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Association between risk of myopathy and cholesterol-lowering effect: A comparison of all statins

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ABSTRACT

In the present study, we examined the mechanisms underlying the cytotoxicity of pitavastatin, a new statin, and we compared the *in vitro* potencies of muscle cytotoxicity using a prototypic embryonal rhabdomyosarcoma cell line (RD cells), a typical side effect of statins and compared the cholesterol-lowering effects of statins using Hep G2 hepatoma cells. Pitavastatin reduced the number of viable cells and caused caspase-9 and -3/7 activation in a time- and concentration-dependent manner. The comparison of cytotoxities of statins showed that statins significantly reduced cell viability and markedly enhanced activity of caspase-3/7 in concentration-dependent manner. On the other hand, the effects of hydrophilic statins, pravastatin, rosuvastatin were very weak. The rank order of cytotoxicity was cerivastatin > simvastatin acid> fluvastatin > atorvastatin > lovastatin acid > pitavastatin ≫ rosuvastatin, pravastatin. Statin-induced cytotoxicity is associated with these partition coefficients. On the other hand, the cholesterol-lowering effect of statins did not correlate with these partition coefficients and cytotoxicity. Thus, it is necessary to consider the association between risk of myopathy and cholesterol-lowering effect of a statin for precise use of statins.

Introduction

All statins (cerivastatin, simvastatin, fluvastatin, atorvastatin, lovastatin, pitavastatin, pravastatin and rosuvastatin) lower cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, thus preventing the formation of the major building block of the cholesterol molecule. However, severe adverse events, i.e., skeletal muscle abnormalities, including myopathy and rhabdomyolysis, associated with lipophilic statins sometimes limit the lipid-lowering therapy with these agents (Hodel, 2002; Thompson et al., 2003). Skeletal muscle abnormalities can range from benign myalgia to myopathy, which is defined as a ten-fold elevation of the serum creatine kinase concentration (Pedersen et al., 2004). When statins are prescribed as monotherapy, the incidence of myopathy is approximately 0.1–0.5% and is dose-related (Garnett, 1995; Maron et al., 2000). The mechanism by which statins cause rhabdomyolysis is not precisely known, though possibilities include, (1) depletion of secondary metabolic intermediates such as coenzyme Q10 (Folkers et al., 1990; De Pinieux et al., 1996), (2) induction of apoptotic cell death (Guijarro et al., 1998; Nakagawa et al., 2001), and (3) participation of chloride ion channels (Pierno et al., 1992; Bramow et al., 2001). Pitavastatin is a new chemically synthesized statin. It has strong HMG-CoA reductase inhibiting activity (Aoki et al., 1997). There has been little investigation of pitavastatin-induced cytotoxicity, and the associations between risk of myopathy and cholesterol-lowering effects of all statins, including pitavastatin, have not been fully investigated.

The aim of this study was to elucidate the mechanisms of the cytotoxicity of pitavastatin and to compare the *in vitro* potencies of muscle cytotoxicity, a typical side effect of statins and to compare the cholesterol-lowering effects of all statins.

Materials and methods

Chemicals

Cerivastatin Na, simvastatin, atorvastatin Ca, lovastatin, pravastatin Na and rosuvastatin Ca were kindly donated by Sankyo (Tokyo, Japan). Fluvastatin Na was kindly supplied by Novartis Pharma (Tokyo, Japan). Pitavastatin Ca was kindly donated by Kowa (Tokyo, Japan). Simvastatin acid and lovastatin acid were prepared from their lactone form by hydrolysis in a 0.05 N NaOH solution with stirring at 20 °C for 30 min. The hydrolyzed solution was adjusted to pH 7.4 with 0.2 N HCl and then stored at 4 °C until use as described previously (Kobayashi et al., 2006). All other reagents were of the highest grade available and used without further purification.

