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Evaluation of the synergistic adverse effects of concomitant therapy with statins and fibrates on rhabdomyolysis

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Abstract

Rhabdomyolysis is a severe adverse effect of hypolipidaemic agents such as statins and fibrates. We evaluated this muscular cytotoxicity with an in-vitro culture system. Cellular apoptosis was determined using phase-contrast and fluorescein microscopic observation with Hoechst 33342 staining. L6 rat myoblasts were treated with various statins and bezafibrate under various conditions. With statins only, skeletal cytotoxicity was ranked as cerivastatin > fluvastatin > simvastatin > atorvastatin > pravastatin in order of decreasing potency. Combined application of fibrates enhanced atorvastatin-induced myopathy, which causes little apoptosis alone. These results suggest that statins and fibrates synergistically aggravate rhabdomyolysis.

Introduction

Hyperlipidaemia is a major risk factor of coronary heart disease (CHD) and the importance of plasma lipid control is increased. A number of hypolipidaemic agents are therefore widely used for the control of plasma lipid levels for preventing CHD. Clinicians need to be alert to appropriate information to ensure patient compliance with this category of drug (Kreisberg & Oberman 2002).

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) is the major rate-limiting enzyme in the pathway of cholesterol biosynthesis at the level of mevalonate production (Rodwell et al 1976). Inhibitors of this enzyme, the statins family, are widely used for hypercholesterolaemia. Fibrates, which diminish plasma triglyceride levels, are also used for hypertriglyceridaemic patients (Kinoshita 1999; Watts & Dimmitt 1999). A number of patients have both hypercholesterolaemic and hypertriglyceridaemic factors and both drugs are frequently administered concomitantly to patients with type IIb hyperlipidaemia (Steiner 2001; Taher et al 2002).

Recently, myopathy has been recognised as a prominent and severe adverse effect of HMG-CoA reductase inhibitors, and has the clinical signs of diffuse myalgia, muscle tenderness and elevation of blood creatinine phosphokinase concentrations (Vu et al 2001). Administration of these drugs in combination with ciclosporin or fibrates (such as bezafibrate and gemfibrozil) causes a higher incidence of this myopathy (Marias & Larson 1990; Smith et al 1991). Meanwhile, other reports describe fibrates themselves as causing myopathy. There are several speculations for the mechanisms of myopathy induction in statins. However, the onset mechanisms of the myopathy are still controversial.

In a previous study, we evaluated the extent of rhabdomyolysis induced by various statins using urethane-anaesthetized rats (Matsuyama et al 2002). However, to clarify the myopathic mechanism and drug interaction in detail, simplification of the assay system is essential. Thus, we have tried to establish an in-vitro assay system for evaluation of the synergistic effects between statins and fibrates on the rhabdomyolysis. We selected the L6 rat skeletal myoblast cell line for the assay, which differentiates fusional muscle fibre under starvation.

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Materials and Methods

Drugs

Chemical structures of statins and bezafibrate used in this study are summarized in Figure 1. They were dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C until use. The final concentration of DMSO in the medium did not exceed 0.3% (v/v).

Cell culture

L6 rat skeletal myoblasts were obtained and then subcloned by limiting dilution in our laboratory. The established subclone, designated G3H6, is referred to simply as L6. The cells were routinely maintained in the growth medium (alpha-minimum essential medium (α-MEM) containing 50 IU mL^{-1} penicillin G, 50 μg mL^{-1} streptomycin and 10% fetal calf serum) and less than 20 passages were used in the experiments.

Determination of lipid biosynthesis

Cholesterol and triglyceride syntheses were determined as described previously (Matzno et al 1997). Briefly, 24-h differentiated cells were washed and fresh differentiation medium containing drugs was added. After 1 h pre-incubation, cells were incubated with 2 mL of α-MEM (without serum) containing each drug and [1-^{14}C]acetate (3 mM, 2×10^6 d min^{-1}/well) for 24 h. Synthesized cholesterol and triglyceride were extracted and separated with thin-layer chromatography and their radioactivity was measured.

Myopathy observation

Cells were seeded on a 12-well plate at a concentration of 3×10^4 cells cm^{-2} and cultured at 37 °C, 5% CO_2 in air. Twenty-four hours after incubation, mononuclear cells were washed twice with Hanks' balanced salt solution (HBSS), and the medium was replaced with differentiation medium (α-MEM supplemented with 0.1% bovine serum albumin, 10 μM insulin, 5 μM transferrin and 10 nM sodium selenite) (Yoshida et al 1996). Each drug was simultaneously added into differentiation medium and medium was replaced freshly every day. Cells were observed under a phase-contrast microscope every two days.

