Efficacy of Simvastatin Treatment of Valvular Interstitial Cells Varies With the Extracellular Environment

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Objective—The lack of therapies that inhibit valvular calcification and the conflicting outcomes of clinical studies regarding the impact of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG)-coenzymeA (CoA) reductase inhibitors on valve disease highlight the need for controlled investigations to characterize the interactions between HMG-CoA reductase inhibitors and valve tissue. Thus, we applied multiple in vitro disease stimuli to valvular interstitial cell (VIC) cultures and examined the impact of simvastatin treatment on VIC function.

Methods and Results—VICs were cultured on 3 different substrates that supported various levels of nodule formation. Transforming growth factor (TGF)-β1 was also applied as a disease stimulus to VICs on 2-D surfaces or encapsulated in 3-D collagen gels and combined with different temporal applications of simvastatin. Simvastatin inhibited calcific nodule formation in a dose-dependent manner on all materials, although the level of statin efficacy was highly substrate-dependent. Simvastatin treatment significantly altered nodule morphology, resulting in dramatic nodule dissipation over time, also in a substrate-dependent manner. These effects were mimicked in 3-D cultures, wherein simvastatin reversed TGF-β1–induced contraction. Decreases in nodule formation were not achieved via the HMG-CoA reductase pathway, but were correlated with decreases in ROCK activity.

Conclusions—These studies represent a significant contribution to understanding how simvastatin may impact heart valve calcification. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: heart valves • valvular interstitial cells • simvastatin • calcification • extracellular matrix

Calcification is the major cause of aortic heart valve failure and is the most common heart valve disorder in developed countries.1 Currently, there are no medical agents that are FDA-approved to halt the progression of aortic valve disease.2 Valvular interstitial cells (VICs) are fibroblast-like cells that comprise the bulk of the valve that are believed to be the predominant cell type involved in valve calcification.1,3 Calcified heart valves are rich in activated VICs, also known as myofibroblasts, as well as osteogenic growth factors and cytokines, including bone morphogenetic proteins (BMPs) and TGF-beta1 (TGF-β1).3–5 Diseased valves also display grossly altered extracellular matrix (ECM) composition and arrangement.6

3-hydroxy-3-methylglutaryl-coenzyme A (HMG)-coenzymeA (CoA) reductase inhibitors have been associated with reduced mortality in patients with atherosclerotic disease.7–9 Despite the intended purpose of lowering LDL serum cholesterol levels, HMG-CoA reductase inhibitors appear to have pleiotropic cholesterol-independent effects such as antioxidant, improvement of endothelial function, and modulation of proinflammatory cytokine production.10–12 In some studies, this type of drug treatment was found to slow the progression of valvular stenosis in patients with atherosclerosis.13–16 However, the numerous—and often conflicting—clinical studies that have examined the relationship between HMG-CoA reductase inhibitor therapy and valvular disease ultimately demonstrate that the scientific and medical communities know relatively little about the nature of the interaction of HMG-CoA reductase inhibitors with heart valves.17,18

The majority of HMG-CoA reductase inhibitor/valve investigations have been retrospective clinical studies, which greatly limit the amount of obtainable information about the structural and molecular properties and characteristics of the diseased or drug-treated valves. Limitations inherent to in vivo studies introduce significant challenges in studying the progression of valvular disease through its intermediate stages. Moreover, complications such as patients with multiple types of cardiovascular disease, variable medication compliance, and a tissue that is difficult to evaluate without explantation, make it exceedingly difficult to characterize the relationship between valves and HMG-CoA reductase inhibitors. These issues highlight the need for a set of controlled in vitro experiments that determine whether and how VICs respond to treatments with HMG-CoA reductase inhibitors of varying duration and timing.

In the current study, we characterize the effects of simvastatin treatment on VIC function in 2-D and 3-D cultures of...
The studies described in the previous section will allow us to develop a better understanding of: (1) how simvastatin regulates VIC dysfunction, (2) the role of the extracellular environment in regulating VIC response to simvastatin, and (3) the limitations/capabilities of simvastatin in preventing or treating valve disease.

