Inhibition of cholesterol biosynthesis by squalene epoxidase inhibitor avoids apoptotic cell death in L6 myoblasts

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Abstract The relationship between the inhibition of cholesterol biosynthesis and occurrence of myopathy was studied in L6 myoblasts using two lines of cholesterol biosynthesis inhibitors, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (simvastatin) and squalene epoxidase inhibitors (TU-2078 and NB-598). All inhibitors completely inhibited the cholesterol synthesis in L6 myoblasts at doses of 1 and 3 μM. Simvastatin (3 μM) inhibited the fusion reaction of L6 myoblasts followed by the severe cellular damage. The myoblasts also had failed actin fiber formation and creatinine phosphokinase (CPK) production. Additionally, this agent also caused apoptotic cell death in differentiated L6 muscle fiber, indicating that skeletal myopathy by HMG-CoA reductase inhibitors seems to occur not only in differentiating immature myoblasts but also in matured skeletal myotubes. In contrast, TU-2078 and NB-598 had no effect on the fusion reaction of differentiating myoblasts or on the cellular viability of muscle fiber at 3 μM, enough to completely inhibit cholesterol biosynthesis. It is conceivable that the mevalonate depletion and subsequent failure of ras farnesylation induced by simvastatin might cause the defects in differentiation and maintenance of the muscle fiber. Squalene epoxidase inhibitors did not show this adverse effect presumably because of the enzyme inhibition downstream of farnesyl synthesis. The present findings suggest the safe use of squalene epoxidase inhibitors in lipid-lowering therapy.—**Matzno, S., T. Yamauchi, M. Gohda, N. Ishida, K. Katsuura, Y. Hanasaki, T. Tokunaga, H. Itoh, and N. Nakamura.** Inhibition of cholesterol biosynthesis by squalene epoxidase inhibitor avoids apoptotic cell death in L6 myoblasts. *J. Lipid Res.* 1997. **38:** 1639–1648.

Supplementary key words HMG-CoA reductase inhibitor • myopathy • squalene epoxidase inhibitor

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 (1). Inhibitors of this enzyme, such as simvastatin, lovastatin, and pravastatin, are widely used for patients with hyperlipidemia. Recently, several investigators described the adverse effects of these drugs (2). Myopathy is a prominent and severe adverse effect of HMG-CoA reductase inhibitors, exhibiting clinical signs of diffuse myalgia, muscle tenderness, and elevation of blood creatinine phosphokinase (CPK) concentrations (3). Administration of these drugs in combination with cyclosporine or gemfibrozil causes a higher incidence of this myopathy (4, 5). The depletion of a certain isoprenoid intermediate may cause the myopathy in HMG-CoA reductase inhibitors treatment (6, 7). However, the onset mechanism of myopathy is still controversial.

More recently, another rate-limiting enzyme in the cholesterol biosynthesis pathway, squalene epoxidase (EC 1.14.99.7), was generated as a target for the inhibition of cholesterol biosynthesis (8, 9). This enzyme catalyzes the oxidation of squalene to 2,3-oxidosqualene in the middle stage of the cholesterol biosynthesis pathway. Squalene epoxidase is located downstream of the branch points for the synthesis of isoprenoid compounds such as farnesylated proteins, ubiquinone and dolichol (10). Thus, the inhibitory agents for this enzyme are expected to reduce the serum lipid levels without inhibiting isoprenoid synthesis (11).

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CPK, creatinine phosphokinase; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; α-MEM, alpha-minimum essential medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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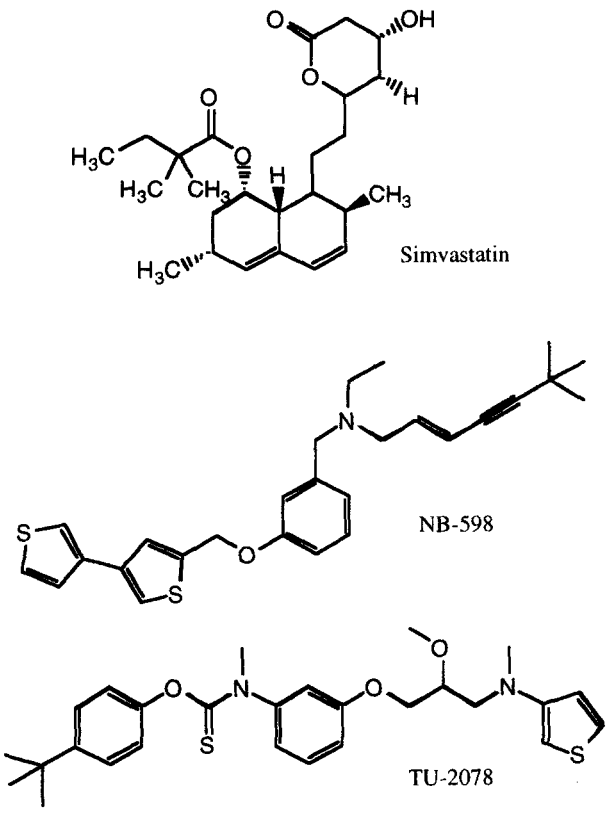

Fig. 1. Chemical structures of simvastatin, TU-2078 and NB-598.

Rat L6 myoblast cell lines have been used for studying myogenesis because these cells start differentiating into skeletal myotube-specific structures when a culture reaches confluence (12). To determine the involvement of the inhibition of cholesterol biosynthesis on skeletal myopathy, we compared the effect of two lines of cholesterol biosynthesis inhibitors, HMG-CoA reductase inhibitor (simvastatin) and squalene epoxidase inhibitors (TU-2078 and NB-598, **Fig. 1**), on the fusion reaction as well as in differentiated skeletal myotubes of L6 myoblasts.