Karyopyknotic observation

Cells were grown and differentiated on the cover glass. Forty-eight hours after differentiation with drugs, cells were fixed with 1% glutaraldehyde for 10 min, stained

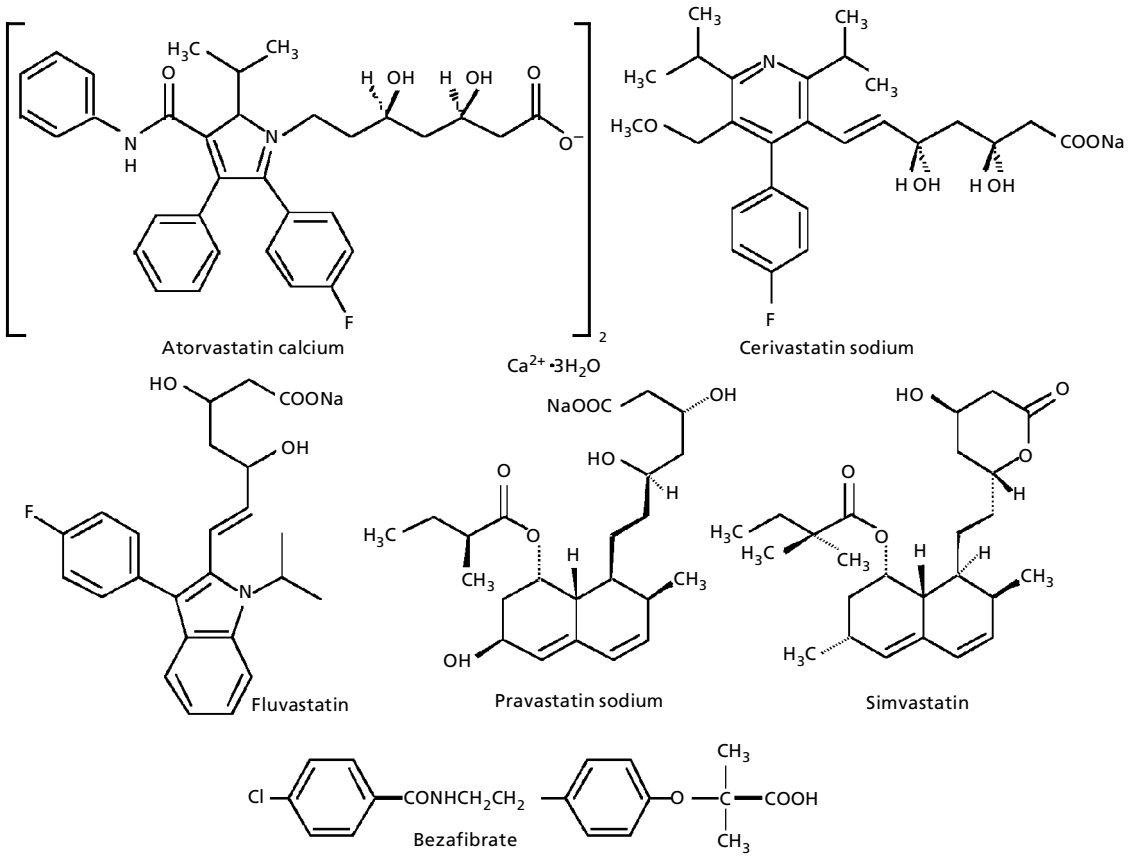

Figure 1 Chemical structure of various statins and bezafibrate.

with 1 mM Hoechst 33342 and examined under fluorescein microscopy (Matzno et al 1997).

Detection of DNA fragmentation

Cells were cultured and differentiated on a 100-mm tissue culture dish (Asahi Techno Glass Ltd., Tokyo Japan). At 8, 16 or 24 h after differentiation with drugs, cells were washed with phosphate-buffered saline (PBS) and scraped by rubber policeman. The cells were then dissolved with lysis buffer (10 mM Tris-HCl, 10 mM EDTA and 0.5% Triton X-100, pH 7.4) at 4 °C for 5 min, treated with 0.2 mg mL^{-1} of RNase A at 37 °C for 60 min, and incubated with 0.2 mg mL^{-1} of proteinase K at 50 °C for 30 min. After phenol–chloroform extraction, DNA fragments were precipitated with 50% isopropanol and then electrophoresed on a 2% agarose gel.

Statistical analysis

Cholesterol and triglyceride synthesis were assessed using a one-way analysis of variance, following which individual differences between treatments were examined using Dunnett's test ($P < 0.05$ denoting significance). Additional information was also obtained by two-dimensional discrimination analysis. Values represent mean ± s.d. of 5 or 6 independent experiments.

Results

Inhibition of lipid biosynthesis

Figure 2 shows the effects of statins, with or without fibrates, on the cholesterol and triglyceride synthesis in differentiating L6 myoblasts. Almost all statins significantly inhibited cholesterol biosynthesis from [1-^{14}C]acetate at 1 mM (pravastatin) or 1 μM (other statins), whereas little effect was observed on triglyceride synthesis (Figure 2B). These effects were not modified by combination with 100 μM bezafibrate.