**Methods**

All reagents were obtained from Sigma-Aldrich unless otherwise noted. Raw data were analyzed via ANOVA with a Tukey HSD post test, and probability values < 0.05 were considered statistically significant. All data are presented as mean ± SD.

**Simvastatin Dose-Response in Varied Culture Environments**

Valvular interstitial cells (VICs) were isolated from porcine aortic valves (Hormel Inc) by collagenase digestion and cultured as previously described.19 VICs (P2-P4) were seeded at a density of 50,000 cells/cm² and cultured in low-serum (LS) medium (1% FBS) on unmodified tissue culture polystyrene (TCPS) or TCPS coated with adsorbed fibrin (FB, 1.5 μg/cm²) or laminin (2 μg/cm²; prepared as in20). These cells were then treated with 0.1 to 1 μM simvastatin (clinical range is approximately 0.1 to 0.3 μM/L), which was supplied in its active form, (EMD Biosciences Inc) in LS medium for 5 days. Addition of TGF-β1 (5 ng/mL) was performed as a positive control, and TGF-β1 (5 ng/mL) was also combined with simvastatin (1 μM/L). Cultures were replenished with simvastatin every 48 hours. The number of calcific nodules formed after 5 days in culture was evaluated via microscopic observation (Olympus IX51) and mineralization staining with Alizarin Red S. A separate set of Day 5 samples was lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl) and cell number was quantified using the QuantIt PicoGreen assay kit (Invitrogen).

A similar simvastatin dose-response study was performed on VICs cultured in type I collagen gels (Inamed Biomaterials). Gels were prepared as described previously22 with a cell density of 1×10⁶ cells/mL and collagen concentration of 2.4 mg/mL. Gels were left in a “stressed” configuration (ie, adherent to the well walls) for 5 days, during which time they received 0.1 to 1 μM/L simvastatin. On Day 5, gels were released from the sides of the wells, and gel contraction was measured every hour for 10 hours, and as needed thereafter.

**Application of Different Simvastatin Treatment Regimens**

VICs were cultured on TCPS, FB, or LN, and fed either regular LS medium or LS medium + 5 ng/mL TGF-β1 for 5 days, at which time nodules were counted. The culture conditions were then switched such that cells continued to receive either plain LS medium, or were administered 1 μM/L simvastatin for another 5 days, at which point nodule counts were performed again (Day 10).

**Nodule Analysis**

VICs were cultured on TCPS surfaces for 5 days in LS medium + 5 ng/mL TGF-β1, and a portion of the samples was harvested on Day 5. The remaining portion of the samples received 4 to 5 additional days of treatment, either in LS medium or in LS medium +1 μM/L simvastatin. On the last day of treatment (Day 5 or Day 10), cells were fixed in 10% formalin, rinsed with PBS, and sputter-coated with 40 ng of gold. Samples were then imaged on a white-light interferometer (New View 6300, Zygo). Images were subjected to a low-pass filter and data fill through the Zygo MetroPro software package. These images were subsequently analyzed for nodule height and area, also with MetroPro.
inhibiting nodule formation on all 3 culture substrates. Medium supplemented with TGF-β1 was used as a positive control, and for all substrates, yielded much greater nodule numbers than any simvastatin condition in Figure 1a (>400% increase in nodule number; not shown).

The dose-dependent trend seen in 2-D experiments was replicated in a 3-D environment, as shown in Figure 1b. VIC-seeded collagen gels exhibited steadily decreasing contraction on receiving increasing dosages of simvastatin. The highest simvastatin dosage (1 μmol/L) completely inhibited gel contraction, whereas gels that received 0.5, 0.1, and 0 μmol/L simvastatin contracted to 95%, 81.3%, and 70.3% of their original sizes, respectively.