MATERIALS AND METHODS

Materials

TU-2078 and NB-598 were synthesized in the Tokyo Research Laboratory, Tosoh Corporation (Kanagawa, Japan). Simvastatin was extracted from Lipovas®-5 tablets (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) in our laboratories. All drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C until use. The final concentration of DMSO in the medium did not exceed 0.3% (v/v).

Sodium acetate and DL-mevalono-1,5-lactone were purchased from Nacalai Tesque (Kyoto, Japan), [1-^{14}C]acetate (sodium salt) was from Du Pont/NEN Research Products (Dreiech, Germany), poly-L-lysine was from Sigma Chemical Co. (St. Louis, MO); Hoechst 33342 was from Molecular Probes (Eugene, OR); anti-myosin antisera was from Chemicon (Temecula, CA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) was from Cappel™ Research Products (Organon Teknika Corporation, Durham, NC). Mevalonate was prepared each day before use from DL-mevalono-1,5-lactone by solubilizing in 0.1 N NaOH, incubating at 50°C for 1 h, and neutralizing with HCl (6).

Cell culture

Rat skeletal myoblast cell line L6 (passage 14) was purchased from Dainippon Pharmaceutical Inc. (Osaka, Japan). They were grown in alpha-minimum essential medium (α-MEM) containing 100 IU/ml penicillin G and 100 μg/ml streptomycin (medium A) and 10% fetal calf serum (FCS). They were subcultured every 3 days, and 17–20 passages were used in the experiments.

Determination of cholesterol synthesis

All following morphological studies were done by triplicate determinations. On day 0, L6 (1.5×10^5 cells/well) were seeded into 6-well tissue culture plates (Falcon®, Becton Dickinson Labware, Lincoln Park, NJ) and cultured. On day 3, the cells were washed twice with Hanks' balanced salt solution (HBSS), and the medium was replaced with medium A containing 1% FCS to synchronize the fusion reaction (12). On day 7, cells were washed and fresh medium containing 1% FCS and drugs were added. They were cultured for 1 h and washed twice with HBSS. Then the cells were incubated with 2 ml of medium A (without serum) containing each drug and [1-^{14}C]acetate (3 mM, 2×10^6 dpm/well) for 24 h. After washing twice with phosphate-buffered saline (PBS), they were scraped with a rubber policeman and suspended in 0.8 ml of PBS. They were then sonicated for 3 min, and their lipid fraction was extracted according to the method of Bligh and Dyer (13). Extracts were evaporated under N_2 gas, the residue was dissolved in 10 μl chloroform, and lipids were separated by thin-layer chromatography using hexane–diethyl ether–acetic acid 70:30:1 (v/v/v) as the solvent. The cholesterol and squalene bands were obtained, and their radioactivity was counted in a liquid scintillation counter.

Indirect immunofluorescence

Indirect immunofluorescence staining was used to determine myosin fiber formation. For this experiment,

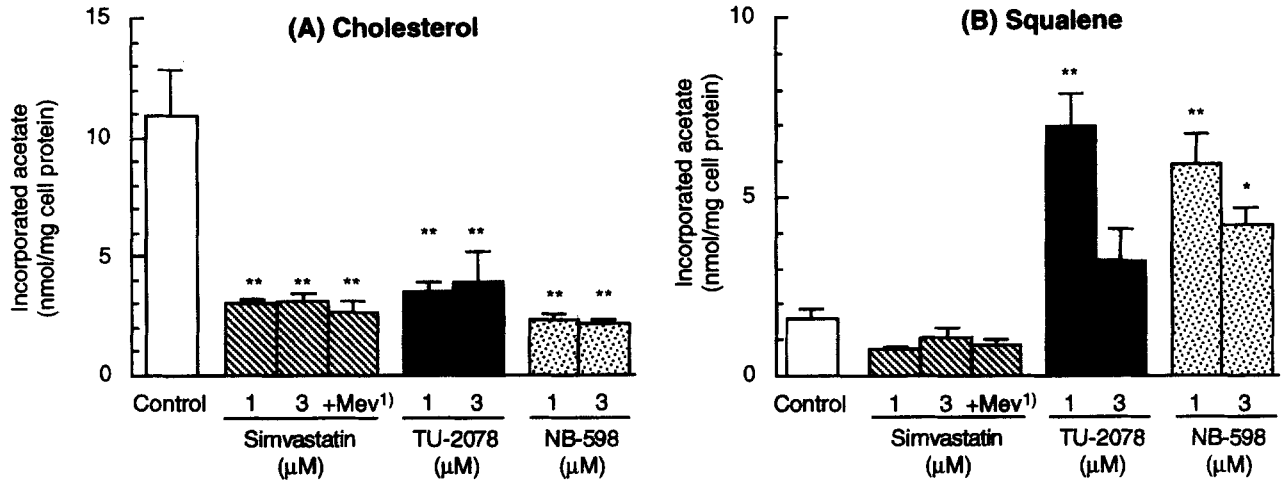

Fig. 2. Effects of simvastatin, NB-598, and TU-2078 on cholesterol (A) and squalene (B) synthesis in differentiated L6 muscle fiber. On day 7, differentiated fibers were incubated with drugs for 1 h, then after washing, [^{14}C]acetate (2×10^6 dpm, 3 mM) was added. After 24-h incubation, lipid-incorporated [^{14}C]acetate was measured. All values represent mean ± SE (n = 4). Significantly different from control, $^*P < 0.05$, $^{**}P < 0.01$. [1]Simvastatin (3 μM) in the presence of mevalonate (0.4 mM).

the cells were grown on the poly-L-lysine-coated cover glass (24 × 24 mm, Matsunami Glass Ind. Ltd., Osaka, Japan).

