Prevention of Simvastatin-Induced Inhibition of Tendon Cell Proliferation and Cell Cycle Progression by Geranylgeranyl Pyrophosphate

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ABSTRACT

Statins have been reported to induce tendinopathy and even tendon rupture. The present study was designed to investigate the potential molecular mechanism underlying the adverse effect of simvastatin on tendon cells. An in vitro tendon healing model was performed using tendon cells isolated from rat Achilles tendons. The viability of tendon cells and cell cycle progression were examined by the MTT assay and flow cytometric analysis, respectively. Immunofluorescent staining for Ki-67 was used to assess the proliferation activity of tendon cells. Western blot analysis and coimmunoprecipitation was used to determine the protein expression of cell cycle-related proteins. To investigate the potential mechanism underlying the effect of statins on tendon cells, mevalonate, farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP) was added to simvastatin-treated tendon cells. Simvastatin inhibited the in vitro tendon healing model and tendon cell proliferation in a dose-dependent manner. Immunofluorescent staining demonstrated reduced ki-67 expression in simvastatin-treated tendon cells. Furthermore, simvastatin induced cell cycle arrest at the G1 phase. The expression levels of cdk1, cdk2, cyclin A, and cyclin E were downregulated by simvastatin in a dose-dependent manner. The inhibitory effect of simvastatin was proved to mediate the reduction of mevalonate, and the addition of exogenous GGPP completely prevented the inhibitory effect of simvastatin on tendon cells. The present study demonstrated, for the first time, the molecular mechanism underlying simvastatin-induced tendinopathy or tendon rupture. GGPP was shown to prevent the adverse effect of simvastatin in tendon cells without interfering with its cholesterol-reducing efficacy.

Key words: simvastatin; tendon cell; proliferation; geranylgeranyl pyrophosphate

Statins are a class of lipid-lowering drugs clinically used to inhibit 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA), a key enzyme in the mevalonate metabolic pathway for endogenous cholesterol production. Mevalonate plays a central role in cholesterol synthesis via squalene, and is a precursor of several isoprenoid derivatives, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Because statins are the most effective therapeutic agents for reducing cholesterol synthesis and serum low-density lipoprotein (LDL) levels, they are widely used by patients with hyperlipidemia to reduce the incidence of cardiovascular events (Kashani et al., 2008; Marschner et al., 2001; Pullatt et al., 2007). However, with the wider use of statins, more adverse effects have been reported, especially musculoskeletal complications, such as myalgia, myositis,
rhabdomyolysis, and myopathies (Kashani et al., 2008; Marschner et al., 2001). Recent case reports have implicated statins in the development of tendinopathy (Carmont et al., 2009; Chazerain et al., 2001; Pullatt et al., 2007). Although all statins may cause tendinous complications, an analysis of statin-attributed tendinous complications showed that 86.2% were associated with simvastatin followed by atorvastatin (10.4%) (Marie et al., 2008). In addition, Marie et al. retrospectively identified cases of statin-associated tendinopathy, and the results suggested that tendinopathies more often occurred within the first year after statin initiation and most were related to atorvastatin and simvastatin use (37% and 31%, respectively). In a case series study, 65.5% of cases were tendinitis and 34.4% were tendon ruptures (Marie et al., 2008), and the Achilles tendon was the most common location of tendon lesions (52%).

Tendon cell proliferation is a principal step in the regeneration phase of tendon healing (Broughton et al., 2006; Leadbetter, 1992). In a previous study, we reported that inhibition of cell proliferation was responsible for ciprofloxacin-associated tendinopathy (Tsai et al., 2008). Cell cycle progression is tightly controlled by the sequential activation of several protein kinases, known as cyclin-dependent kinases (cdks), through the formation of complexes with various cyclins (Pines, 1994). Statins have been shown to inhibit cell cycle progression. However, the exact mechanism varies among cell types (Denoyelle et al., 2001; Ghosh et al., 1999) and the effect of simvastatin on tendon cell proliferation has not yet been investigated.