Cell culture

RD cells, a prototypic embryonal rhabdomyosarcoma cell line, are tumor cells of skeletal muscle origin that affect children and young

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adults and express a number of muscle-specific proteins (Knudsen et al., 1998), and these cells have been used as a model for studying the myotoxicity effects of statins (Nishimoto et al., 2003). RD cells were maintained in plastic culture flasks (Corning Incorporated Corning) as described previously (Kobayashi et al., 2005).

Hep G2 cells were kept in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH) and 1% penicillin-streptomycin at 37 °C under 5% CO_2.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay was performed as described previously (Mosmann, 1983). MTT is normally reduced by dehydrogenases of viable cells and transformed to formazan. The assay detects living, but not dead, cells and the signal generated is dependent on the degree of activation of cells. For the MTT assay, RD cells were seeded on 96-well plastic plates. Following cell attachment (24 h), various concentrations of statins were added for the times indicated. At 4 h before the end of treatment, 10 µL of PBS-containing MTT solution (0.5%) was added, and the cells were incubated for a further 4 h. The MTT medium was then replaced with 0.2 mL dimetylsulfoxide, and absorbance was read at 590 nm. Absorbance measured in MTT assays was expressed as percent of the control (defined as 100%).

Caspase assay

The caspase assay was performed as described previously (Kobayashi et al., 2007a). Cells were lysed with a cell culture lysis reagent (Promega, Madison, WI). Protein concentration of the cell lysate was adjusted to 10 µg/mL, and the cell lysate was assayed for caspase-3/7 and caspase-9 colorimetric protease assays measuring Ac-DEVD-pNa and Ac-LEHD-pNa cleavage (Promega, Madison, WI) as described in the manufacturer's protocol (Promega, Madison, WI).

Uptake experiments

The uptake experiments were performed as described previously (Kobayashi et al., 2007a,b). Since we previously examined the accumulation of 100 µM cerivastatin Na, pravastatin Na and rosuvastatin Ca in RD cells, we selected other statin concentrations at 100 µM in order to compare all statins.

In the experiments on uptake of cerivastatin Na, simvastatin acid, fluvastatin Na, atorvastatin Ca, lovastatin acid, pitavastatin Ca, pravastatin Na and rosuvastatin Ca, after removal of the growth medium, cells were washed with HEPES buffer (25 mM D-glucose, 137 mM NaCl, 5.37 mM KCl, 0.3 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 10 mM HEPES, pH 7.4) and preincubated at 37 °C for 10 min with 1 mL of HEPES buffer (pH 7.4). Uptake was initiated by applying HEPES buffer (pH 7.4) containing 100 µM statins. The uptake study was performed at 37 °C. After a predetermined time period, uptake was terminated by suctioning off the applied solution and immersing the plates in ice-cold HEPES buffer (pH 7.4) and then suspending in 0.5 mL of ice-cold water. The cellular protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

All statins were determined using an HPLC system equipped with a UV detector. Cerivastatin, pravastatin and rosuvastatin concentrations