On day 3, the cells were washed and incubated for 96 h with drugs under the fusion reaction as described above. On day 7, they were washed twice with PBS, fixed with 50% methanol for 30 sec, and washed. The cells were blocked with 2 ml of PBS containing 5% skim milk for 1 h at room temperature and rinsed twice with PBS. Then, the cells were incubated with anti-myosin antisera that was diluted 1:100 with PBS containing 0.1% bovine serum albumin (BSA-PBS) for 2 h and rinsed. They were incubated under dark condition with FITC-conjugated goat anti-rabbit IgG (diluted 1:100 with BSA-PBS) for 2 h at room temperature. Immunostained cells were then washed twice with PBS and observed by a fluorescence microscope.

CPK assay

On day 3, the cells were washed and incubated for 96 h with drugs under the fusion reaction. On day 7, they were washed twice with PBS, 1 ml of PBS was added, and cells were scraped with a rubber policeman and suspended in PBS. They were sonicated for 3 min, and their CPK activity was measured using a CPK assay kit (Wako Pure Chemical, Osaka, Japan). One mU of CPK is equal to 1 mmole NADPH formed/min at 30°C. Protein concentration was determined by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA) using human IgG as a standard.

Karyopyknotic observation

Cells were grown and differentiated on the poly-L-lysine-coated cover glasses as described above. On day 7, the differentiated fibers were washed and treated with medium A containing 1% FCS and drugs for 24 or 48 h. Then, they were fixed with 1% glutaraldehyde, stained with hematoxylin–eosin and/or 1 mM Hoechst 33342, and examined under light and/or fluorescein microscopy.

DNA fragmentation assay

Cells were grown and differentiated in 100-mm tissue culture dishes in this assay. On day 7, the fibers were washed and treated with medium A containing 1% FCS and drugs for 24 or 48 h. Then, they were washed with PBS and scraped with a rubber policeman. Chromosomal DNA was extracted using the TurboGen™ Genomic DNA Isolation Kit (Invitrogen Corp., San Diego, CA). DNA was electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV fluorescence.

Analysis of data

Statistical significance was assessed by one-way analysis of variance and Dunnett's multiple comparison method.

RESULTS

Inhibition of cholesterol synthesis.

Figure 2 shows the effects of NB-598, TU-2078, and simvastatin on cholesterol synthesis in differentiated L6 muscle fibers. All compounds inhibited cholesterol synthesis at 1 and 3 μM. Coexistence of mevalonate (0.4

