caspase-8, and caspase-9 after exposure to simvastatin (A) and possible involvement of both caspase-9 and caspase-8 in the activation of caspase-3 (B) in cultured human hepatocytes. Cells were incubated with 30 μM simvastatin for 24 h. Control cells were treated with 0.1% DMSO. Then, peptide substrates specific for the respective caspase subtypes were added to determine the caspase activity, and incubated for further 2 h in the absence or presence of the respective caspase inhibitors (3 μM). Each column represents the mean ± S.E.M. of four experiments. $^{**}P < 0.01$ as compared with simvastatin alone (Dunnett's test).

3.5. Decrease in the protein content and mRNA expression for bcl-2 after exposure to simvastatin

It has been demonstrated that the caspase activities are regulated negatively by bcl-2 and positively by bax, thus the ratio of bax to bcl-2 is considered to be one of determinants of caspase activation. Thus, the effects of simvastatin on bcl-2 protein and mRNA expression for bcl-2 and bax were examined by an enzyme immunoassay and RT–PCR, respectively. As shown in Fig. 8A, 30 μM simvastatin significantly reduced the content of bcl-2 protein in hepatocytes at 12 h after incubation. In addition, the bcl-2 mRNA but not bax mRNA was reduced by simvastatin treatment, thereby resulting in a significant elevation of the ratio of bax mRNA/bcl-2 mRNA (Fig. 8B). Both the simvastatin-induced decrease in bcl-2 content and the elevation of bax mRNA/bcl-2 mRNA ratio were almost completely reversed by 30 μM MVA.

3.6. Effects of various lipophilic and hydrophilic stains on the cellular cholesterol content

To determine whether the lack of hepatotoxic effect of pravastatin observed in the present study results from the limited access to cells, the effect of pravastatin on the cellular cholesterol content were examined in comparison with various lipophilic statins. As shown in Fig. 9, pravastatin as well as other statins significantly reduced the endogenous cholesterol contents, although pravastatin was slightly less potent than lipophilic statins in reducing the endogenous cholesterol.

4. Discussion

In the present study, a variety of lipophilic statins but not the hydrophilic statin reduced the viability of cultured human hepatocytes as assessed by WST-8 assay. WST-8 is taken up into living cells and reduced to the water-soluble formazan by the mitochondrial NADH enzymes, thus is widely used for the cell viability assay [29]. Although the WST-8 assay also reflects cell proliferation, the rate of proliferation of the cultured hepatocytes used in the present study was extremely low and no significant increase in the amount of formazan was observed during the course (24 h) of incubation (data not shown). Thus, it is unlikely that the reduction in WST-8 assay induced by statins is due to the inhibition of cell proliferation.

The hepatotoxic actions of lipophilic statins were augmented by fenofibrate. Therefore, our present data are similar to the synergistic hepatotoxicity observed in clinical

