Simvastatin, a potent HMG-CoA Reductase Inhibitor, inhibits the Proliferation of Human and Bovine Endothelial Cells in vitro

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We investigated in vitro effect of simvastatin, a potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on the proliferation of human and bovine endothelial cells (EC) compared to that of bovine smooth muscle cells (SMC). Cells were cultured in a medium supplemented with 10% normal serum (FBS). Simvastatin at concentrations ranging from 0.1 μg/ml to 10 μg/ml were used. In each kind of cells the proliferation was markedly reduced at 1 μg/ml of simvastatin (P < 0.01) which was accompanied by morphological changes in the cell shape. We conclude that simvastatin inhibits the proliferation of not only the smooth muscle cells but also the endothelial cells. J Atheroscler Thromb., 1998; 4 : 102-106.

Key words : Simvastatin, HMG-CoA reductase inhibitor, Endothelial cells, Proliferation

Introduction

Simvastatin is one of the most potent inhibitors of cholesterol synthesis both in vitro and in vivo that competitively suppress 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme for the mevalonate pathway (1). Besides regulating cholesterol synthesis, HMG-CoA reductase plays an important role in cell proliferation as the mevalonate pathway yields both cholesterol and non-sterol metabolites (such as geranylaeraniol and farnesol) which are essential for the DNA replication (2). Pharmacological approaches have shown that simvastatin inhibits proliferation of rat smooth muscle cells (SMC) (3).

Atherosclerosis is characterized not only by the enhanced proliferation of SMC but also that of endothelial cells (EC) (4, 5). The enhanced SMC proliferation is important in the development of atherosclerosis while that of EC is essentially required for the repair in this pathological condition (4, 6).

Materials and Methods

1. Preparation of chemicals

The following chemicals were used: Simvastatin (produced by Merk-Banyu Co., Ltd., Tokyo, Japan) was a generous gift of Sankyo Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was from Hyclone Sterile Systems Inc. (Logan, UT, USA). Medium MEM and RPMI 1640, trypsin were from GIBCO Lab. (Grand Island, NY, USA). Endothelial cell growth supplement (ECGS) was from Collaborative Res. Inc. (Lexington, MA, USA). Tissue culture plasticwares were from Corning Glass Works (Corning, NY, USA). All other chemicals were purchased from Wako Chemical Co. (Osaka, Japan).

2. Culture of human umbilical vein endothelial cells (HUVEC)

HUVEC were obtained from the inner lumen by a
modified method of Jaffe et al (9). Cells were grown in collagen-coated dishes with a medium of RPMI 1640 supplemented with 20% FBS, 30 μg/ml ECGS and 5 μg/ml heparin at 37°C in an incubator with a humidified atmosphere of 95% air, 5% CO₂ and passaged weekly at a density of 1x10⁵ cells per 10 cm² (78.5 cm²) plastic plates (10). HUVEC showed the typical cobblestone patterned monolayer and stained positively with 1,1'-dioctadecyl-3,3',3',3'-tetramethylindo-carbocyanine perchlorate labeled acetylated-LDL. Cells of the 4th passage were used in a series of this study.

3. Culture of bovine EC

Bovine EC were isolated from bovine carotid arteries and maintained as described previously (11). Briefly, cells were grown in a monolayer at 37°C in a humidified atmosphere of 95% air, 5% CO₂ in MEM containing 10% FBS and passaged weekly at a density of 1x10⁵ cells per 10 cm² (78.5 cm²) plastic plates. Bovine EC showed the typical cobblestone patterned monolayer and stained positively with 1,1'-dioctadecyl-3,3',3',3'-tetramethylindo-carbocyanine perchlorate labeled acetylated-LDL. Cells used in this study had undergone approximately 15 passages.

4. Culture of bovine SMC

SMC were isolated from medial explants of bovine carotid arteries by the method of Ross (12). Small pieces of explants were incubated in an MEM containing 20% FBS, and cells began to grow out from the explants after 7-10 days. When cells became confluent, they were subcultured and maintained in a MEM with 10% FBS. The cultured cells showed typical characteristics of SMC, forming multilayered hills and valleys and having abundant myofilaments in their cytoplasm. Cells at the 4th passage were used in a series of this study.

5. Treatment of cells

Cells were subcultured on the 3.5 cm diameter plastic plates and then grown sparsely in monolayers. After the cells became confluent, they were treated with various concentrations of simvastatin (0.01-10 μg/ml) in the culture medium which was supplied with 10% FBS. Cells were further incubated at 37°C, 95% air, 5% CO₂ for 1-4 days.

6. Determination of cellular proliferation

Cellular proliferation was determined by measuring cell number, before and after the simvastatin treatment. For measurement, each culture was washed with 1 ml phosphate buffered saline. Then, a mixture of 0.5 ml of 0.05% trypsin and 0.05% EDTA was used to harvest the cells. The trypsinization was terminated by the addition of 0.5 ml medium MEM to the culture. Duplicate cultures of each treatment were measured with a Coulter Counter ZBI (Coulter Electronics Inc., Healeah, FL, USA).

7. Statistical analysis

All values in the figures are expressed as mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher’s test to correct for multiple comparisons between treatments.