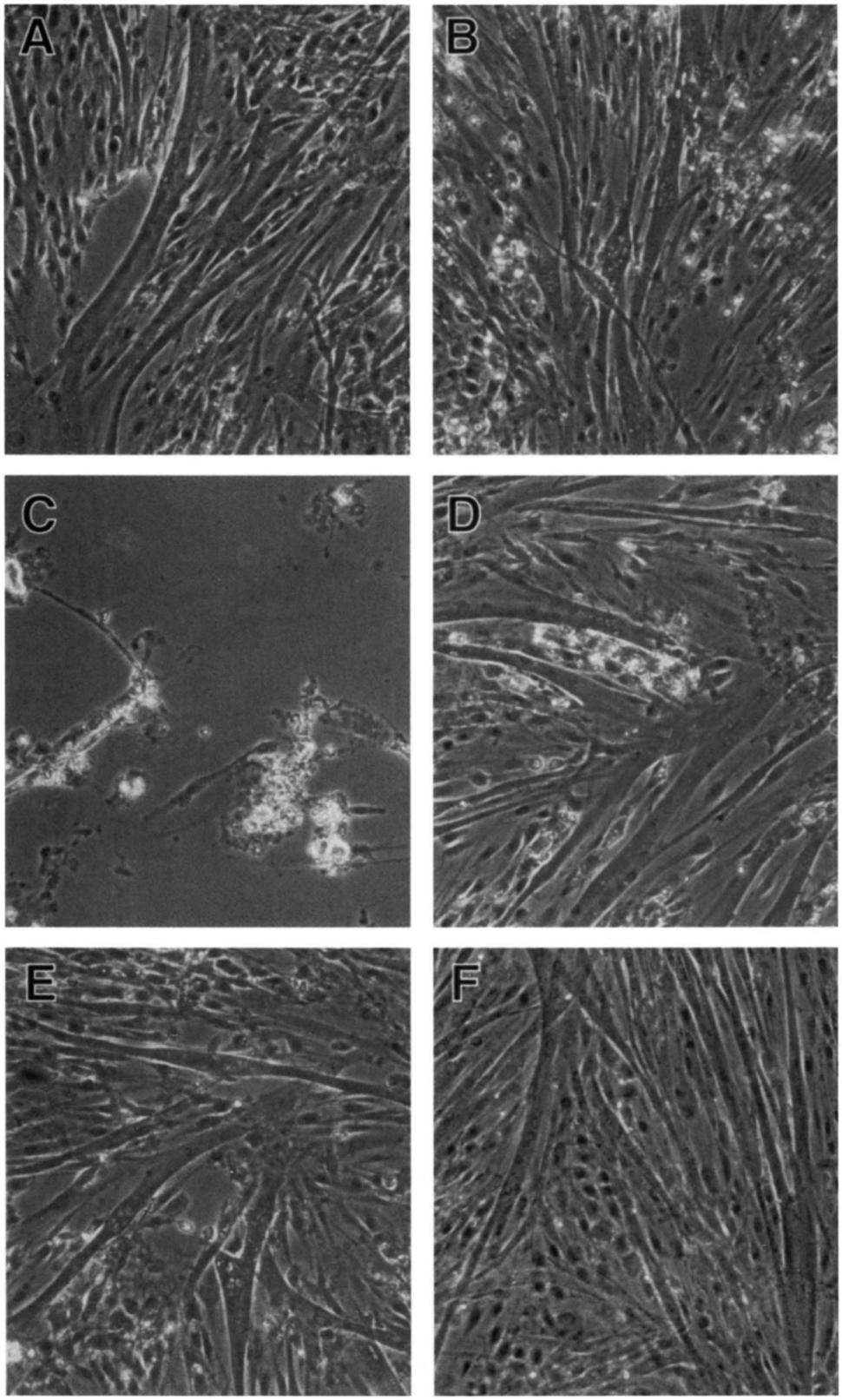

Fig. 3. Effects of simvastatin, TU-2078 and NB-598 on the differentiation of L6 myoblasts into muscle fiber. After confluence on day 3, medium was changed to contain 1% FCS and drugs. After 96-h incubation, the cells were observed under phase contrast microscopy. A: control, B: simvastatin (1 μM), C: simvastatin (3 μM), D: simvastatin (3 μM) in the presence of mevalonate (0.4 mM), E: TU-2078 (3 μM), F: NB-598 (3 μM). Magnification: ×400.

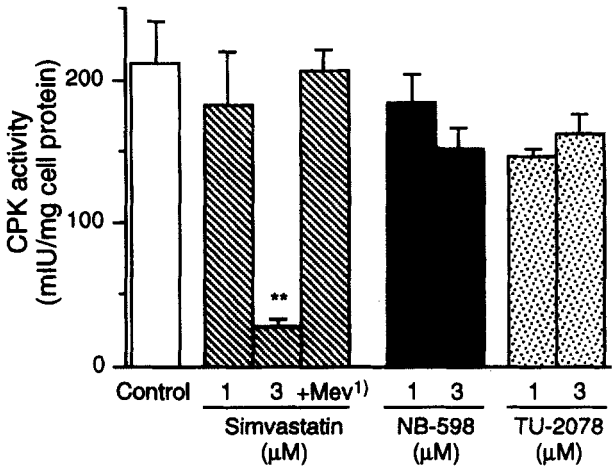

Fig. 4. Effects of simvastatin, TU-2078, and NB-598 on cellular CPK activities in cultured L6 myoblasts. Intracellular CPK in the cells of Fig. 3 were measured. All values represent mean ± SE (n = 3). Significantly different from control, $^{**}P < 0.01$. [1]Simvastatin (3 μM) in the presence of mevalonate (0.4 mM).

mM) did not affect the inhibitory activity of simvastatin on cholesterol synthesis. On the other hand, the cells treated with TU-2078 and NB-598 markedly increased their squalene content (Fig. 2B), whereas simvastatin-treated fibers did not.

Effects of compounds on the differentiation process

We investigated the effects of simvastatin on the fusion reaction of L6 myocytes. Each typical pattern of triplicate determinations is shown in **Fig. 3.** Although L6 cells treated with a low dose of simvastatin (1 μM) during differentiation showed slight inhibition of the fusion reaction (Fig. 3B), a high dose (3 μM) resulted in the severe cellular damage by day 7 (Fig. 3C). Interestingly, mevalonate (0.4 mM) completely abolished the simvastatin-induced cellular damage (Fig. 3D), whereas two squalene epoxidase inhibitors, TU-2078 (Fig. 3E) and NB-598 (Fig. 3F), at a concentration of 3 μM did not affect the fusion reaction of myocytes during differentiation.

Figure 4 shows the cellular CPK activities of L6 myocytes at day 7. Cellular CPK was markedly depressed in simvastatin (3 μM)-treated cells, and it was recovered by mevalonate (0.4 mM) application. We also detected the skeletal actin fiber by indirect immunofluorescence staining (**Fig. 5**). On day 7, multi-fused L6 myoblasts strongly expressed intracellular actin fiber (A). Simvastatin (3 μM)-treated cells (B) mostly eliminates intracellular actin, and mevalonate (0.4 mM) addition completely recovered the actin formation (C). TU-2078 (D) and NB-598 (E) treatment at a concentration of 3 μM did not affect actin fiber formation under fluorescence microscopic observation.

Effects of compounds in differentiated L6 muscle fibers

We also studied the effects of drugs in skeletal myotubes differentiated from L6 myoblasts. For this evaluation, drugs were added to the medium at day 7. **Figure 6** shows each typical pattern of triplicate determinations. After 72-h treatment, simvastatin (1 μM) caused the dysfunction of muscle fiber, and drug increments up to 3 μM led to severe muscular damage (Fig. 6C). Mevalonate (0.4 mM) addition completely abolished the simvastatin-induced cellular damage (D). However, no abnormal morphologies were observed in L6 myotubes treated with TU-2078 (E) or NB-598 (F).