The present study was designed to investigate whether simvastatin, a commonly prescribed statin, exerts any adverse effect on tendon cell proliferation, to explore the underlying molecular mechanisms, and to determine whether mevalonate, GGPP, and FPP can reverse the inhibition of tendon cell proliferation.

**MATERIALS AND METHODS**

**Primary culture of rat Achilles tendon cells.** Achilles tendons were obtained from Sprague–Dawley rats (weighing 200–250 g). The excised tendons were cut into small pieces (1.5–2.0 mm³) and placed in culture plates. The tendon tissues were incubated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies, Grand Island, New York, USA) containing 20% fetal bovine serum (FBS; Gibco, Life Technologies) and 100 U/ml penicillin/100 µg/ml streptomycin (Gibco, Life Technologies) at 37°C in a humidified atmosphere with 5% CO₂. After migrating from the explants, the cells started to grow rapidly. The confluent cells were sub-cultured by digestion with 0.05% trypsin/EDTA (Gibco, Life Technologies). Tendon cells between passages 2 and 4 within the proper growth range and with normal fibroblast shapes were used in the experiments.

**In vitro tendon healing model.** Tendon cells were grown on plastic dishes in DMEM with 10% FBS. The tendon cell monolayer in the plastic dishes was scraped with a sterile pipette tip to produce a linear cell-free zone (1-mm in diameter). Tendon cells were either untreated or treated with 1, 5, or 10 µM simvastatin (Sigma-Aldrich, St. Louis, Missouri, USA) for 24 h. During the 24-h treatment period, tendon cell outgrowth began, and the cells migrated into the cell-free zone, which was considered as the beginning of the in vitro healing process. The in vitro wound was photographed after 24 h of treatment. Relative wound closure was quantified using NIS Elements BR software (Nikon, Japan).

**Cell viability testing.** Cell viability was assessed by the MTT assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) reagent. After the tendon cells were treated with 1, 5, or 10 µM simvastatin for 24 h (or left untreated), MTT reagent (50 µg/ml) was added to the culture plate, which was incubated at 37°C for 1 h. The supernatant was discarded, and 200 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Aliquots were transferred to a 96-well plate, and the absorbance at 595 nm was immediately read using a multi-well spectrophotometer (VICTORTM X3; Perkin Elmer Inc., Waltham, Massachusetts, USA).

**Lactate dehydrogenase (LDH) cytotoxicity assay.** Tendon cells were either left untreated or treated with 1, 5, or 10 µM simvastatin for 24 h, and then the culture medium was transferred to another 96-well plate. The presence of LDH in the conditioned medium was detected using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wisconsin, USA) according to the manufacturer’s instructions. Briefly, LDH reaction mix was added to the microplate and incubated for 30 min at room temperature. Then, the absorbance at 490 nm was read with a multi-well spectrophotometer (VICTORTM X3).

Mevalonate analysis by ultra-performance liquid chromatography tandem mass-spectrometry (UPLC–MS/MS). The cell pellet was dissolved in 1 ml methanol and vortex on ice. The cell suspension was stand on ice for 5 min, and then centrifuged at 14 000 g for 30 min at 4°C. The supernatant was transferred to new vial and completely dried under nitrogen gas. The residue was dissolved in 90 µl 10 mM ammonium formate (pH 8.0) for UPLC–MS/MS analysis. The Acquity UPLC system (Waters Corporation, Milford, Massachusetts, USA) consisted of a binary solvent manager, a vacuum degasser, a column heater and sample manager. The column temperature was maintained at 35°C. The samples were injected onto an Acquity UPLC HSS T3 C18 column, 100 × 2.1 mm, 1.8 µm particle diameter (Waters Corporation). The mevalonate was separated by a linear gradient between solution A (10 mM ammonium acetate, pH 8.0) and acetonitrile as a solution B. The flow rate was 3 ml/min and the gradient was as follows: 0–1.5 min, 1% B; 1.5–2.0 min, 1–99% B; 2.0–4 min, 99% B; 4.0–4.5 min, 1% B. For each analysis, 7 µl of sample was injected onto the column, and the total analysis time, including equilibration, was 8 min. A Xevo TQMS (Waters Corporation) was used in the negative electrospray ionization mode. Nitrogen was used as desolvation gas (1000 l/h) and cone gas (150 l/h). The desolvation temperature was 500°C. The cone voltage was 2 V and the collision energy was 12 eV. The capillary voltage was set at 0.5 kV, and the source temperature was 150°C. The mevalonate was detected in multiple reaction monitoring (MRM) mode with a dwell time of 0.045 s. The parent and product ions (m/z) were 147.03 and 59.05, respectively.