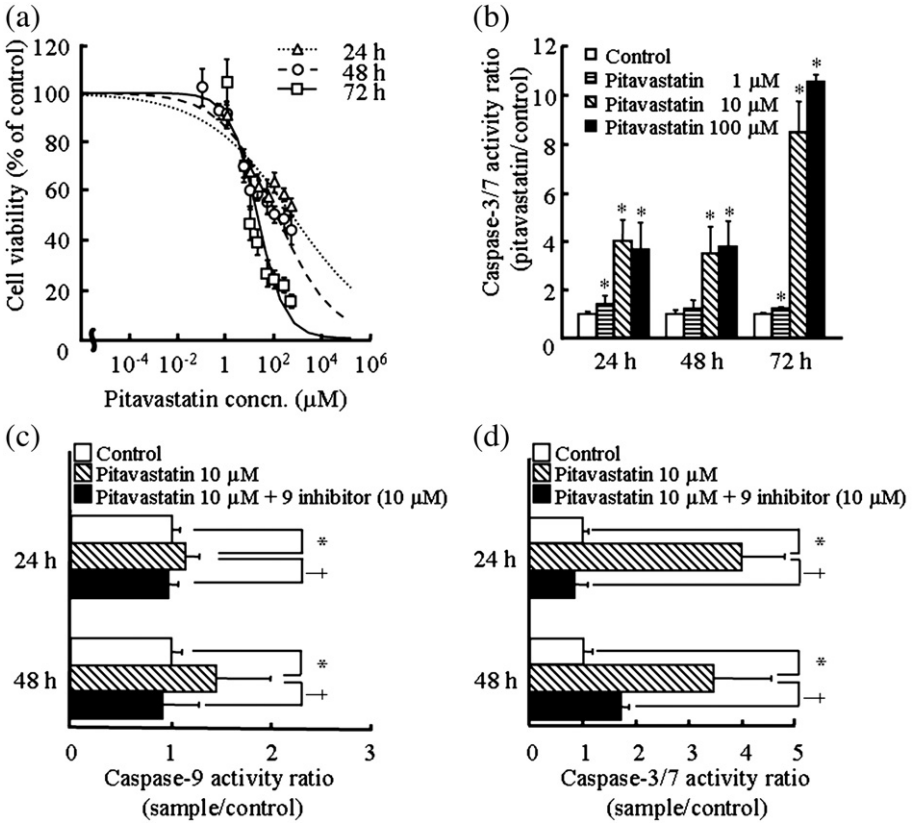

Fig. 1. Effect of pitavastatin on viability of RD cells (a). Cell viability was measured by the MTT assay. RD cells were exposed to various concentrations of pitavastatin for 24–72 h. Each point represents the mean±S.D. of six–twelve determinations. Effect of pitavastatin on caspase-3/7 activity ratio in RD cells (b). RD cells were exposed to various concentrations of pitavastatin for 24–72 h, and the cell lysate was used to measure caspase-3/7 activity ratio. Each column represents the mean with S.D. of three-eighteen determinations. *; significantly different from control (no addition) at $p<0.05$. Effect of caspase-9 inhibitor on caspase-9 (c) and caspase-3/7 (d) activity ratio in pitavastatin-treated RD cells. RD cells were exposed to 10 µM pitavastatin in the absence or presence of 10 µM caspase-9 inhibitor for 24–48 h, and the cell lysate was used to measure caspase-9 and -3/7 activity ratio. Each column represents the mean with S.D. of three–eighteen determinations. *; significantly different from control (no addition) at $p<0.05$, †; significantly different from pitavastatin alone at $p<0.05$.

were determined using an HPLC system as described previously (Kobayashi et al., 2007a,b). The columns were Mightysil RP-8 GP (5 μm, 250 mm × 4.6 mm i.d.). The mobile phase of cerivastatin Na and simvastatin acid consisted of 2.5 mM CH_3COONH_4:CH_3CN (1:4, v/v), that of fluvastatin Na, atorvastatin Ca and lovastatin acid consisted of 2.5 mM CH_3COONH_4:CH_3CN (1:2, v/v), and that of pitavastatin Ca, pravastatin Na and rosuvastatin Ca consisted of 2.5 mM CH_3COONH_4:CH_3CN (1:1, v/v).

Measurement of cellular cholesterol contents

HepG2 cells were seeded at a density of 5×10^5 cells/mL on 12-well plastic plates. Following cell attachment (24 h), 1 μM statins were added for 24 h or 48 h. After the medium had been removed, the cells were washed two timees with PBS and cellular cholesterol were extracted with 1.5 mL of *n*-hexane:isopropanol (3:2, v/v) for 30 min. The lipid phase was evaporated to dryness and resuspended in 300 μL of hexane. Cellular total cholesterol was measured by using a Cholesterol assay kit (Cayman Chemical).

Statistical analyses

Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA).

