Simvastatin Exerts Favourable Effects on Neointimal Formation in a Mouse Model of Vein Graft

L. Zhang*, H. Lu, J. Huang, Y. Guan, H. Sun

Department of Cardiology, The Second Affiliated Hospital of Nantong University, Nantong University, Nantong 226001, PR China

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Abstract  Background: Simvastatin inhibits human saphenous vein neointima formation in human saphenous vein organ cultures. However, it is not known if simvastatin actually inhibits vein graft intima hyperplasia in vivo, and the underlying mechanisms behind that. In this study, we used a murine vein graft model to address these issues.

Methods and results: Vein grafting was performed among C57BL/6 J mice treated with low-dose (2 mg kg⁻¹) or high-dose (20 mg kg⁻¹) simvastatin or vehicle subcutaneously 72 h before and then daily after surgery. As compared to the vehicle, simvastatin dose-dependently significantly inhibited vein graft intima hyperplasia 4 weeks after surgeries. Immunohistochemistry studies suggested that vein graft neointima was mainly composed of vascular smooth muscle cells (VSMCs), and the rate of proliferating cell nuclear antigen (PCNA)-positive cells in the intima of vein grafts was significantly lower in simvastatin-treated groups than in control group. We isolated VSMC from mouse vena cava, simvastatin significantly reduced VSMC proliferation, and platelet-derived growth factor (PDGF)-induced VSMC migration in a dose-dependent manner.

Conclusion: Simvastatin inhibits neointima formation of mouse vein graft under normocholesterolaemic condition in vivo, the mechanisms might be associated with inhibitory effects of simvastatin on VSMC proliferation and migration.

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reduce SVG occlusions and improve cardiovascular outcome after CABG, irrepre-spective of whether treatment is initiated pre- or post-operatively. Statins are a class of drugs that are used to reduce plasma cholesterol levels in patients with hypercholesterolaemia. A number of large clinical trial results have related statin-induced cholesterol lowering to the significant improvement in cardiovascular morbidity and mortality. Moreover, their clinical benefits appear greater than would be expected from simply lowering cholesterol, and it is becoming increasingly clear that these agents have favourable direct vascular effects independent of cholesterol lowering. For example, a number of animal studies have demonstrated that statins reduce neointima formation following arterial injury independently of effects on cholesterol synthesis. Statins act by inhibiting synthesis of intracellular mevalonate which, in addition to its central role in cholesterol synthesis, is also a precursor of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), isoprenoids required for prenylation of Ras and Rho-family guanosine triphosphatases (GTPases), respectively. This ability of statins to inhibit the prenylation and resultant activity of small GTPases may underlie many of their cholesterol-independent effects.

The molecular and cellular processes that regulate intimal hyperplasia within VG are poorly understood and significantly different from those that regulate intimal hyperplasia in native arteries. Hence, mechanistic studies are needed to define the pathogenesis of VG intimal hyperplasia and develop new preventive and therapeutic strategies. It had been reported that simvastatin inhibits human SV neointima formation in human SV organ cultures. However, it is not known if simvastatin actually inhibits VG intima hyperplasia under normocholesterolemic condition in vivo, and the underlying mechanisms behind that. In this study, we used a murine VG model to address these issues.

Materials and Methods

Mice

Male C57BL/6 J (25–30 g, 12–16-week-old) mice, provided by the Animal House, Nantong Medical College, were caged in groups of three with normal chow and water given ad libitum. The animals were kept in a temperature-controlled environment (21 °C) on a 12-h light–dark cycle. All animal experiments were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All surgical procedures and morphometric and immunohistochemical analyses were performed with the investigators blinded to different mouse groups.

Simvastatin treatment

Simvastatin was purchased from Merck & Co., Inc. Treatments were via subcutaneous injection 72 h before and daily after surgery. C57BL/6 J mice were divided into three treatment groups: phosphate-buffered saline (PBS) vehicle (control group, n = 8), or 2 mg kg⁻¹ (low-dose, n = 9) or 20 mg kg⁻¹ (high-dose, n = 9) alkaline-hydrolysed simvastatin.