**Cell cycle analysis.** Tendon cells that were either untreated or treated with simvastatin or GGPP for 24 h were washed twice in phosphate-buffered saline (PBS) and fixed with 1 ml of 70% ethanol in PBS for 1 h at –20°C. The cells were then centrifuged at 3000 × g for 5 min, resuspended in 1 ml of 0.5% Triton X-100 (Sigma-Aldrich) and 0.05% RNase A (Sigma-Aldrich) in PBS, and incubated at 37°C for 1 h. The cell suspension was centrifuged, washed, and resuspended in 1 ml of 50 µg/ml propidium iodide (PI) (Sigma-Aldrich) solution in PBS. Cells were incubated overnight at 4°C, and then analyzed by flow cytometry (FACScan; Becton Dickinson, San Francisco, California, USA).

**Immunofluorescence staining.** Tendon cells grown on glass coverslips in the bottom of plastic dishes were either treated with or
without 1, 5, or 10 μM simvastatin for 24 h. Tendon cells were fixed with 4% paraformaldehyde in PBS (pH 7.5) for 15 min. After washing 3 times in PBS, the glass coverslips were incubated in blocking solution (5% BSA in PBS) at room temperature for 1 h, and the incubated in blocking solution containing a mouse monoclonal anti-ki67 antibody (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 2 h. After washing, the glass coverslips were incubated with PBS containing fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Leinco Technologies, Inc., St. Louis, MI, USA) for 1 h. Finally, chromosomes were stained with propidium iodide (PI), and the cells were observed under a fluorescence microscope (Eclipse Ni-U; Nikon, Japan).

Western blot analysis. Total protein in tendon cells was extracted in lysis buffer (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na3VO4, 1 mM Na2P2O7, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1% Triton X-100). The protein concentration of the cell extracts was determined using the Bradford assay (Bio-Rad Laboratories, Richmond, California, USA). Then, 10 μg of total protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane. The membrane was incubated in blocking solution (5% BSA in TBST) at room temperature for 1 h, and then incubated for 2 h in blocking solution containing an appropriate dilution of primary antibody (i.e. mouse monoclonal anti-tubulin (Thermo Fisher Scientific), rabbit polyclonal anti-cdk1, anti-cdk 2, and anti-cyclin B1 (Abclonal, Cambridge, MA, USA), and rabbit polyclonal anti-cyclin E1, anti-cyclin A2 (Santa Cruz Biotechnology, Santa Cruz, California, USA), and phosphorylated cdk1 (Abcam, Cambridge, UK), and phosphorylated cdk2 (Cell Signaling Technology, Danvers, Massachusetts, USA)). After washing, the membranes were incubated in TBST containing a horseradish peroxidase-conjugated goat anti-mouse IgG (Leinco Technologies, Inc.) or goat anti-rabbit IgG (Cell Signaling Technology, Danvers, Massachusetts, USA) at 4°C. The membranes were washed 3 times in TBST and developed with Luminata Crescendo Western HRP Technology for 1 h. The membranes were incubated in blocking solution (5% BSA in TBST) at room temperature for 1 h, then incubated for 2 h in blocking solution containing an appropriate dilution of primary antibody (i.e. mouse monoclonal anti-tubulin (Thermo Fisher Scientific), rabbit polyclonal anti-cdk1, anti-cdk 2, and anti-cyclin B1 (Abclonal, Cambridge, MA, USA), and rabbit polyclonal anti-cyclin E1, anti-cyclin A2 (Santa Cruz Biotechnology, Santa Cruz, California, USA), and phosphorylated cdk1 (Abcam, Cambridge, UK), and phosphorylated cdk2 (Cell Signaling Technology, Danvers, Massachusetts, USA)). After washing, the membranes were incubated in TBST containing a horseradish peroxidase-conjugated goat anti-mouse IgG (Leinco Technologies, Inc.) or goat anti-rabbit IgG (Cell Signaling Technology, Danvers, Massachusetts, USA) at 4°C. The membranes were washed 3 times in TBST and developed with Luminata Crescendo Western HRP Technology for 1 h. The membranes were washed 3 times in TBST and developed with Luminata Crescendo Western HRP Technology for 1 h. The membranes were washed 3 times in TBST and developed with Luminata Crescendo Western HRP Technology for 1 h.