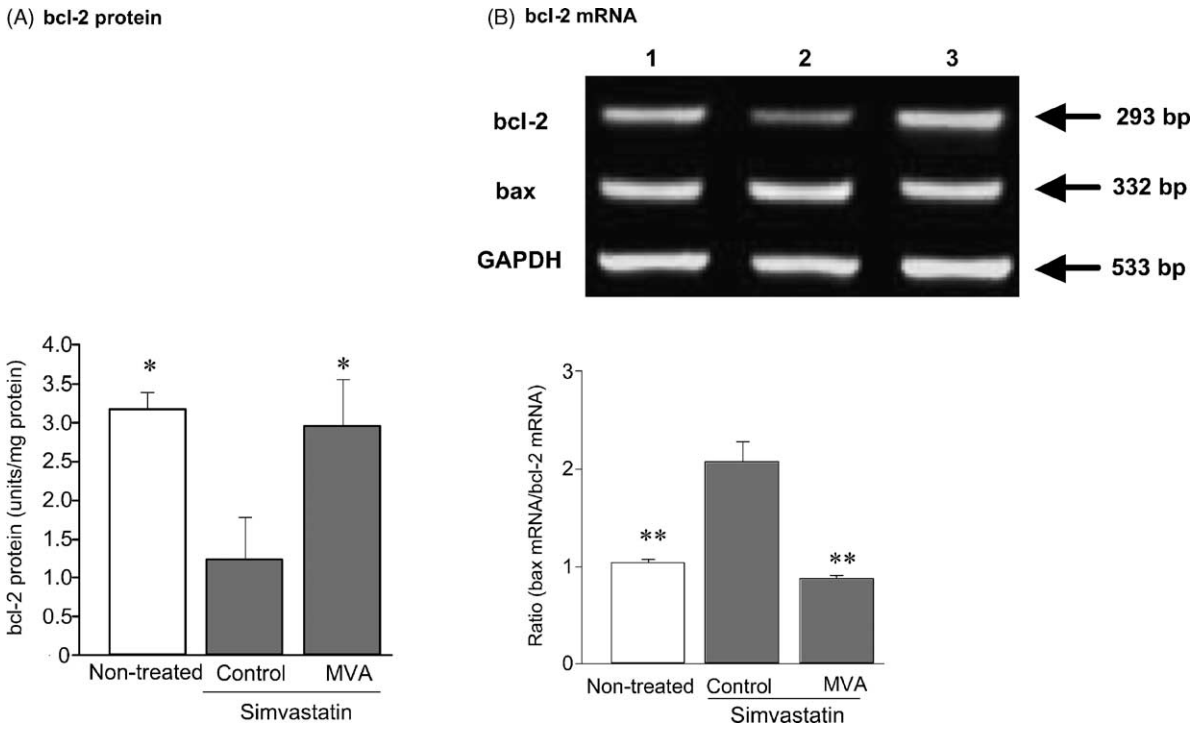

Fig. 8. Decrease in the protein content (A) and mRNA expression for bcl-2 with no change in bax mRNA (B) after exposure of cultured human hepatocytes to simvastatin and the reversal by MVA. In (A), cells were incubated with 30 μM simvastatin for 12 h in the absence or presence of 30 μM MVA and the bcl-2 content was determined by an enzyme immunoassay. In (B), cells were treated with 0.1% DMSO for control (lane 1), or 30 μM simvastatin alone (lane 2) or in combination with 30 μM MVA (lane 3) for 24 h. The mRNAs for bcl-2, bax and GAPDH were determined by RT–PCR. The density of bands of the RT–PCR products was quantitated and the ratio of bax/bcl-2 is shown in the lower figure. Data represent the mean ± S.E.M. of four experiments (A) or three separate experiments (B). $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control (Dunnett's test).

setting during the combination therapy with statin and fibrate [6,7,11].

It is conceivable that the injury of cultured human hepatocytes induced by lipophilic statins is due to apoptosis, since a number of annexin V-stained cells were observed after exposure to a variety of statins except for pravastatin. During the process of apoptosis, phosphatidylserine is exposed to the cell surface due to the loss of membrane integrity [26,30]. Annexin V binds with high affinity to phosphatidylserine, thus is used for the detection of apoptosis [31]. In addition, simvastatin caused the nuclear damage, as assessed by TUNEL stain and DNA fragmentation.

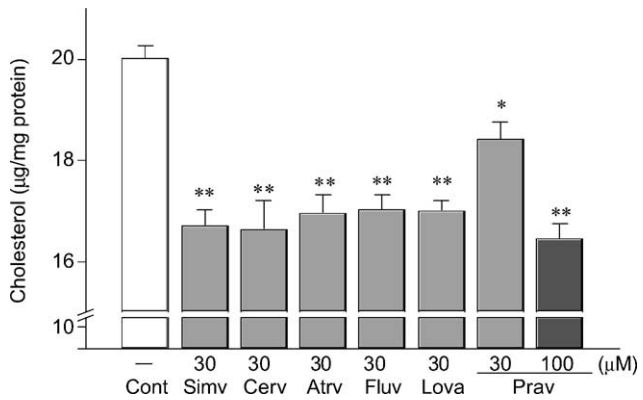

Fig. 9. Comparative effects of various statins on the cellular cholesterol content in cultured human hepatocytes. Cells were incubated with 30 μM of lipophilic statins or 30 and 100 μM of pravastatin for 12 h. The cholesterol content was determined by fluorometric enzyme immunoassay. Each column represents the mean ± S.E.M. of eight experiments. For control group, cells were exposed to 0.1% DMSO in serum-free medium. Abbreviations: Atrv, atorvastatin; Cerv, cerivastatin; Fluv, fluvastatin; Lova, lovastatin; Simv, simvastatin; Prav, pravastatin. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control group (Dunnett's test).