Statistical significance was defined as $p<0.05$. The IC_{50} value for each drug was determined from the dose–response curve generated by using Origin® (version 6.1J).

Results

Mechanism underlying the cytotoxicity of pitavastatin in RD cells

To clarify the mechanism of the cytotoxicity of pitavastatin, we performed an MTT assay and examined the effects of pitavastatin, a new statin, on RD cell viability. As shown in Fig. 1(a), pitavastatin reduced the number of viable cells in a time- and concentration-dependent manner. To clarify whether pitavastatin-induced cytotoxicity is associated with apoptosis, we examined the activation of caspases. The effector caspase-3/7 plays a central role in apoptosis since it translocates from the cytosol into the nucleus upon activation (Ferri and Kroemer, 2001). As shown in Fig. 1(b), pitavastatin markedly enhanced the activity of caspase-3/7 in a time- and concentration-dependent manner. Moreover, the activation of caspase-9 and that of caspase-3/7 were blocked by the caspase-9 inhibitor zLEHD-fmk (Fig. 1(c,d)). Accordingly, pitavastatin mainly induced apoptosis via a mitochondrial stress-induced cascade in RD cells. These results are consistent with the results for another statin, cerivastatin (Kobayashi et al., 2007a).

Statin-induced skeletal muscle toxicity: a comparison of all statins

To compare the cytotoxicities of statins, we examined the effects of all statins on RD cell viability. As shown in Fig. 2(a), lipophilic statins, cerivastatin, simvastatin acid, fluvastatin, atorvastatin, lovastatin acid and pitavastatin, significantly reduced cell viability in a concentration-dependent manner. On the other hand, the effects of hydrophilic statins, pravastatin and rosuvastatin were very weak (Table 1). As