RESULTS

Simvastatin Slowed Down the In Vitro Tendon Healing Model
To study whether simvastatin exerts an inhibitory effect on tendon cell growth, we used an in vitro wound healing model. A confluent monolayer of tendon cells was wounded and treated with or without simvastatin for 24 h. As shown in Figure 1, wound closure of simvastatin-treated tendon cells appeared to be slower than that of control cells (Fig. 1A). The relative wound closure in cells treated with 0, 1, 5, and 10 μM simvastatin was 100.0 ± 0%, 78.3 ± 0.4%, 63.7 ± 6.2%, and 52.8 ± 3.2%, respectively (Fig. 1B), and the differences between the groups were statistically significant (P < .05).

Simvastatin Reduced the Number of Viable Tendon Cells
Tendon cells were treated with various concentrations of simvastatin for 24 h, and then both the MTT assay and LDH cytotoxicity assay were used to examine differences in the number of viable tendon cells. The relative viability (percentage) of 0, 1, 5, and 10 μM simvastatin-treated tendon cells as determined by the MTT assay was 100.0 ± 0%, 74.6 ± 0.7%, 88.6 ± 2.4%, and 74.6 ± 4.0%, respectively (Fig. 2A), and the differences between groups were statistically significant (P < .05). Furthermore, the relative LDH released from 0, 1, 5, and 10 μM simvastatin-treated tendon cells was 0.0%, 7.8 ± 2.2%, 7.8 ± 0.7%, and 4.6 ± 0.8%, respectively (Fig. 2B), and the differences between groups were not statistically significant. These results indicated that the simvastatin-induced decrease in the number of viable tendon cells was not due to cytotoxicity under these assay conditions. Therefore, the mechanism likely involves cell proliferation.

Simvastatin Altered the Cell Cycle of Tendon Cells
Tendon cells were incubated with simvastatin for 24 h, and then cell cycle progression was analyzed by flow cytometry. The results showed dose-dependent G1 phase arrest and a decreased number of cell in S phase for simvastatin-treated cells (Fig. 3A). The percentage of 0, 1, 5, and 10 μM simvastatin-treated tendon cells in G1 phase was 69.7 ± 6.2%, 74.6 ± 4.0%, 80.6 ± 1.8%, and 84.0 ± 2.6%, respectively (Fig. 3B), and the differences between groups were statistically significant (P < .05). In contrast, the percentage of 0, 1, 5, and 10 μM simvastatin-treated tendon cells in S phase was 17.1 ± 1.7%, 14.8 ± 2.2%, 7.8 ± 0.7%, and 4.6 ± 0.8%, respectively (P < .05; Fig. 3B). The percentage of tendon cells in G2/M phase with and without simvastatin treatment was nearly identical. Immunofluorescent staining demonstrated reduced ki67 expression in simvastatin-treated tendon cells, indicating inhibition of cell proliferation (Fig. 3C).