Although both studies showed that fluvastatin significantly elevated triglyceride synthesis but did not affect cholesterol, two-dimensional discrimination analysis (Figure 2C, D)

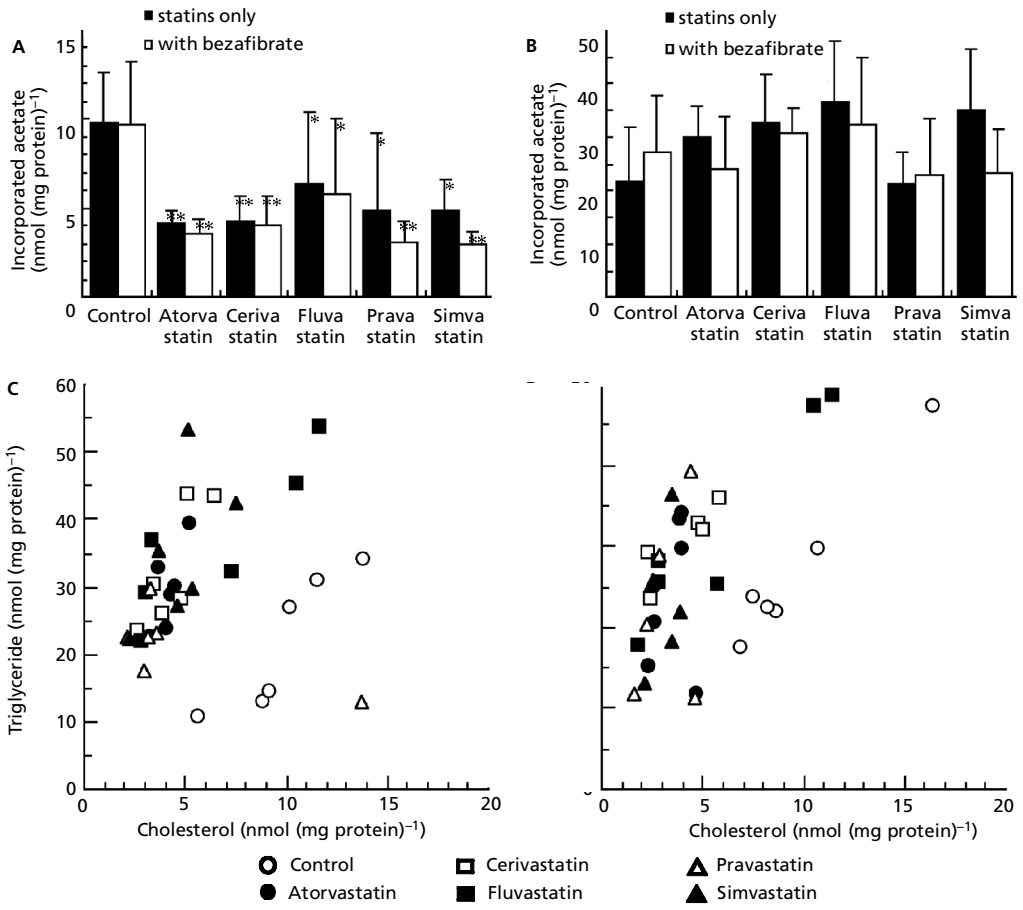

Figure 2 Effects of various statins on the syntheses of cholesterol (A) and triglyceride (B) in L6 myoblasts. Values represent mean ± s.d. (n = 5 or 6). The relationship of cholesterol and triglyceride syntheses was also analysed by two-dimensional discriminant analysis by administration of each statin alone (C) or with bezafibrate (D). Cells were incubated with drugs for 1 h and subsequent 24-h-synthesized lipids were measured. The incubated concentration of each drug was as follows: atorvastatin 1 μM, cerivastatin 1 μM, fluvastatin 1 μM, simvastatin 1 μM, pravastatin 1 mM and bezafibrate 100 μM. *$P < 0.05$, **$P < 0.01$, compared with control.