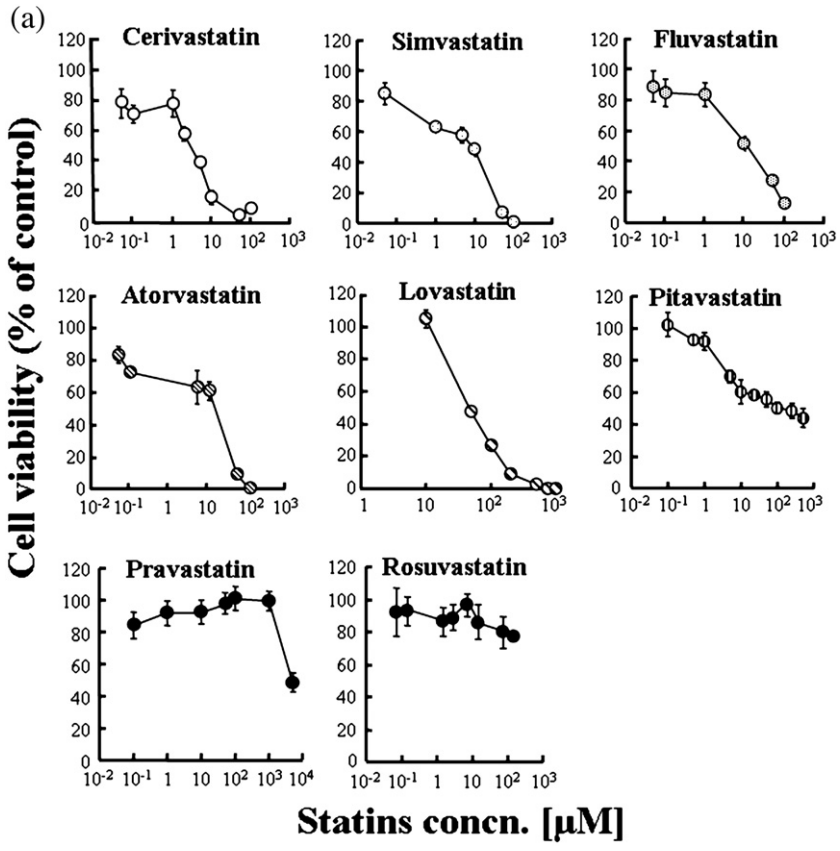

Fig. 2. Effects of all statins on viability of RD cells (a). Cell viability was measured by the MTT assay. RD cells were exposed to various concentrations of all statins for 48 h. Each point represents the mean ± S.D. of three–eighteen determinations. Effect of cerivastatin on the viability of RD cells was investigated in our previous studies (Kobayashi et al., 2007a). Correlation between the cytotoxicity and partition coefficients of statins (b). Effects of all statins on caspase-3/7 activity ratio in RD cells (c). RD cells were exposed to various concentrations of statins for 48 h, and the cell lysate was used to measure caspase-3/7 activity ratio. Each column represents the mean with S.D. of three-eighteen determinations. *; significantly different from control (no addition) at $p<0.05$. Effects of cerivastatin and rosuvastatin on caspase-3/7 activity ratio were investigated in our previous studies (Kobayashi et al., 2007a,b).

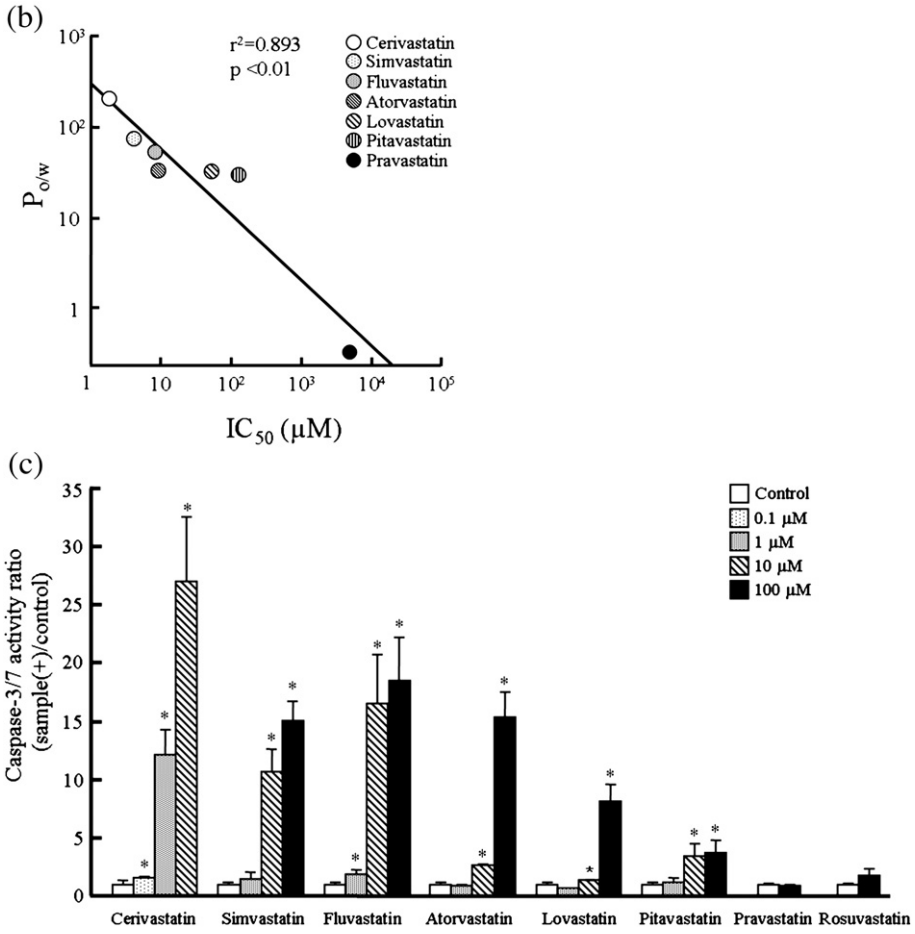

Fig. 2 (*continued*).

shown in Fig. 2 (b), statins induced the reduction of cell viability correlated with these partition coefficients. Next, we compared the caspase-3/7 activation potencies of all statins. As shown in Fig. 2(c), lipophilic statins significantly enhanced the activity of caspase-3/7 in a concentration-dependent manner. On the other hand, the effects of hydrophilic statins were very weak. These results suggest that statin-induced apoptosis is associated with these partition coefficients.