Muscle fiber chromosomes were stained with Hoechst 33342. Conspicuous changes were not found in any 24 h-treated fibers; however, after 48-h treatment with simvastatin (3 μM), many karyopyknotic cells were observed (**Fig. 7**). Agarose gel electrophoresis (**Fig. 8**) revealed that 24- and/or 48-h treatment with simvastatin (3 μM) caused 180–200 bp (one chromatin-unit) interval chromosomal DNA fragmentation (lanes 2 and 7), whereas TU-2078 (lanes 4 and 9) and NB-598 (lanes 5 and 10) did not. Coexistence of mevalonate (0.4 mM) with simvastatin avoided the DNA fragmentation (lanes 3 and 8).

DISCUSSION

This study evaluated the skeletal myopathy of cholesterol biosynthesis inhibitors with different target points, that is, HMG-CoA reductase inhibitor and squalene epoxidase inhibitor, in the cultured skeletal myoblast cell line L6.

Currently, the family of HMG-CoA reductase inhibitors is in widespread clinical use and can lower LDL cholesterol by 45% with a very low incidence of adverse effects (14). However, morbidity is increased when inhibitors are administered in combination with a number of other drugs, such as cyclosporin, niacin, and fibrates. Skeletal myopathy is one of the most serious adverse effects of HMG-CoA reductase inhibitors. Ghirlanda et al. (15) reported that treatment with HMG-CoA reductase inhibitors lowered both plasma cholesterol and ubiquinone levels in normal volunteers and in hypercholesterolemic patients, suggesting that a depletion of ubiquinone availability caused membrane alteration with consequent cellular damage. Belo, Jamieson, and Wright (12) indicated that HMG-CoA reductase inhibitors obstructed L6 myoblast fusion by sup-

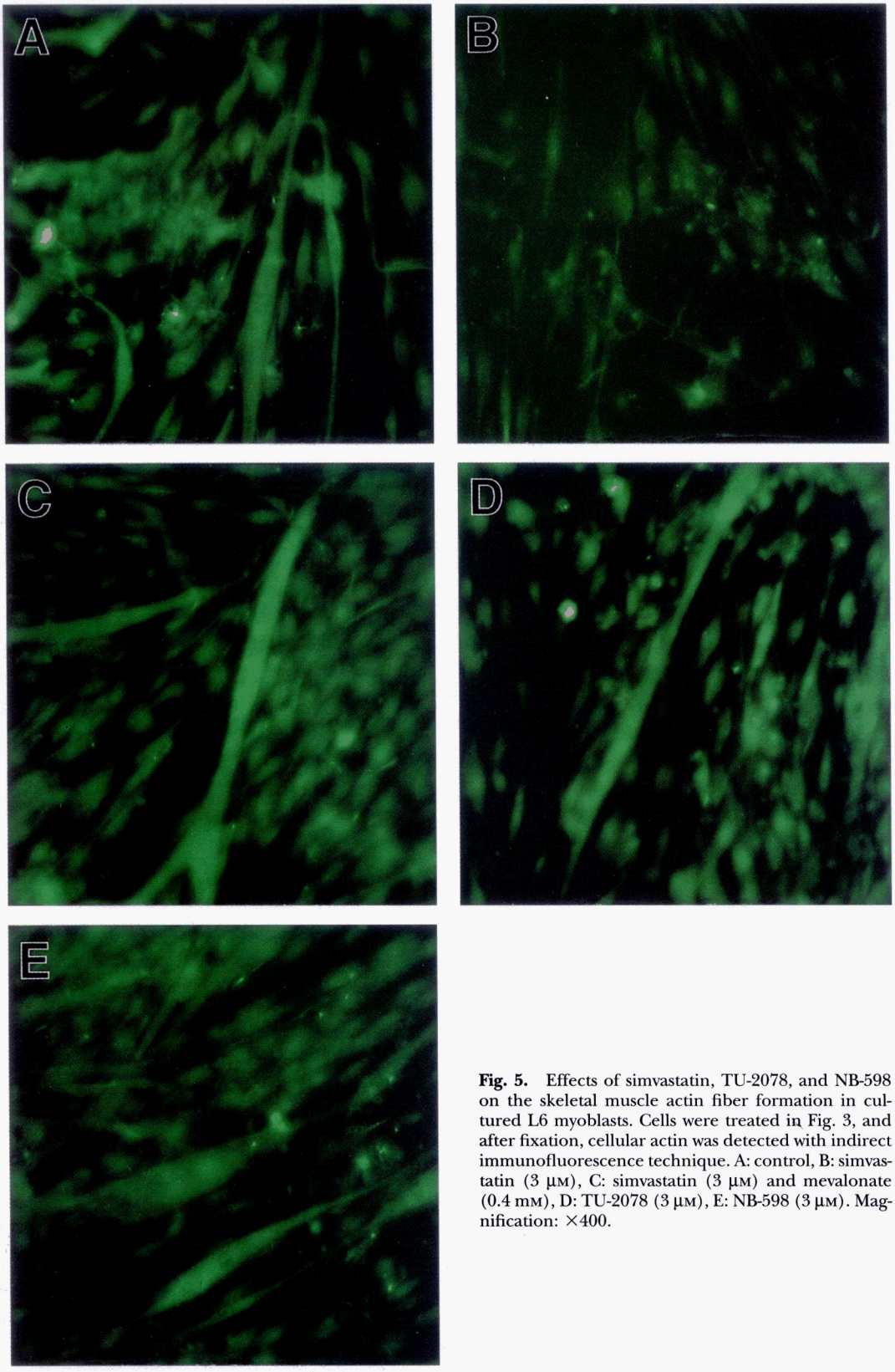

Fig. 5. Effects of simvastatin, TU-2078, and NB-598 on the skeletal muscle actin fiber formation in cultured L6 myoblasts. Cells were treated in Fig. 3, and after fixation, cellular actin was detected with indirect immunofluorescence technique. A: control, B: simvastatin (3 μM), C: simvastatin (3 μM) and mevalonate (0.4 mM), D: TU-2078 (3 μM), E: NB-598 (3 μM). Magnification: ×400.