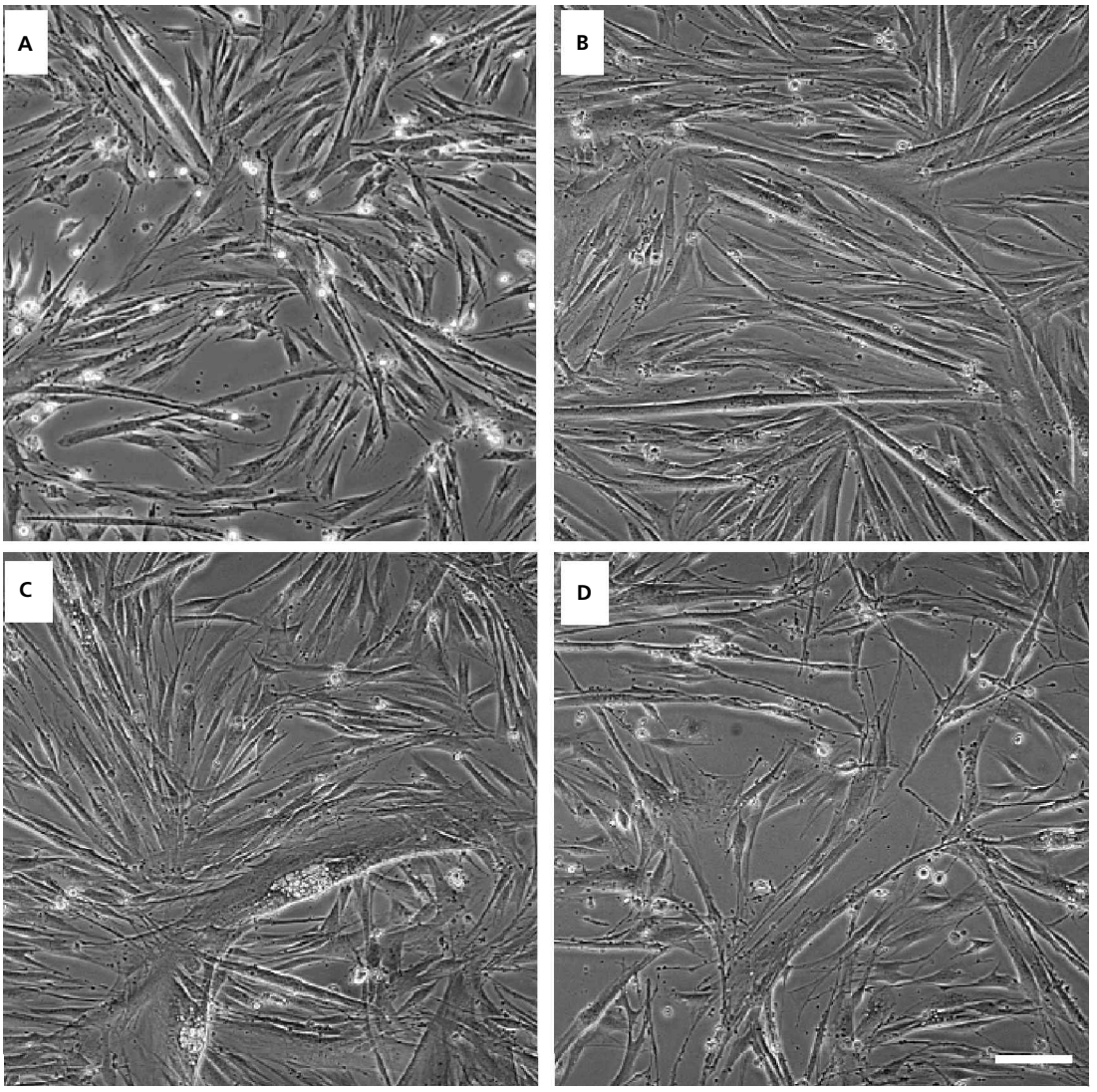

Figure 3 Typical morphology of myopathy in atorvastatin-treated L6 myoblasts. Cells were seeded on the 12-well plate, and 24 h after seeding medium was replaced with differentiated medium and incubated for 48 h with drug (control (A), 1 μM (B), 3 μM (C), 10 μM (D)). Bar = 100 μm.

clearly shows that fluvastatin (closed square) was separated from control and also categorized under the same group of other statins.

Statin-induced myopathy

We evaluated statin-induced myopathy using differentiating myoblasts. Figure 3 shows the typical morphology of atorvastatin-induced myopathy in L6 cells. Atorvastatin had no toxicity at a concentration lower than 1 μM, slight injury occurred at 3 μM and severe myopathy was observed at 10 μM in differentiating L6 myoblasts. Figure 4 shows phase-contrast microscopic observation of differentiating myoblasts in the presence of various statins. Their cytotoxicity was ranked as cerivastatin > fluvastatin > simvastatin > atorvastatin > pravastatin in order of decreasing potency. We also stained differentiating myoblasts with Hoechst 33342 (Figure 5), showing that each statin caused nuclear fragmentation. These photos suggest that statin-induced myopathy was caused by cellular apoptotic pathway.

We also examined statin-induced DNA fragmentation with agarose gel electrophoresis. Although a DNA ladder was not observed at 6 and 18 h (data not shown), this fragmentation pattern was observed at 24 h (Figure 5G). This result indicated the same order as the results in the karyopyknotic assay (Figure 5).