Accumulation of statins in skeletal muscle cells: a comparison of all statins

To clarify the difference in the cytotoxicities of statins, we examined the accumulation of statins in RD cells. As shown in Fig. 3, the time courses of accumulation of all statins reached an equilibrium in 30 min, and accumulation of lipophilic statins was greater than that of hydrophilic statins. Moreover, accumulation of statins correlated with cytotoxicity of all statins. These results suggest that statin-induced cytotoxicity is associated with intracellular accumulation of statins.

Table 1
IC_{50} values of statins on the growth of RD cells

Statins IC_{50}	Values (μM)
Cerivastatin	1.77
Simvastatin	3.99
Fluvastatin	8.34
Atorvastatin	9.26
Lovastatin	52.2
Pitavastatin	117
Pravastatin	4890
Rosuvastatin	>100

Data were taken from Fig. 2(a).

Association between risk of myopathy and cholesterol-lowering effect: a comparison of all statins

The association between risk of myopathy and cholesterol-lowering effect must be clarified for more precise use of statins. We examined the cholesterol-lowering effects of all statins using an *in vitro* hepatocyte model, HepG2 cells. All statins significantly lowered intracellular cholesterol amounts after 24 or 48 h of incubation (Tables 2 and 3). As shown in Fig. 4, cholesterol-lowering effect of statins did not correlate with cytotoxicity.

Discussion

HMG-CoA reductase inhibitors such as statins are the most widely used cholesterol-lowering agents for prevention of cardiovascular disease (Havel and Rapaport, 1995; Jukema et al., 1995; Downs et al., 1998). However, severe adverse events, including myopathy and rhabdomyolysis, associated with lipophilic statins sometimes limit the lipid-lowering therapy with these agents (Hodel, 2002; Thompson et al., 2003). There have been many studies on statin-induced myopathy in animals and humans (Smith et al., 1991; Baker and Tarnopolsky, 2001; Evans and Rees, 2002; Thompson et al., 2003; Schaefer et al., 2004), but the precise mechanism has not been elucidated. Pitavastatin is a new chemically synthesized statin. There has been little