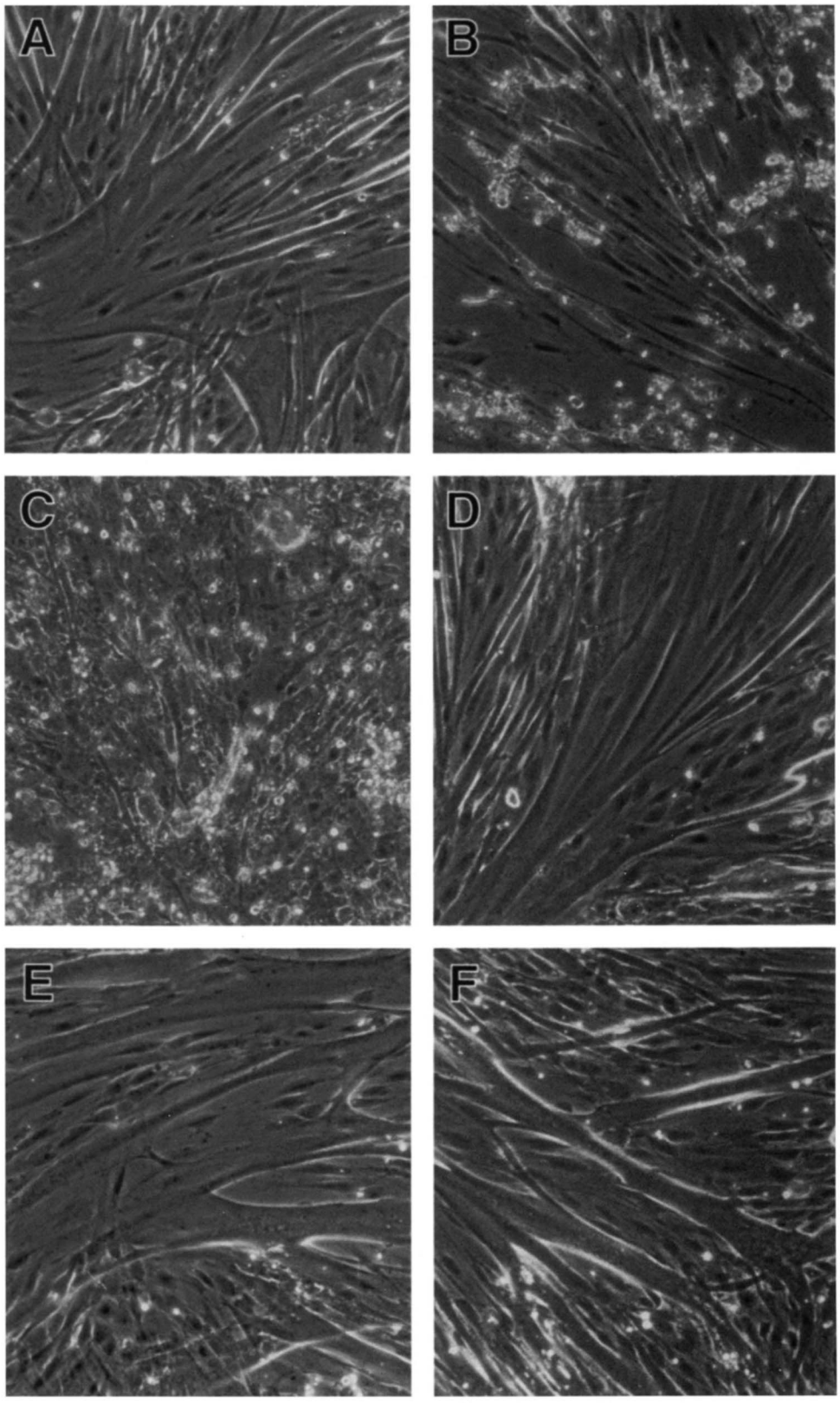

Fig. 6. Effects of simvastatin, TU-2078, and NB-598 on the L6 differentiated previously into muscle fiber. After differentiation (on day 7), medium was changed to contain 1% FCS and drugs. After 72-h incubation, the cells were observed under phase contrast microscopy. A: control, B: simvastatin (1 μM), C: simvastatin (3 μM), D: simvastatin (3 μM) in the presence of mevalonate (0.4 mM), E: TU-2078 (3 μM), F: NB-598 (3 μM). Magnification: ×400.