Simvastatin Altered the Expression of Cyclins and cdkss in Tendon Cells
To determine how the cell cycle was modulated by simvastatin, we investigated the expression of cell cycle-related protein. Tendon cells were treated with 0, 1, 5, and 10 μM simvastatin, and protein expression in cell extracts was analyzed by western blotting. The protein expression of cyclin E1, cyclin A2, cyclin B1, cdk1, and cdk2 is shown in Figure 4A. The results of the western blot and band intensity analysis revealed dose-dependent inhibition of cyclin E1, cyclin A2, cdk1, and cdk2 protein expression, but not cyclin B1 protein expression by simvastatin.

Mevalonate, GGPP, and FPP Reversed Simvastatin-Mediated Inhibition of Tendon Cell Proliferation
To examine whether simvastatin-induced inhibition of tendon cell proliferation was mediated by the depletion of mevalonate, tendon cells were treated with simvastatin in the presence of mevalonate and examined by the MTT assay. The results showed that exogenous addition of mevalonate could reverse the inhibitory effect of simvastatin on cell proliferation. The percentage of viable cells was 100.0 ± 0%, 74.6 ± 4.0%,
FIG. 1. In vitro tendon healing model. A monolayer of tendon cells grown on plastic dishes was scratched to produce a linear, cell-free zone. Tendon cells were untreated or treated with 1, 5, or 10 μM simvastatin for 24 h, and then wound was photographed at 200×. The cell-free zone is indicated by the arrows (A). Relative wound closure is shown in (B). Data are the mean ± SEM of 3 independent experiments. *p < .05.

FIG. 2. Simvastatin decreased the number of viable tendon cells in a dose-dependent manner. Tendon cells were untreated or treated with 1, 5, or 10 μM simvastatin for 24 h, and cell viability was determined by the MTT assay (A) and the LDH cytotoxicity assay (B). Data are the mean ± SEM of 3 independent experiments. *p < .05.
FIG. 3. Simvastatin caused G1 phase arrest of tendon cells. Tendon cells were treated with 0, 1, 5, or 10 μM simvastatin for 24 h, and subjected to cell cycle analysis by flow cytometry (A). The percentage of tendon cells in the G1 phase increased, whereas the percentage in the S phase decreased in a dose-dependent manner (B). Ki67-positive cells were stained with FITC (green) and cell nuclei were stained with PI (red) (C). Data are the mean ± SEM of 3 independent experiments. *p < .05. Full color version available online.

FIG. 4. Simvastatin modulated cyclin and cdk levels in tendon cells. Tendon cells were treated with 0, 1, 5, or 10 μM simvastatin for 24 h, and the protein levels in cell extracts were analyzed by western blotting (A). The relative band intensity is shown in (B). Tubulin was used as an internal control. Data are the mean ± SEM of 3 independent experiments. *p < .05.
86.5 ± 3.3%, 101.5 ± 3.6%, and 104.8 ± 3.1% for the untreated control cells, 10 μM simvastatin-treated cells, and cells treated with 10 μM simvastatin in the presence of 1, 10, and 100 μM mevalonate, respectively (Fig. 5A). GGPP and FPP, 2 downstream intermediates in the mevalonate pathway, also reversed the inhibitory effect of simvastatin on tendon cell proliferation in a dose-dependent manner (Fig. 5B). It is worth noting that 0.5 μM GGPP alone (black bar) could completely reverse the adverse effect of simvastatin on tendon cell proliferation. FPP also reversed the adverse effect of simvastatin on tendon cell proliferation; however, complete reversal required concentrations greater than 1 μM. We measured the intracellular mevalonate level by UPLC-MS/MS in tendon cells, simvastatin-treated tendon cells, and simvastatin-treated tendon cells with 1 μM GGPP. The peak area of mevalonate was determined to be 1648.4 in control cells, 1035.6 in simvastatin-treated cells, and 1054.3 in cells treated with simvastatin and GGPP. Simvastatin decreased the mevalonate level in tendon cells even in the presence of GGPP.