Vein grafting surgery

Surgery was performed as described. In brief, the right common carotid artery of an anaesthetised male mouse was ligated proximally and distally and transected at its mid-portion. The transected ends were each passed through polyethylene cuffs, everted back over the cuffs, and tied to them with 8.0 silk ligatures. A segment (1 cm) of inferior vena cava (IVC) was cut from a different (donor) male mouse injected with 0.2 ml of saline solution containing 100 units of heparin by mouse tail vein, and rinsed with saline containing 100 U ml⁻¹ heparin, after which the vena cava from donor mice was grafted between the two ends of the carotid artery by ‘sleeving’ the ends of the vein over the artery cuffs and secured with 8/0 silk sutures. Blood flow was restored by removing the haemostats from the carotid artery, after which the wound was closed and sutured and the animal was allowed to recover from anaesthesia.

Morphometric analyses

Four weeks after surgery, the mouse was anaesthetised and the VG was surgically exposed. The vasculature was perfused with PBS and 4% phosphate-buffered paraformaldehyde for 2 and 5 min, respectively. The VG was embedded in paraffin. Three cross sections (5 μm thick, 70 μm between sections) from its mid-portion were prepared, mounted and stained with haematoxylin and eosin. Microscopic images were imported into Image-Pro Plus (Media Cybernetics) and lumen-vascular wall and tunica media–adventitia interfaces were traced. Neointima area was defined as the difference between the two traced cross-sectional areas. Mean neointima area value of cross sections from each VG was calculated. Intimal thickness was measured at four pre-specified locations – that is, 3, 6, 9 and 12 o’clock – in three cross sections of each VG and calculated mean intimal thickness for each graft.

Immunohistochemistry

Cross sections obtained from the mid-portions of VGs were mounted on slides, de-paraffinised, hydrated, incubated for 10 min in methanol containing 3% H₂O₂ and rinsed with PBS. Antigens were detected using the Histomouse-MAX Kit (Invitrogen). The following primary antibodies were used: (1) mouse anti-smooth muscle α-actin monoclonal antibody (clone 1A4, Santa Cruz Biotechnology) and (2) mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Santa Cruz Biotechnology). After washing, cross sections were incubated with secondary antibody (included in the kit), washed, incubated with peroxidase substrate (DAB), counterstained with haematoxylin and examined by light microscopy. The percentage of PCNA-positive cells was determined from analysis of >50 cells per cross section; mean values for each group were calculated. Negative control reactions lacking primary antibodies confirmed the specificity of each antibody. The same antibody dilutions, incubation times and washing conditions were used for all samples.
Isolation of venous SMC

Lines of cultured venous SMC, each isolating from the IVC of a single mouse, were established by digesting veins with collagenase (Worthington) after carefully removing loose adventitia, as described. Venous SMCs were grown under standard conditions in Dulbecco’s modified eagle medium (DMEM)/F12 medium supplemented with 20% foetal bovine serum (FBS). Immunohistochemical analyses of established cell lines revealed homogeneous, smooth-muscle-α-actin-positive cells. Cells were passaged a maximum of 5 times before being used in experiments.

SMC proliferation MTT assay

For the preparation of proliferation experiments, SMCs were seeded onto 96-well plates at a density of 1 x 10^4 per well in the culture medium DMEM/F12, which was supplemented with 5% or 10% FBS and without or with simvastatin at different concentrations (0.3, 1, 3 μM), at 37°C in a 5% CO2 incubator for 3 days. Cell Proliferation Kit I (Roche) was used for MTT mitochondrial function assay. Insoluble purple formazan crystals form if the cell mitochondria are functioning and the cells are viable. On the day of assay, the culture media were replaced with serum-free medium. SMCs were incubated with MTT labelling reagent for 4 h at 37°C in a 5% CO2 incubator. The formed purple formazan crystals were solubilised with solubilisation solution and mixed thoroughly with a pipette. Optical density of converted dye was measured at a wavelength of 550 nm and a reference wavelength of 650 nm with a spectrophotometer (SpectraMax 340, Molecular Devices). Each determination was performed in triplicate. Results were expressed as the relative percentage of viability as follows: relative percentage of viability = mean OD550/650 in test/control x 100. The concentrations of simvastatin tested were 0.3, 1 or 3 μM. Simvastatin was dissolved in ethanol and vehicle was added to the control group at the same concentration. The final concentration of ethanol in both the treated and control groups was <0.1%.