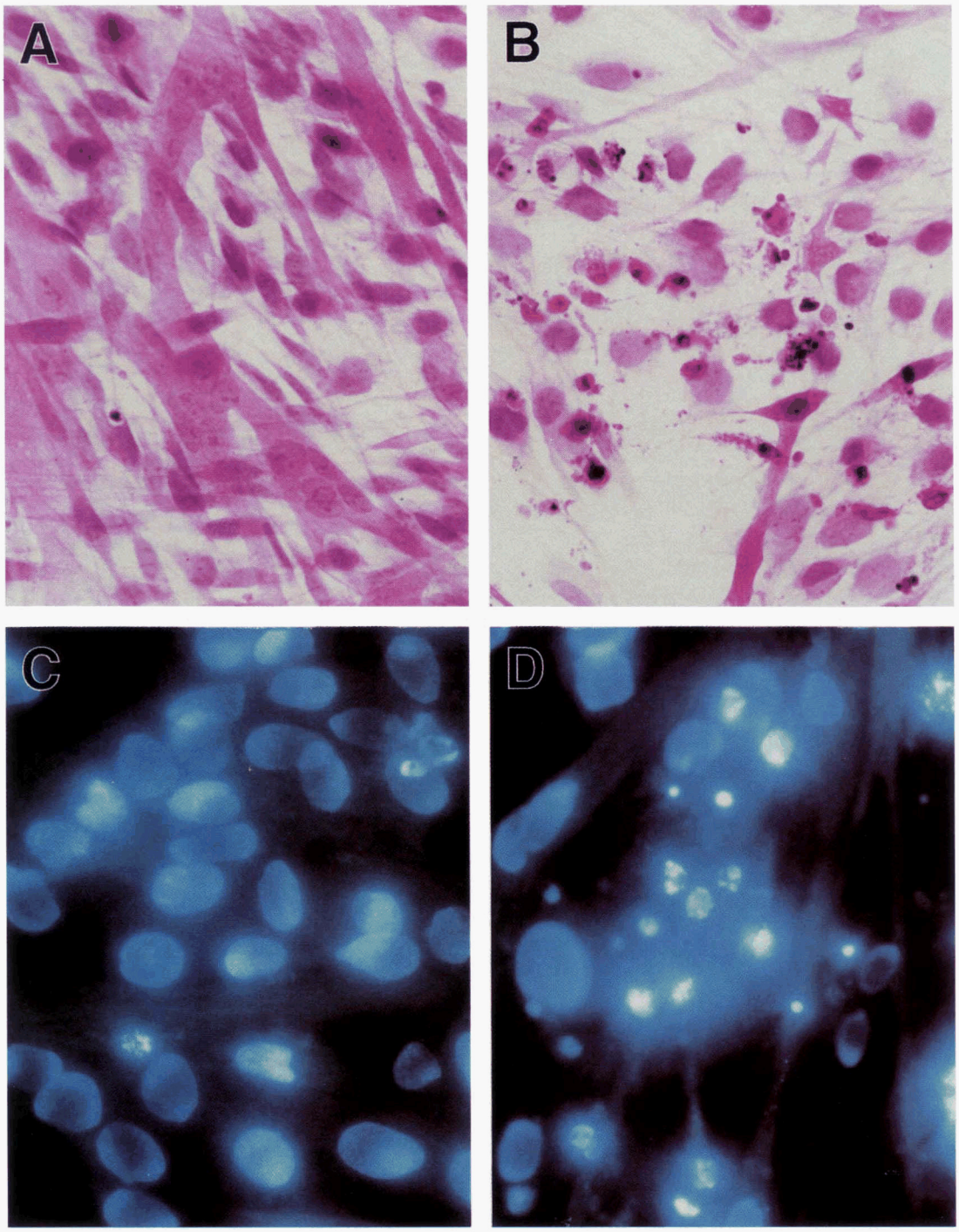

Fig. 7. Light and fluorescent microscopic observation of differentiated L6 muscle fiber with or without treatment with simvastatin (3 μM). After fixation cells were stained with hematoxylin–eosin (A and B) or Hoechst 33342 (C and D). A and C: control; B and D: simvastatin. Magnification: ×400.

pressing the dolichol synthesis. Munro et al. (6) observed that the antiproliferative effect of lovastatin in vascular smooth muscle cells was reversed by adding farnesol, a isoprenoid precursor derived from mevalonate. These studies suggest that the HMG-CoA inhibitor-induced myopathy can be attributed to the depletion of non-cholesterol isoprenoid intermediates. In contrast, earlier electromyographic study (16) indicated that the administration of HMG-CoA reductase inhibitors in rabbits led to lesions of the muscle surface membrane. They also showed that membrane lesions were due to the depletion of membrane cholesterol content, sug-

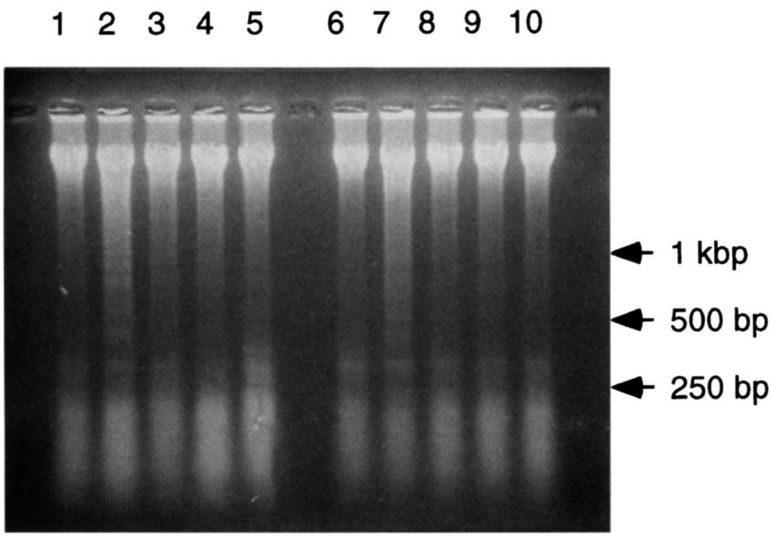

Fig. 8. Chromosomal DNA examination using agarose gel electrophoresis. Chromosomal DNA from 24 (lanes 1–5) or 48 (lanes 6–10) h-treated cells (see Materials and Methods) was extracted. Lanes 1 and 6: control; 2 and 7: simvastatin (3 μM); 3 and 8: simvastatin (3 μM) with 0.4 mM mevalonate; 4 and 9: TU-2078 (3 μM); 5 and 10: NB-598 (3 μM).

gesting that myopathy from exposure to HMG-CoA reductase inhibitors is caused by its cholesterol-lowering effect.