Synergistic application

We also evaluated the statin-induced myopathy with an application of bezafibrate. Figure 6 shows the result of

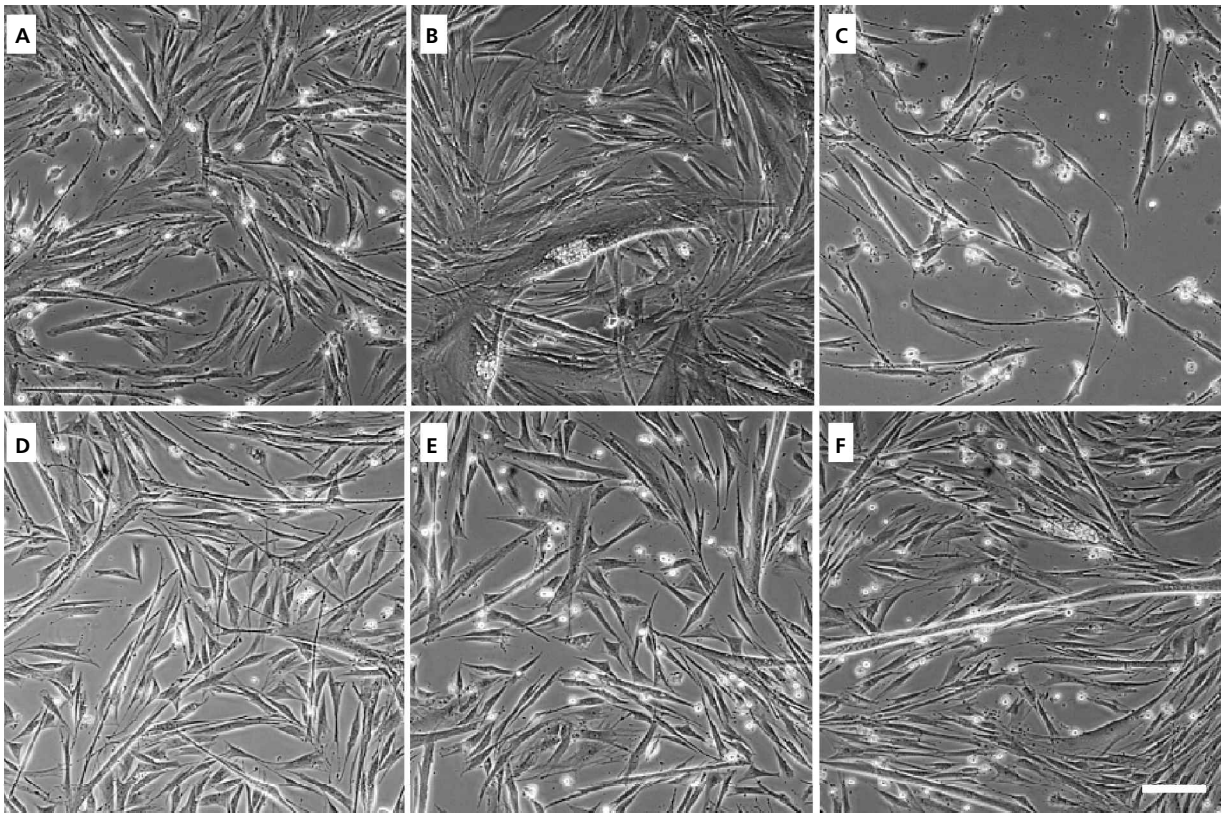

Figure 4 Cytotoxic effects of various statins in L6 myoblasts. Cells were seeded on the 12-well plate and 24 h after seeding, medium was replaced with differentiated medium and incubated for 48 h with drugs (control (A), atorvastatin 3 μM (B), cerivastatin 3 μM (C), fluvastatin 3 μM (D), simvastatin 3 μM (E), pravastatin 3 mM (F)). Bar = 100 μm.

combination with atorvastatin. Atorvastatin had no toxicity to L6 myoblasts at a concentration of 1 μM (Figure 6A) as already shown in Figure 1. However, upon addition of bezafibrate (100 μM), atorvastatin at only 1 μM caused myoblast injury (Figure 6B), showing that bezafibrate enhanced the atorvastatin-induced apoptosis. Hoechst-staining analysis was also performed for those myoblasts 24 h after combined drug treatment (Figure 6C, D). Interestingly, karyopyknotic observation further demonstrated that synergistic myopathy was found significantly at lower concentration; administration of 0.3 μM atorvastatin and 30 μM bezafibrate apparently fragmented their nuclei. These results indicated that simultaneous use of statins and fibrates causes a severe myopathy, not caused by administration of either drug alone.

Discussion

In this study, we tried to establish an evaluation system of drug interactions using the L6 myoblasts; certainly, statin-induced apoptosis was clearly reinforced.