SMC migration assay

Migration assays were performed using gelatin-coated modified Boyden chambers (Costar) with 8-μm pores in 24-well tissue culture plates. SMCs were quicked with serum-free DMEM/F12 containing insulin—transferrin—selenium-A supplement (GibcoBRL) for 72 h, trypsinised lightly with Tryple™ Express (GibcoBRL) and resuspended in DMEM/F12 supplemented with 0.25% FBS (migration medium). Platelet-derived growth factor (PDGF, R & D Systems) was prepared at 10 ng ml⁻¹ in migration medium and loaded (600 μl) into the lower wells. SMCs were subsequently loaded into the upper wells (2 x 10⁴ cells in 100 μl). Simvastatin at appropriate concentrations (0.3, 1 or 3 μM) was added to the upper and lower chambers of the experimental wells and incubated for 9 h at 37°C. The control wells contained PDGF only. The cell suspension was aspirated and uninvaded cells were scrubbed and rinsed twice from the top of the membranes with cotton swabs and PBS. Diff-Quick Stain Kit (Siemens Healthcare Diagnostics) was used to stain the membrane. The membranes were then detached from the inserts and mounted for microscopic examination. Cell nuclei on the underside of each membrane were counted in four random high power fields (~200) under a light microscope. SMC migration in the experimental groups was expressed as mean number of migrated cells per high power field.

Statistical analyses

Results are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance with multiple comparison procedures (Holm–Sidak method) and the Student’s t-test were used, as appropriate, to compare differences between groups. A probability (P) value of <0.05 was considered to indicate a statistically significant difference.

Results

Simvastatin decreases neointimal thickening

Vein grafting surgeries were performed in C57BL/6 J mice treated with 2 or 20 mg kg⁻¹ simvastatin or vehicle subcutaneously 72 h before and then daily after surgery until euthanasia. In mice receiving vehicle, intimal area after surgery progressed significantly at 28 days (0.19 ± 0.010 mm²). As compared to vehicle, low- and high-dose simvastatin significantly reduced intimal area at 28 days (0.12 ± 0.0054 and 0.077 ± 0.0078 mm², P < 0.05, respectively). The neointima area of VG was significantly less in high-dose simvastatin-treated group than in low-dose treated group (0.12 ± 0.0054 vs. 0.077 ± 0.0078 mm², P < 0.05). The results revealed that simvastatin dose-dependently decreased intimal area in this VG model (Fig. 1D). Simultaneously, we measured intimal thickness at four pre-specified locations and calculated mean intimal thickness for each graft; the results were concordant with the intimal area data. It was shown that intimal thickness in simvastatin groups (40 ± 1.8 and 31 ± 3.4 μm) significantly reduced compared to that in the control group (56 ± 3.7 μm, P < 0.05, respectively) in a dose-dependent manner (Fig. 1E).

A SMC-rich neointima developed within 4 weeks of vein grafting

Significant intimal hyperplasia was observed in all VGs at 4 weeks after initial surgery (Fig. 1). Smooth muscle α-actin immunostaining displayed that VG neointima was composed mainly of SMCs (Fig. 2A). Thrombus formation was not observed within VGs at 4 weeks after surgery.

Effects of simvastatin on cell proliferation in VG neointima

The % of actively proliferating (PCNA-positive) cells in the VG neointima was significantly higher in control group mice (52 ± 2.5%) than in the simvastatin-treated group mice (39 ± 3.9% and 27 ± 2.6%, P < 0.05, respectively), and the mean percentage of PCNA-positive cells in the low-dose simvastatin-treated group was significantly higher than in
the high-dose simvastatin-treated group (39 ± 3.9% vs. 27 ± 2.6%, \( P < 0.05 \)), suggesting that simvastatin dose-dependently inhibited cell proliferation within neointima of the VG wall (Fig. 2B and C).