Results

1. Effects of simvastatin on HUVEC

HUVEC were plated onto 3.5 cm diameter plates at a density of 1-2x10⁵ cells/plate. When HUVEC were grown in a medium for 4 days, they increased 4-fold in cell number (Fig. 1). Inclusion of simvastatin in the culture medium at concentration of 1 μg/ml significantly inhibited this proliferation.

2. Effects of simvastatin on bovine EC

Bovine EC were plated onto 3.5 cm diameter plates at a density of 20x10⁴ cells/ml. When bovine EC were grown in a medium for 2 days, they increased 4.7-fold in cell number (Fig. 2). One μg/ml of simvastatin inhibited the proliferation by 18% which was significant (P<0.01).

3. Effects of simvastatin on bovine SMC

Bovine SMC were plated onto 3.5 cm diameter plates at a density of 20x10⁴ cells/ml. When bovine SMC were grown in a medium for 2 days, they increased 3.6-fold in cell number. Similarly to EC, when the simvastatin concentration was raised up to 1 μg/ml, SMC proliferation were inhibited by 45% (Fig. 3, P<0.01).

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Fig. 1. Effects of simvastatin on the growth of HUVEC. When HUVEC were grown for 4 days, they increased 4-fold in cell number. One μg/ml of simvastatin significantly inhibited this proliferation. Values represent the means of triplicate cultures.
Simvastatin caused a rounding of cell shape both in EC (Fig. 4) and SMC (data not shown). The cell-rounding of EC was clearly induced at the lowest concentration tested (0.1 μg/ml). Light micrograph observation showed that the frequency for cell-rounding was approximately 20% at 1 μg/ml of simvastatin whereas more than 80% at 10 μg/ml. The cell-rounding accompanied a lower ability of adhesion since the cells were easily removed by trypsin treatment compared to those of normal shaped cells.

Discussion

We demonstrated that simvastatin inhibits the proliferation of endothelial cells. The inhibitory effect of simvastatin on cell growth was evident regardless of species of EC. Our observation that HMG-CoA reductase inhibitors inhibit EC proliferation is consistent with our previous study (8) and the study by Falke et al. who reported the inhibitory effects on proliferation of bovine EC and SMC by mevinolin, another kind of HMG-CoA reductase inhibitor (6). Although they reported a less sensitivity to mevinolin in EC than SMC, the minimum concentration of simvastatin needed for the inhibition of proliferation was the same in EC and in SMC in the present study. The
disparate results could be due to the different inhibitors used since there may be some differences in the activity of cell membrane penetration between simvastatin and mevinolin. In this respect, Sviridov et al. reported the higher sensitivity of EC than SMC to the inhibitory effect of mevinolin on cholesterol synthesis (13, 14).

Consistent with previous studies, we suggest that simvastatin inhibits the proliferation by inhibiting non-sterol metabolites from the mevalonate pathway since the growth inhibition occurred regardless of the endogenous- and exogenous-derived cholesterol in this study (15, 16). This hypothesis derived from two lines of evidence. First, the mevalonate-derived cholesterol was not essential for the proliferation both of EC and SMC. We previously confirmed that 0.1 μg/ml of simvastatin significantly reduces the cellular cholesterol level, i.e., the mevalonate-derived cholesterol level, in EC (7). Similar concentration has been reported by others in SMC (17, 18). However, this concentration did not affect the proliferation of EC and SMC in the present study. The second evidence is that higher concentrations of simvastatin transiently inhibited the proliferation both in EC and SMC, the inhibition of which was occurred regardless of the existence of cholesterol (data not shown). This may further suggest that cholesterol has also few roles on the inhibitory effect on proliferation induced by simvastatin. We are currently investigating the effects of non-sterol metabolites on the endothelial cell growth.

Simvastatin non-specifically changed the cell–shape, i.e., the cell–rounding, of both EC (Fig. 5) and SMC. The morphological change was definitely observed when the concentration of simvastatin was raised up to 10 μg/ml which was 100-fold higher than those required for inhibiting the cholesterol synthesis (7). Thus, the cell–rounding induced by simvastatin may be due to the deprivation of non-sterol metabolites similar to that of the proliferation. The morphological change induced by simvastatin is consistent with our previous study in neuronal cells (19) and other studies in human renal carcinoma cells and human fibroblasts (20, 21). Fenton et al. demonstrated that the prenylated protein which is a non-sterol product from mevalonate, plays a critical role in the cell–rounding by regulating the states of the intracellular actin (21). Thus, the morphological changes in EC and SMC might reflect the result of depolymerization of the actin cables.

In spite of the widely use in clinical treatment of hypercholesterolemia, simvastatin treated patients sometimes suffer from serious side effects such as rhabdomyolysis or suicidal tendencies, therefore, careful use of this drug has been suggested (22–25). The reason for these side effects are yet unclear, however, these could be due to a decrease in brain serotonin by lowering the serum cholesterol level (25) as well as the decrease of coenzyme Q10, an essential compound of the mitochondrial respiratory chain (25). We have recently found that simvastatin inhibits the proliferation of neuronal cells which may somewhat account for these side effects (19). Further studies are needed to rule out the possibility that simvastatin on long-time exposure in vivo might have negative effects on either neuronal cells or EC.

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References

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