Our recent in-vivo study (Matsuyama et al 2002) showed that cerivastatin caused myopathy most severely, though the present study showed that simvastatin exerted the most drastic effect on L6 myoblasts. In the previous report, the main reaction of enzymatic inhibition of simvastatin was attributed to its main metabolite, the open acid form, not intact simvastatin. Thus we considered that the toxicity decrement of simvastatin in the present study depended on the lack of first-pass reaction in the liver. For the evaluation of metabolic effect, a co-culture study of hepatocytes and myoblasts is needed.

We also previously mentioned the drug interaction between statins and urethane (Matsuyama et al 2002). Interestingly, this myopathy was observed specifically under the condition of urethane-induced anaesthesia (data not shown), indicating that statin-induced myopathy is aggravated by urethane. Since other anaesthetics did not have any effect, it was noteworthy that a specific mechanism exists for statin–urethane potentialization. The specific mechanism might be likewise observed in statin–fibrate interaction in the present observation, so our next goal is the elucidation of this interaction. Therefore, our in-vitro system is also useful for the anaesthetized effect in myopathy.

Several investigators have suggested mechanisms for statin-induced myopathy. In a previous study (Matzno et al 1997), we reported that HMG-CoA reductase inhibitor simvastatin caused severe cytotoxicity in differentiating L6 myoblasts, accompanied by apoptosis. In contrast, skeletal myopathy in L6 did not occur with squalene