**Effects of simvastatin on venous SMC proliferation**

We analysed the effects of simvastatin on the growth of venous SMC by MTT assay. The concentrations of simvastatin tested were 0.3, 1 and 3 \( \mu \text{M} \) in DMEM supplemented with 5% or 10% FBS, respectively. The relative percentage of venous SMCs viability in groups treated with simvastatin at the above concentrations decreased to 85 ± 5.4%, 95 ± 3.1%; 55 ± 1.6, \( P < 0.01 \), 64 ± 2.0%, \( P < 0.05 \); and 49 ± 6.7%, \( P < 0.05 \), 55 ± 3.5%, \( P < 0.05 \) in comparison with those in the control group (100 ± 0.6% and 100 ± 5.7%). Simvastatin had a significant inhibitory effect on venous SMC growth in a dose-dependent manner under different FBS concentrations as compared to vehicle (Fig. 3).

**Effects of simvastatin on venous SMC migration**

SMC migration is an important determinant of intimal hyperplasia. We studied the functional effect of simvastatin on venous SMC migration through gelatin-coated membranes. Cell migration was dose-dependently inhibited with simvastatin. Results represented three independent experiments. Compared with that in the control (245 ± 7), the mean number of migrated venous SMCs per high power field was significantly reduced to 213 ± 9 \( P < 0.05 \), 159 ± 5 \( P < 0.05 \) and 122 ± 6 \( P < 0.05 \) in the groups treated with simvastatin at the concentrations of 0.3, 1 and 3 \( \mu \text{M} \), respectively (Fig. 4).
Statins are a class of drugs prescribed to individuals with hypercholesterolaemia. Aggressive lipid controlling therapy is quite attractive to avoid post-CABG VG disease and the studies indicated that it may be effective to maintain the long-term graft patency. In addition to their primary function of lowering plasma cholesterol levels, statins also have beneficial effects on the cardiovascular system through lipid-independent mechanisms. There is increasing clinical evidence that statins can reduce the incidence of CABG occlusion in SV conduits, which is independent of cholesterol levels. Recently, Antoniades and colleagues reported that short-term treatment with atorvastatin before CABG improves redox state in SVGs, by inhibiting vascular Rac1-mediated activation of NADPH-oxidase. Indeed, statin therapy can retard the progression of VG disease and reduce the need for repeat revascularisation procedures, but the exact mechanisms are still under investigation. The underlying pathology of SV graft occlusion is intimal hyperplasia, a process initiated by endothelial damage during vein harvesting and implantation. The resultant increase in the expression of inflammatory mediators such as PDGF and interleukin-1 (IL-1) induces matrix metalloproteinase (MMP)-2 and activity, which permits intimally directed SMC migration and proliferation. However, it is unknot known if simvastatin actually inhibits VG intima hyperplasia under normocholesterolaemic condition in vivo, and the roles of simvastatin in mouse venous SMC proliferation and migration.

Our study provided definitive evidence that simvastatin caused a dose-dependent reduction of neointima formation in a murine model of VG disease. In this study, we chose low dose (2 mg kg\(^{-1}\)) or high dose (20 mg kg\(^{-1}\)) as simvastatin treatments following the published paper. But for a translational study, a mouse dose of 2 mg kg\(^{-1}\) is equivalent to a human dose of 0.14 mg kg\(^{-1}\) (about 10 mg day\(^{-1}\)), and 20 mg kg\(^{-1}\) equals 1.4 mg kg\(^{-1}\) (about 100 mg day\(^{-1}\)).

**Figure 2** Immunohistochemical analyses of cross sections from vein grafts 4 weeks after implantation. Anti-smooth-muscle-\(\alpha\)-actin immunostaining (A); anti-proliferating cell nuclear antigen (PCNA) immunostaining (B); Quantification of PCNA-positive nuclei: from left to the right, control group, \(n = 8\); low-dose simvastatin-treated group, \(n = 9\); high-dose simvastatin-treated group, \(n = 9\); \(* P < 0.05\) versus control group, \(\dagger P < 0.05\) versus low-dose treated group (C).