**Mevalonate and GGPP Reversed the Inhibitory Effect of Simvastatin on Cyclin and cdk Expression**

To determine whether the simvastatin-mediated inhibition of cell cycle-dependent protein expression could also be reversed by mevalonate and GGPP, tendon cells were treated with 10 μM simvastatin alone or in the presence of 100 μM mevalonate or 1 μM GGPP for 24 h, and protein expression in the cell extracts was analyzed by western blot analysis. The results showed that both mevalonate and GGPP could reverse simvastatin-mediated inhibition of protein expression (including cyclin E1, cyclin A2, cdk1, and cdk2 (Figs. 6A and B). The levels of phosphorylated and native forms of cdk1 and cdk2 in tendon cells as analyzed...
FIG. 6. Mevalonate and GGPP reversed the simvastatin-mediated inhibition of cyclin and cdk expression. Tendon cells were untreated or treated with 10 μM simvastatin (SIM) in the presence or absence of 100 μM mevalonate or 1 μM GGPP for 24 h, and then protein expression in the cell extracts was analyzed by western blotting (A). The relative band intensities of cyclin E1, cyclin A2, cyclin B1, cdk1, and cdk2 were shown in (B). Tubulin was used as the internal control. The levels of phosphorylated and native forms of cdk1 and cdk2 in tendon cells were shown in (C). The complex formation of cyclin/cdk were measured by co-immunoprecipitation (D). Tendon cells were treated with simvastatin or both simvastatin and GGPP (SIM+GGPP) for 24 h, and subjected to cell cycle analysis by flow cytometry. GGPP could partially reverse simvastatin-inhibition of cell cycle progression from G1 to S phase (E). Data were presented as mean±SEM of 3 independent experiments. *p < .05.)
by western blot were shown to be reduced by simvastatin and reversed by GGPP in a similar pattern (Fig. 6C). We measured cyclin/cdk complex by co-immunoprecipitation. The results revealed that the formation of cyclin E1/cdk2, cyclin A2/cdk2 or cyclin B1/cdk1 complexes remained the same ratio with different treatments compared with control (Fig. 6D). Cell cycle analysis showed GGPP could partially reverse simvastatin-inhibition of cell cycle progression from G1 to S phase. The S phase was 14.5 ± 2.1 % in control group, 4.0 ± 3.3 % in simvastatin-treated group, and 7.2 ± 0.6 % in tendon cells treated with both simvastatin and GGPP. The difference between simvastatin-treated group and simvastatin and GGPP group was statistically significant (Fig. 6E). These data indicated that the inhibition of tendon cell proliferation by simvastatin is mediated mainly through the down-regulation of cell cycle related protein expressions rather than inhibition of phosphorylated cdk5 or binding capacity of cyclins and cdk5.

**DISCUSSION**

Tendons consist mainly of dense collagen arranged in a linear structure with a basic cellular component, fibroblasts or tendon cells. The tendon cell, which appears stellate in cross-section and in rows in longitudinal section, is the source of collagen, protein mediators of repair, and matrix proteoglycans (Broughton et al., 2006; O'Brien, 1992). In injured tendons, the healing process can be divided into 3 overlapping phases: (1) inflammation, (2) regeneration, and (3) remodeling and maturation. The healing process can be divided into three overlapping phases: (1) inflammation, (2) regeneration, and (3) remodeling and maturation (Broughton et al., 2006). In the regenerative phase, tendon cells migrate into the injury/repair site, actively proliferate, and deposit abundant extracellular matrix (ECM) in the tissue. Therefore, tendon cell proliferation plays a pivotal role in tendon healing. Simvastatin, which inhibits tendon cell proliferation, obviously has a negative impact on tendon healing and may lead to statin-induced tendinopathy.