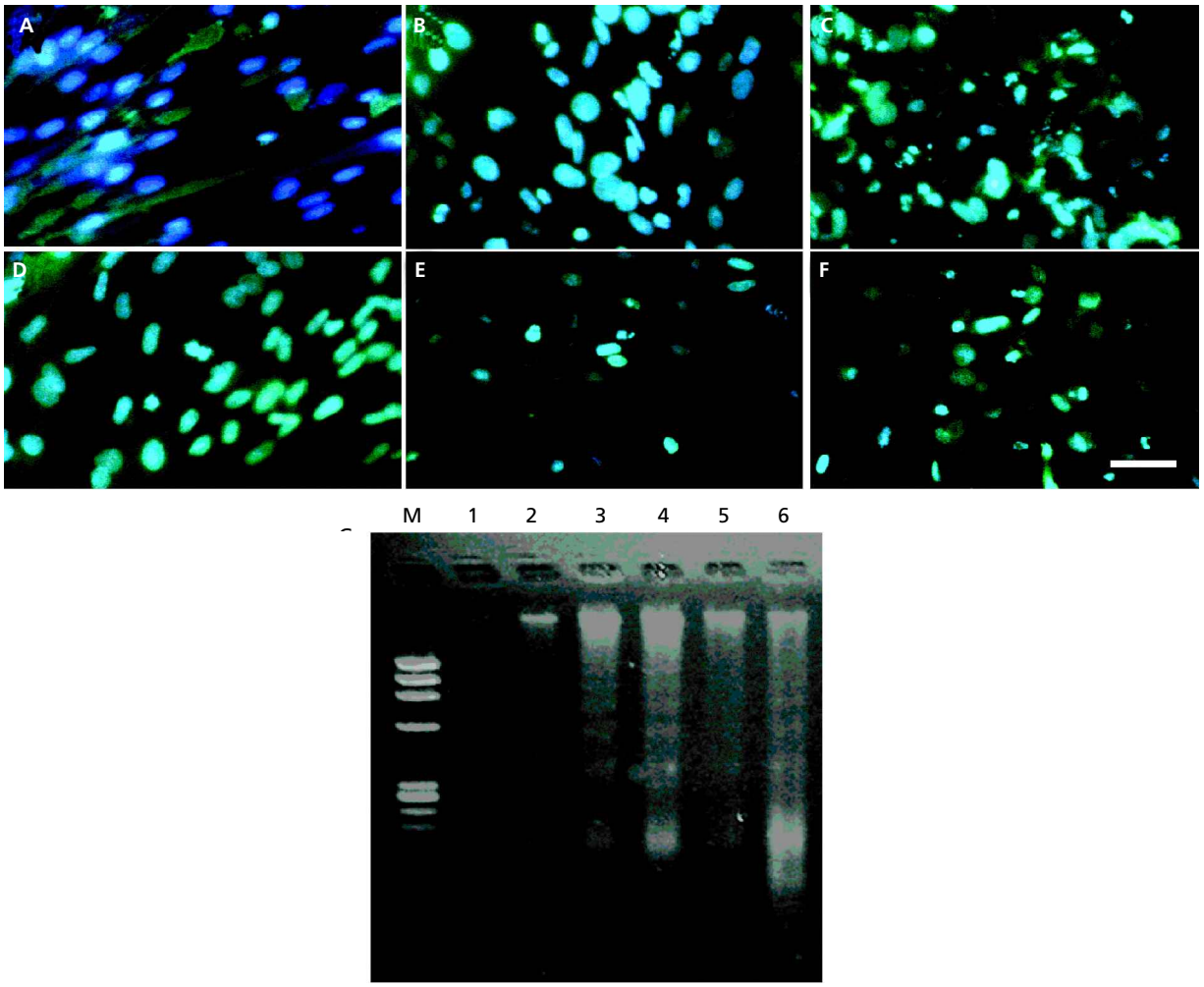

Figure 5 Fluorescent microscopic observation of differentiated L6 muscle fibre after treatment with statins (control (A), atorvastatin 1 μM (B), cerivastatin 1 μM (C), fluvastatin 1 μM (D), simvastatin 1 μM (E), pravastatin 1 mM (F)). After fixation, cells were stained with Hoechst 33342. Bar = 50 μm. (G) DNA fragmentation of L6 myoblasts treated with various statins. Fragmented DNA was electrophoresed on a 2% agarose gel. M, marker; lane 1, control; 2, atorvastatin 3 μM; 3, cerivastatin 3 μM; 4, fluvastatin 3 μM; 5, simvastatin 3 μM; 6, pravastatin 3 mM.

epoxidase inhibitors (Hidaka et al 1991), another cholesterol biosynthesis inhibitor that affects the downstream cholesterol synthesis pathway, suggesting that this myopathy might result from the depletion of a certain isoprenoid, not cholesterol per-se. Supporting this idea, our previous study (Matzno et al 1997) showed that simvastatin-induced myopathy was completely abolished by supplementation with mevalonate.

The cholesterol synthesis pathway is summarized in Figure 7. The intermediate metabolites, geranyl and farnesyl pyrophosphate, facilitate membrane association of several proteins, such as p21Ras and lamin B. Pérez-Sala & Mollinedo (1994) reported that isoprenoid depletion by lovastatin caused DNA fragmentation in HL-60, suggesting that protein isoprenylation is essential for cellular maintenance. The same mechanism was also suggested in vascular smooth muscle (Takemoto & Liao 2001). Hess et al (2000) suggested that the effect on smooth muscle might aid in prevention of strokes. In contrast, Weiss et al (1999) described how short-term pravastatin mediates growth inhibition and apoptosis, independently of Ras, via the signaling proteins p27Kip1 and P13 kinase. Interestingly, no investigations have been undertaken on the apoptotic mechanism in skeletal muscle, the main target tissue in the adverse effect of statins. Therefore, our system should be useful to provide direct evidence of statin-induced myopathy.

On the other hand, the mechanism of fibrate-induced apoptosis is not clearly understood. Recently, Zhou & Wallace (1999) reported that fenofibrate, clofibrate and ciprofibrate caused a direct dose-dependent depolarization of mitochondrial membrane potential. Likewise, Ikemoto & Endo (2001) reported that clofibrate induced calcium release from intracellular calcium stores. These investigations might generate the mechanism of fibrate-induced apoptosis, such as mitochondrial- or endoplasmic-