Combined Effects of Simvastatin Treatment and the ECM on VIC Nodule Formation

Further experiments were performed to explore administration of various simvastatin treatment regimens in combination with nodule-inducing stimuli. Specifically, VICs were cultured on TCPS (Figure 2a), FB (Figure 2b), or LN (Figure 2c) with or without TGF-β1 supplementation for 5 days, followed by either simvastatin or no simvastatin treatment for the subsequent 5 days. When the nodule number on Day 10 was significantly less than the nodule number on Day 5, this occurrence was labeled “regressive.” This regressive phenomenon was observed on all 3 surfaces (TCPS, FB, and LN), but only when the cells had received TGF-β1 for the first 5 days; no regressive effects were observed in cultures that were untreated during the initial culture period. An assay for apoptosis in nodule-regressive cultures (supplemental Figure I, available online at http://atvb.ahajournals.org) verified that the observed dissipation of nodules was not attributable to an increase in apoptotic events. The magnitude of the regressive effects also differed across the different substrates. Notably, simvastatin was most effective when applied to TGF-β1-treated VICs on LN, where nodule number was reduced by approximately 10-fold between days 5 and 10. In comparison, the regressive action was much more modest on TCPS and FB, with 1.3-fold and 2-fold reductions in nodule number, respectively. In one instance (FB without TGF-β1 pretreatment, Figure 2b), statin treatment did not lower
nodule numbers compared to Day 5 values, but did result in less nodule formation than found on the untreated control at Day 10; this occurrence was labeled an “inhibitory” action of simvastatin.

**Analysis of Nodule Characteristics and Topography**

The regressive results in Figure 2 raised questions regarding the fate of nodules after statin treatment. Thus, topographical imaging techniques were used to examine nodule height and area over time, and time-course photomicrographs were captured to track nodule morphology on simvastatin treatment. Figure 3 shows the gradual dissipation of a representative nodule from cultures treated with 1 μmol/L simvastatin for 5 days. In this experiment, nodules were formed during an initial 5-day TGF-β1 pretreatment, mimicking the regressive conditions documented in Figure 2.

A topographical analysis of nodules was performed on Day 5 and Day 9 samples from the aforementioned experiment. As seen in Figure 4a, the topography of nodules treated with 1 μmol/L simvastatin changed dramatically when compared to both the original Day 5 nodules and Day 9 nodules that did not receive simvastatin. This morphological observation is confirmed quantitatively in Figure 4b, in which the simvastatin-treated nodules exhibited a more dramatic drop in maximum height when compared to their untreated counterparts. This drop in height was accompanied by an increase in area (Figure 4c). These data validate the images in Figure 3 and 4a in which simvastatin-treated nodules appear to flatten and dissipate over the course of statin treatment.

**Impact of Simvastatin on Cell Contractility**

The impact of simvastatin treatment on VIC contractility was also measured in an effort to better understand the mechanism behind simvastatin’s inhibition of nodule formation. It is clearly shown in Figure 5a that simvastatin application, either alone or in conjunction with TGF-β1, drastically reduced contraction. To test the reversibility of this contraction, simvastatin was applied at various time points to the gels that received an initial 5 days of TGF-β1 treatment. This condition was intended to mimic the regressive scenarios described above for 2-D conditions. Figure 5b and 5c show that simvastatin application at either 1 or 4 hours led to a partial reversal of gel contraction, whereas simvastatin applied at 24 hours (Figure 5d) was not able to reverse contraction. To test the reversibility of simvastatin treatment (ie, contraction resistance), TGF-β1 treatment was applied to the gels that had initially received simvastatin. However, application of TGF-β1 at either 1, 4, or 24 hours after gel release did not cause contraction of the gels initially treated with simvastatin.

**Mechanism of Simvastatin Regulation of VIC Function**

To investigate the mechanism of simvastatin’s inhibition of VIC nodule formation, mevalonate, a downstream metabolite of the HMG-CoA reductase pathway, was added to VIC cultures. Addition of mevalonate represents “blocking” simvastatin’s action by reinitiating the HMG-CoA reductase pathway. However, Figure 6a shows that addition of mevalonate to simvastatin-treated cultures on TCPS did not reverse the nodule-inhibiting actions of simvastatin, indicating that nodule inhibition is not dependent on the HMG-CoA reductase pathway. Similar results were obtained on FB and LN surfaces (data not shown).