It has been demonstrated that caspases are important mediators of apoptosis. Various apoptotic stimuli activate the pro-apoptotic machinery; in which the initiator caspase-8 is activated by death receptor, while caspase-9 is activated by cytochrome *c* released from mitochondria. The activated caspase-9 converts the inactive pro-caspase-3 into active caspase-3, which leads to chromosomal DNA fragmentation and cellular morphologic changes characteristic of apoptosis [32,33]. In the present study, the increase in the number of cells positively stained with annexin V or TUNEL as well as the nuclear fragmentation (DNA ladder) induced by simvastatin were almost completely reversed by a caspase-3 inhibitor zDEVD-fmk and partially inhibited by caspase-9 inhibitor zLEHD-fmk or a caspase-8 inhibitor zIETD-fmk, thereby indicating that both caspase-9 and caspase-8 are involved in the activation of caspase-3. This idea was confirmed by the present findings indicating that simvastatin stimulated the activities of caspase-3, caspase-9 and caspase-8 and that the activation of caspase-3 was partially blocked by caspase-9

inhibitor or caspase-8 inhibitor but completely reversed by their combination. It has been reported that caspase-8 stimulates directly as well as indirectly the activity of caspase-3. Stennicke et al. [34] have reported in purified system that caspase-8 directly facilitates the conversion of pro-caspase-3 into caspase-3. On the other hand, caspase-8 has been shown to interact with bid, a bcl-2 family protein, which in turn, mediates the release of cytochrome *c* from mitochondrial membrane, leading to the activation of caspase-3 subsequent to activation of caspase-9 [35–37]. It has also been shown in hepatocytes that the mitochondrial pathway is critical for the death receptor-mediated apoptosis [38].

Various ligands for the death receptors such as Fas-ligand, p53 and tumor necrosis factor-α (TNF-α) cause the recruitment of adaptor molecules including Fas-associated death domain and TNF receptor 1-associated death domain, which leads to the activation of caspase-8 by self-cleavage [39]. It has been shown that lipophilic statins increase the sensitivity of vascular smooth muscle cells to Fas-ligand or cytokines to induce apoptosis [40]. Moreover, statins have been reported to increase the expression of p53 in cultured neurons [41]. The statin-induced caspase-8 activation through Fas is considered to be due to the reduction in the isoprenylation of small G proteins such as Rho due to the decrease in geranylgeranyl pyrophosphate (GGPP), an intermediate of cholesterol biosynthesis from HMG-CoA [40,41].

In the present study, simvastatin-induced hepatocytes apoptosis as well as the cellular events such as reduction in the content and mRNA expression for bcl-2 and caspase activation were all reversed by MVA. Moreover, the simvastatin-induced loss of cell viability was attenuated by GGPP. Therefore, it is suggested that the caspase-dependent apoptosis induced by lipophilic statins results predominantly from the inhibition of HMG-CoA reductase and subsequent decrease in GGPP.

On the other hand, in the mitochondrial membranes, an anti-apoptotic protein bcl-2 inhibits, while bax facilitates, the activity of caspase-9 by modulating cytochrome *c* release [42–45]. The ratio of bax to bcl-2 is considered to be an important determinant of caspase activation and induction of apoptosis [46]. In the present study, simvastatin reduced the protein content and mRNA expression for bcl-2 without changing bax mRNA expression. Thus, the ratio of bax mRNA/bcl-2 mRNA was markedly elevated. It has been demonstrated that lipophilic statins down-regulate the bcl-2 expression by inhibiting the isoprenylation of Rho A in vascular smooth muscle cells [47]. Indeed, the inactivation of Rho with clostridium difficile toxin B reduces the expression of bcl-2 and causes an apoptosis in endothelial cells [48] or lymphocytes [49].