**Figure 3** Effect of simvastatin on the viability of venous SMCs was measured by the MTT assay. Cells were exposed to various concentrations of simvastatin (0.3, 1, 3 \(\mu\)M) for 72 h with 5% (left) or 10% (right) foetal bovine serum (FBS). \(* P < 0.05\) compared with control group; SV, simvastatin.
the latter looks too high, as higher doses of simvastatin (160 mg day \(^{-1}\)) have been found to be too toxic in clinic, while giving only minimal benefit in terms of lipid lowering. In secondary prevention, 80 mg day \(^{-1}\) reduced major cardiovascular events by an absolute rate of 1.2% compared to 20 mg day \(^{-1}\) in a randomised controlled trial.\(^2\) The male C57BL/6 J mice were used, and no significant changes could be observed in plasma lipid level after simvastatin treatments (data not shown); the results supported that the effect of simvastatin on neointima formation was in a lipid-independent manner. Similarly, Steinmetz and colleagues reported that treatment with simvastatin (2 mg kg \(^{-1}\) day \(^{-1}\), subcutaneously) suppresses the development of experimental abdominal aortic aneurysms in C57BL/6 J wild-type mice.\(^2\) The molecular and cellular processes that regulate intimal hyperplasia within VGs are poorly understood. In this study, immunohistochemical analyses revealed that VG neointima was mainly composed of SMCs, and the rate of proliferating (PCNA-positive) cells in the intima of VGs was significantly lower in the simvastatin-treated groups than in the control group. Statins inhibit the synthesis of isoprenoid intermediates that are important lipid attachments for signalling proteins, including Ras and Rho family of small guanosine triphosphate (GTP)-binding proteins (e.g., Rho, Rac and Cdc42).\(^2\) Rho is implicated in various biological functions, including cellular migration, proliferation and survival.\(^2\) Statins attenuate vascular SMC proliferation in vitro by decreasing Rho geranylgeranylation and membrane localisation and inhibiting Cdk activity.\(^2\) It had been reported that PDGF-induced phosphorylation of Akt is inhibited in aortic tissue from simvastatin-treated mice. The effects of statins on Akt signalling seem to be tissue specific. Statins rapidly activate Akt signalling in endothelial cells, enhance phosphorylation of endothelial NO synthase and inhibit apoptosis.\(^2\) By contrast, statins impair Akt activation in SMCs, leading to diminished SMC proliferation and induction of apoptosis via effects on phosphatidylinositol-3 kinase or Rho.\(^2\) These divergent actions of statins on Akt activation in endothelial cells and SMCs may act in synchrony to diminish neointimal thickening after VG surgery. We isolated VSMC from mouse vena cava; simvastatin significantly inhibits venous SMC proliferation and PDGF-induced venous SMC migration.

In conclusion, we found for the first time that simvastatin dose-dependently inhibited the neointima formation in a mouse VG model with normal plasma cholesterol level, and reduced mouse VSMC proliferation and migration in vitro. These novel findings may have major clinical implications, because they support the notion that even in patients with relatively low low-density lipoprotein (LDL), treatment with statins has direct beneficial effects on the biology of SVGs used in CABG and may have an impact on the patency of these grafts. However, the model that we used is not an experimental system for studying lumen stenosis, but rather early intimal hyperplasia, and this process of early intima hyperplasia, in itself, rarely produces significant stenosis, which represents the foundation for later development of graft atheroma. Conversely, in this mouse VG model, the inferior vena cava was grafted into the common carotid artery by using ‘cuffs’, thus avoiding direct anastomosis. The operation procedure of this model is relatively simple as compared to other VG models, and the traumatic and ischaemic injuries to the grafts are minimal. The major disadvantage of this model is that cuff anastomoses are not used clinically. We tried to anastomose the ends of the vein segment and arteries using a suture, and found that the cuff technique was easier to obtain a reproducible result. In the near
future, additional research may lead to further understanding of the actions of statins, and will support the proposal of an earlier and more aggressive use of statins for patients with CABG.

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Conflict of Interest

None.

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None.

References