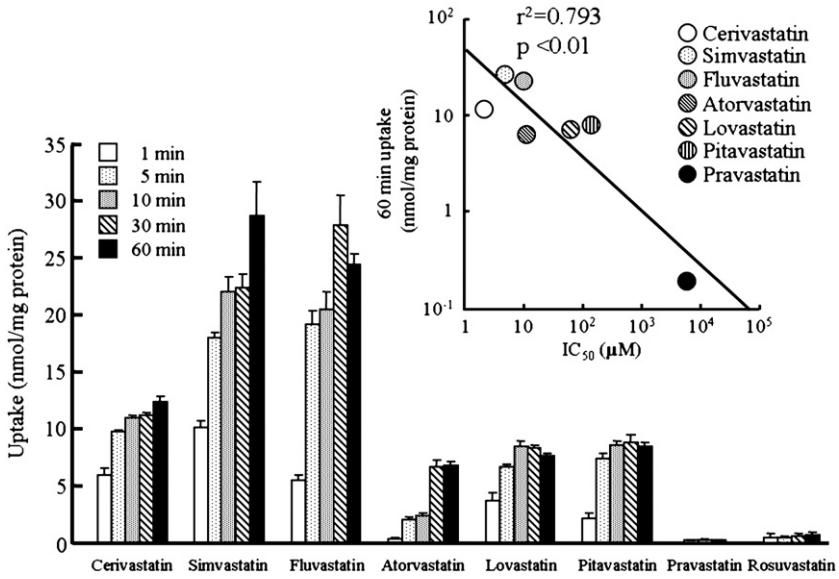

Fig. 3. Accumulation of all statins in RD cells. The accumulation of 100 μM statins was measured at pH 7.4. Each column represents the mean with S.D. of three–eight determinations. Insert: correlation between a 60-min uptake and the cytotoxicity of statins. The accumulation of cerivastatin, pravastatin and rosuvastatin was investigated in our previous studies (Kobayashi et al., 2007a,b).

investigation of pitavastatin-induced cytotoxicity. Therefore, we examined the mechanism of the cytotoxicity of pitavastatin. Pitavastatin reduced the number of viable cells and elevated caspase-3/7 activity in a time- and concentration-dependent manner (Fig. 1(a,b)). Caspases, a family of cysteine proteases, is one of the best-studied apoptotic pathways. Caspases are synthesized in the cytosol as inactive proenzymes and, in response to severe stress, become activated in a particular sequential cascade. Once activated, executioner caspases contribute to dismantle the cell through direct proteolysis of cell structures and repair enzymes and regulatory proteins through initiator caspases (i.e., caspase-2, -8, -9, or -12) or effector (downstream) caspases (i.e., caspase-3, -6, or -7). Activation of caspase-3 represents a commitment for cell disassembly and is a hallmark for apoptosis. Three different pathways are recognized as the initiator caspases involved in activating apoptosis (Loeffler and Kroemer, 2000; Walczak and Krammer, 2000). The receptor-mediated pathway includes the Fas/Fas-ligand cell-surface receptors, which are activated and subsequently lead to caspase-8 activation (Chang et al., 1995). Alternatively, the mitochondrial pathway includes the activation of caspase-9, is associated with mitochondrial stress, and leads to cytochrome *c* release (Cecconi et al., 1998). The newly recognized caspase-12 is activated by endoplasmic reticulum stress and induces the cleavage of caspase-3 in a cytochrome *c*-independent manner (Nakagawa and Yuan, 2000). The activation of caspase-9 and -3/7 was blocked by the caspase-9 inhibitor zLEHD-fmk (Fig. 1(c,d)). Accordingly, we speculate that pitavastatin mainly induced apoptosis via a mitochondrial stress-induced cascade in RD cells. Next, to compare the cytotoxicities of statins, we examined the effects of all statins on RD cell viability. Lipophilic statins, cerivastatin, simvastatin acid, fluvastatin, atorvastatin, lovastatin acid and pitavastatin, significantly reduced cell viability in a concentration-dependent manner. On the other hand, the effects of hydrophilic statins, pravastatin and rosuvastatin, were very weak (Fig. 2(a), Table 1). The rank order of the cytotoxicity was cerivastatin > simvastatin acid> fluvastatin > atorvastatin > lovastatin acid > pitavastatin ≫ rosuvastatin, pravastatin and cytotoxicity of statins correlated with these partition coefficients (Fig. 2(b)). We also examined whether all cytotoxicity of statins was associated with apoptosis. Lipophilic statins significantly enhanced the activity of caspase-3/7 in a concentration-dependent manner (Fig. 2(c)). The rank order of caspase-3/7 activation was cerivastatin > simvastatin acid > fluvastatin > atorvastatin > lovastatin acid > pitavastatin ≫ rosuvastatin, pravastatin. These results suggest that statin-induced apoptosis is associated with these partition coefficients. To clarify the difference in the cytotoxicities of statins, we examined the accumulation of all statins in RD cells. Accumulation of statins correlated with the cytotoxicity of all statins (Fig. 3). These results suggest that statin-induced skeletal muscle cytotoxicity is associated with intracellular accumulation of statins.