Several hypotheses could explain the relationship between statin use and tendon injury. Although the exact cause remains is not yet known, its suppressive effects on matrix metalloproteinase activity and prostaglandin E2 activity are thought to play a major role (Magra and Maffulli, 2005; Pullatt et al., 2007; Riley et al., 2002). These enzymes are active in the remodeling process, and when disrupted, could potentially lead to tendon weakening or even rupture. In addition, statin use has also been shown to promote apoptosis in fibroblasts (Yokota et al., 2008), further suggesting an association between statin use and tendon rupture. Taken together, these factors can weaken the integrity of tendon tissue and predispose the tendon to rupture.

This study, to our knowledge, is the first to demonstrate simvastatin-induced inhibition of tendon cell proliferation and cell cycle arrest at G1/S transition. In addition, the underlying molecular mechanisms were shown to be related to the down-regulation of cdk1 and cdk5. Cyclin E is induced at the G1/S boundary, whereas cyclin A is induced at a later phase of the cell cycle and is required for progression through S phase (Girard et al., 1991). Cyclin B1 regulates the transition through the G2/M checkpoint, whereas cyclin B associates with cdk1 and regulates the transition through the G2/M checkpoint of the cell cycle (Draetta and Beach, 1988; Minshull et al., 1989). The activity of cdk2 is restricted to G1-S phase, and it is essential for the transition from G1 to S phase. Cdk2 is regulated by the regulatory subunits of the complex, including cyclin E or A. In G1 phase, cyclin E binds to cdk2, which is required for the transition from G1 to S phase, whereas cyclin A binding is required for progression through S phase. In this study, simvastatin-treated tendon cells were found to be arrested at G1/S phase, suggesting that cyclin-cdk complexes, which play an important role in regulating cell cycle progression, are modulated. The results of this study showed down-regulation of cdk1, cdk2, cyclin A, and cyclin E in simvastatin-treated cells. These results provide clues to the molecular mechanism of cell cycle arrest and inhibition of tendon cell proliferation by simvastatin.

Simvastatin is a powerful lipid-lowering drug that effectively reduces LDL-cholesterol concentration by up to 50%, and it is widely used in clinical practice to treat patients with hyperlipidemia. It also has life-saving potential in some patients, particularly those with established or at high risk for coronary heart disease. By inhibiting HMG-CoA reductase, simvastatin depletes the cellular isoprenoid biosynthetic pathway products, including FPP and GGPP. Depletion of FPP, but not GGPP, inhibits cholesterol production. In our study, we demonstrated that the adverse effect of simvastatin on tendon cell proliferation could be fully prevented by the addition of exogenous GGPP. Our result also indicated that the addition of GGPP could not reverse the changes caused by simvastatin in the up-stream pathway and the reduction of mevalonate level remained nearly the same and as low as in simvastatin-treated cells. It was unlikely that the addition of GGPP could return the up-stream changes. More importantly, GGPP proceeds through a pathway that is different from FPP-mediated cholesterol biosynthesis (Schonbeck et al., 2004). Together, it suggests that GGPP could reverse the inhibitory effect of simvastatin on tendon cells without affecting the reduction of cholesterol biosynthesis. Our study provides an alternative way to prevent the potential side effect of simvastatin on tendons without affecting its lipid-lowering effect. Our study indicated that the inhibition of simvastatin in tendon cell proliferation was due to down-regulation of cdk1, cdk2, cyclin A2, and cyclin E1, rather than phosphorylation of cdk5 or binding capacity of cyclins and cdk5.

In conclusion, simvastatin inhibited tendon cell proliferation and caused cell cycle arrest at the G1/S transition. The underlying molecular mechanism in simvastatin-treated tendon cells seems to be related to the down-regulation of cdk1, cdk2, cyclin A2, and cyclin E1. These findings may provide novel information about simvastatin-induced tendinopathy or tendon rupture, which could be prevented by the addition of GGPP without affecting the lipid-lowering effect.

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