The present study shows that skeletal myopathy in L6 did not occur after administration of squalene epoxidase inhibitors at the concentration of 3 μM (Fig. 6), a dose sufficient for complete inhibition of cholesterol synthesis (Fig. 2). Consequently, this myopathy might result from the depletion of a certain isoprenoids, not cholesterol per se. Supporting this idea, simvastatin-induced myopathy was completely abolished by the supplementation of mevalonate, an immediate metabolite of HMG-CoA and a precursor of all isoprenoids (**Fig. 9**).

Simvastatin abolished the differentiation process of L6 cells (Fig. 3) with the inhibition of actin filament formation (Fig. 4) and cellular CPK expression (Fig. 5). Thus, isoprenoids production is essential for the functional differentiation of L6 into muscle fiber. In addition, simvastatin also damaged the post-differentiated myotubes (Fig. 6), indicating that isoprenoids production is also necessary for the maintenance of skeletal muscle function. As a common signal of differentiation and functional maintenance, we noted the isoprenylation of ras protein, which is required for its biological function.

Ras protooncogene plays a crucial role in cell growth and differentiation. For the activation and signal re-

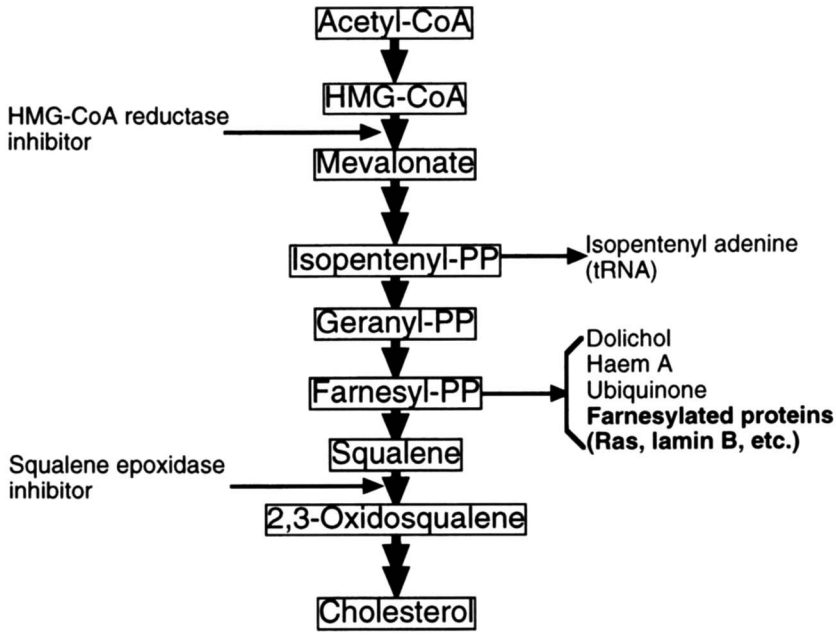

Fig. 9. The cholesterol biosynthesis pathway.

sponse of ras protein, farnesyl residue, which is derived from farnesyl pyrophosphate, must be covalently bound to the C-terminal of $p21^{ras}$ protein and anchored to the plasma membrane (17). In the pheochromocytoma cell line (PC12), ras protein promoted the morphological differentiation into neuron-like cells (18, 19). In many instances, the ras signal is essential to maintain cell function. In HL-60 cells, the lovastatin-treated cells died via apoptosis by an impairment of the membrane association of ras protein (20). Likewise, our observation in the differentiated muscle fiber showed karyopyknosis (Fig. 7) and typical DNA laddering (Fig. 8). Therefore, we concluded that simvastatin-treated muscle fiber failed to farnesylate the ras protein, compromised the membrane anchoring, and made DNA accessible to fragmentation. In anaplastic myoblasts, dysfunction of ras protein also hindered the differentiation process, and the cells were detached from dishes. Further investigation for cellular ras-protein distribution would clarify this hypothesis.

Mevalonate (0.4 mM) supplementation did not restore the [^{14}C]cholesterol production in L6 myocytes (Fig. 2) regardless of the resolution of cellular function (Figs. 3 and 6). It seems that the addition of excess amount of non-labeled mevalonate competed with [^{14}C]acetate-specific cholesterol production and decreased [^{14}C]cholesterol radioactivity. Further investigations are needed to reveal the detail of cholesterol synthesis pathway.

In conclusion, simvastatin-induced myopathy is caused by mevalonate depletion. Subsequent failure of ras farnesylation might be responsible for the effect in differentiation and maintenance of the muscle fiber. Squalene epoxidase inhibitors did not show this adverse effect presumably because of the enzyme inhibition downstream of farnesyl synthesis. The present findings suggest the safe use of squalene epoxidase inhibitors in lipid-lowering therapy.

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