In terms of the more precise use of statins, we examined the association between risk of myopathy and cholesterol-lowering

Table 2
Cholesterol-lowering effect of statins in Hep G2 cells

Control	Cholesterol (% of control)
	100.0±25.8
Cerivastatin	72.6±7.0
Simvastatin	83.0±10.0
Fluvastatin	68.3±11.1
Atorvastatin	59.6±7.0
Lovastatin	71.1±16.5
Pitavastatin	59.3±10.1
Pravastatin	62.9±9.7
Rosuvastatin	62.1±2.3

Hep G2 cells were exposed to 1 μM statins for 24 h. Each value represents the mean±S.D. of 4–16 measurements.

Table 3
Cholesterol-lowering effect of statins in Hep G2 cells

Control	Cholesterol (% of control)
	100.0±23.8
Cerivastatin	93.0±29.5
Simvastatin	62.7±16.4
Fluvastatin	91.1±26.5
Atorvastatin	93.7±36.5
Lovastatin	55.1±30.7
Pitavastatin	63.1±18.7
Pravastatin	97.1±44.9
Rosuvastatin	92.1±41.5

Hep G2 cells were exposed to 1 μM statins for 48 h. Each value represents the mean±S.D. of 13–31 measurements.

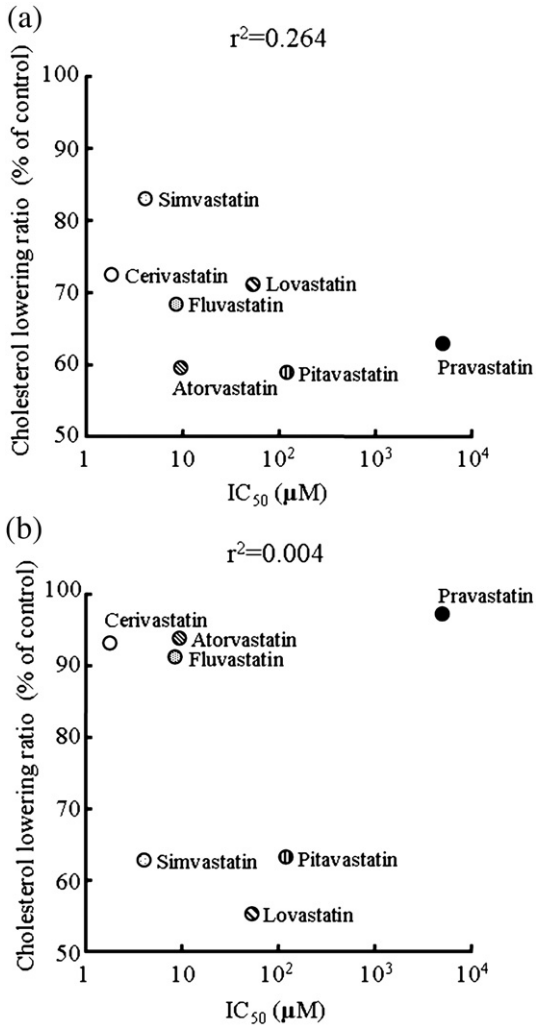

Fig. 4. Correlation between cholesterol-lowering effect after 24-h (a) and 48-h (b) incubation and the cytotoxicity of all statins.

effect. All statins significantly lowered intracellular cholesterol amounts after 24 or 48 h of incubation (Tables 2 and 3). However, the rank order of cholesterol-lowering effect of all statins was different between 24-h and 48-h incubation. Further investigations to clarify the reason are in progress.

The cholesterol-lowering effect of statins did not correlate with this cytotoxicity (Fig. 4). We suggested that it should be selected the statin with low toxicity and high cholesterol-lowering effect for hyperlipidemia.

Thus, it is necessary to consider the association between risk of myopathy and cholesterol-lowering effect for precise use of statins. Finally, this approach may prove to be safe and useful to increase the efficacy of statins for treatment of patients with hyperlipidemia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lfs.2008.02.019.

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