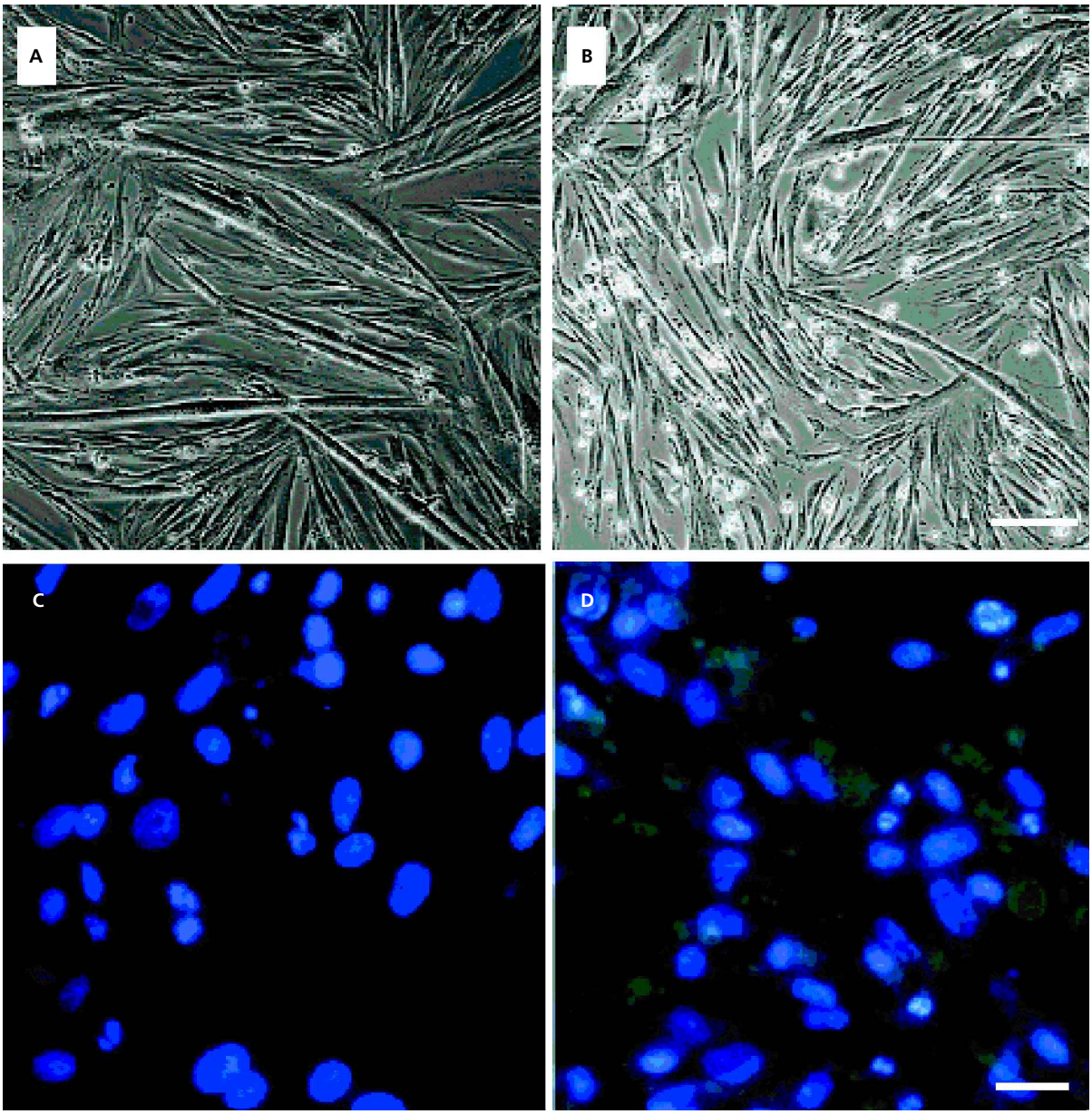

Figure 6 Drug interaction between atorvastatin and bezafibrate on the skeletal myopathy in L6 myoblasts. Twenty-four hours after seeding, the medium was replaced with differentiation medium containing 3 μM atorvastatin and incubated for 48 h in the absence (A) or presence (B) of 100 μM bezafibrate. Bar = 100 μm. In the karyopyknotic evaluation of drug interaction, cells were incubated with 0.3 μM atorvastatin in the absence (C) or presence (D) of 30 μM bezafibrate for 24 h.

reticulum-dependent apoptosis (Rao et al 2001). On the other hand, Inoue et al (2002) reported that fibrates and statins synergistically increase the transcriptional activity of PPARα/RXRα. Thus, the combined activity of these receptors seems also important. Taken together, we offer that the in-vitro system described in this study can confirm the possibility of an apoptosis mechanism of various lipid-lowering agents applied alone or concurrently.

Conclusions

In this study, we newly established a rapid evaluation system for rhabdomyolysis in-vitro. This system should enable rapid gathering of precise information about adverse effects in clinical situations. In practical use, it clearly demonstrated that the simultaneous use of statins and fibrates causes a severe myopathy, which was provided to a lesser extent by administration of each drug

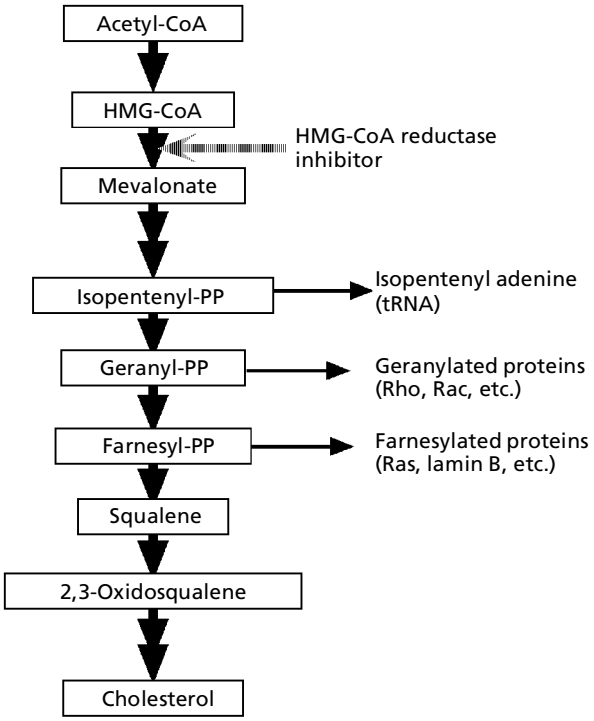

Figure 7 Cholesterol synthesis pathway.

alone. Moreover, this system has wide application for the prediction of other adverse effects, such as neuroleptic malignant syndrome, by changing cell lines. Further investigation is needed to improve the precision of side-effect prediction in the system.

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