Taken together, lipophilic statins may stimulates caspase-9 activity by reducing the expression of bcl-2 on one hand, and enhance caspase-8 activity through activation of Fas/FADD system on the other, both of which may be associated with apoptotic hepatocytes injury through activation of caspase-3 (Fig. 10).

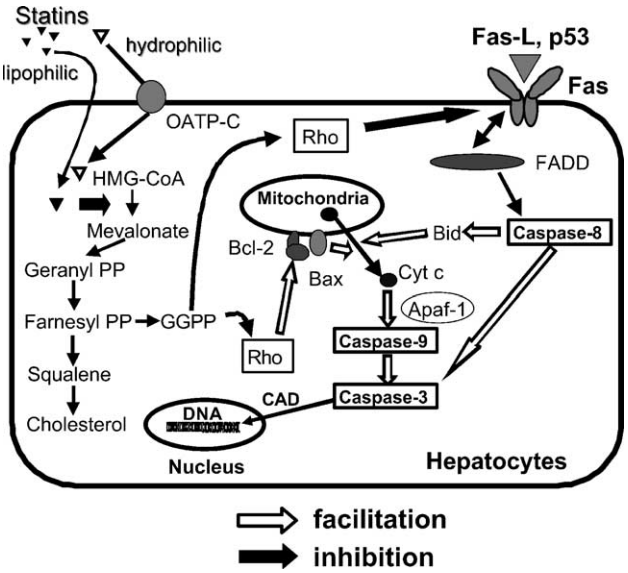

Fig. 10. Schematic drawing for possible mechanisms underlying lipophilic statin-induced hepatotoxicity. Lipophilic statin-induced hepatotoxicity is mediated through activation of caspase-3 subsequent to the enhancement of caspase-9 and caspase-8 activities, in which the reduction of intermediate products in cholesterol biosynthesis such as GGPP after inhibition of HMG-CoA reductase may be involved. Abbreviations: GGPP: geranylgeranyl pyrophosphate; FADD: Fas-associated death domain; Cyt *c*: cytochrome *c*.

In the present study, pravastatin did not reduce the cell viability, nor did it have any influence on annexin V stain. It is unlikely that the lack of hepatotoxic effect of pravastatin results from the limited access of this statin to the cultured cells, since pravastatin, like lipophilic statins, significantly decreased the cellular cholesterol content. It has been shown that pravastatin can enter the hepatocytes via the organic anion transporter such as LST-1, which distributes specifically to hepatocytes [19], while lipophilic stains can easily penetrate the membranes by passive diffusion [50,51]. At present, we cannot explain why pravastatin did not cause hepatocyte injury irrespective of the reduction in cellular cholesterol content.

The extent of the reduction in GGPP may be different between the hydrophilic and lipophilic statins. Further studies are required to elucidate the difference in the cellular action between pravastatin and lipophilic statins.

On the other hand, it has recently been shown that membrane cholesterol plays an important role in the hepatotoxic action of anandamide, an endogenous cannabinoid that is known to be elevated in plasma of patients with severe hepatitis [52]. The decrease in membrane cholesterol with mevastatin, an HMG-CoA inhibitor, reverses the anandamide-induced apoptosis of hepatoma cell line Hep G2 cells [52]. Therefore, it should be noticed that the inhibition of HGM-CoA reductase shows bi-directional actions on the viability of hepatocytes, in which the reduction in geranylgeranyl pyrophosphate causes

apoptosis while the decrease in membrane cholesterol enhances the resistance of hepatic cells to some irritant stimuli including anandamide.

In conclusion, a variety of lipophilic statins reduced the cell viability, when treated alone or in combination with fenofibrate. The cell injury seemed to be due to apoptosis since these statins markedly increased the number of annexin V- or TUNEL-stained cells and induced DNA fragmentation. The simvastatin-induced cell injury was reversed by MVA and GGPP, thereby suggesting the involvement of HMG-CoA reductase inhibition followed by the decrease in GGPP in the apoptotic cell injury. Simvastatin-induced cell injury as well as the DNA fragmentation were reversed by the inhibitors of caspase-3, caspase-9 and caspase-8. Consistently, the activities of these subtypes of caspases were remarkably enhanced after exposure to simvastatin. The statin-induced caspase activation was due in part to the reduction in the expression for bcl-2 mRNA.

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