Quantification of Rho kinase (ROCK), an enzyme in the Rho contraction pathway, confirmed a positive correlation between VIC contraction and ROCK. The results in Figure 6b show that increased concentrations of simvastatin led to decreased ROCK expression. Because decreased ROCK ex-
pression generally correlates with decreased contraction, these results are complementary to the 3-D dose-dependence in Figure 1b, wherein simvastatin treatment decreased VIC contractility.

Discussion
Currently, few studies have investigated simvastatin treatment of VICs, and this work extends those initial findings through a more in-depth in vitro study involving multiple simvastatin dosages and treatment regimens, as well as several different nodule-conducive environments. This work is notable in its exploration of multiple types of culture environments, findings of statin-induced nodule regression and accompanying morphological analysis, and investigation of potential mechanisms.

We have shown that VICs respond to simvastatin treatment in a dose-dependent manner, both in 2-D and 3-D environments. Application of increasing concentrations of simvastatin resulted in decreased formation of nodules, with simvastatin treatment most effective on laminin-coated surfaces, followed by fibrin-coated surfaces, and finally, least effective on TCPS. The dose-dependent effect of simvastatin on nodule formation was consistent with results obtained for aortic valve myofibroblasts (AVMFs) on TCPS. However, it is interesting to note that all previous studies of statins applied to VIC cultures were done exclusively on TCPS, a culture condition which we found to be the least responsive to simvastatin treatment in terms of nodule reduction.

Culturing cells on TCPS alone cannot paint an accurate picture of cell behavior, as cells in their native environment are surrounded by ECM proteins that continuously modulate cellular responses. The ECM components studied in the present investigation were not intended to mimic native conditions; rather, they were selected for their ability to stimulate nodule formation in VIC cultures. However, use of these ECM molecules did illustrate an important point with respect to HMG-CoA reductase inhibitor efficacy: the composition of the culture substrate can greatly alter the response of VICs to simvastatin treatment. The significant substrate-dependence of simvastatin efficacy highlights the importance of performing such experiments in varied culture environments and provides motivation for future evaluations of pharmacological efficacy to be executed in more bioinspired environments.

The findings described herein reveal the unexpected, yet exciting, finding that simvastatin was capable of not only inhibiting the formation of nodules in VIC cultures, but also reducing nodule number after the nodules had formed. When TGF-β1 was applied for 5 days to induce nodule formation, simvastatin treatment was most effective at reversing nodule formation on laminin, followed by fibrin, and finally, least effective on TCPS—a trend consistent with that seen in Figure 1. The aforementioned nodule-regressive effects of simvastatin were noted only in conditions pretreated with TGF-β1. Although unusual, these results are also consistent with those obtained with concurrent TGF-β1+statin treat-
been widely used. Thus, because of historical limitations in studying in vivo valve composition over time, the exact fate of calcific nodules during clinical studies of statins is not known. The clinical studies that have tracked the condition of a patient’s heart valve over time tend to examine stenosis, which is generally characterized by blood flow measurements, and may or may not be directly related to calcification.

Although previous studies have examined some effects of HMG-CoA reductase inhibitor treatment on other cell types, VICS are a unique cell type, both in their physiological function and their response to statin treatment. Unlike smooth muscle cells, which exhibit decreased proliferation with HMG-CoA reductase inhibitor treatment, VICS did not experience any changes in proliferation on simvastatin treatment. This result illustrates the uniqueness of VICS and the possibility that their response to treatment with HMG-CoA reductase inhibitors will be significantly different in comparison to other cell types. Additionally, previous HMG-CoA reductase inhibitor studies performed with other cell types did not investigate the possibility of a differential cellular response depending on the ECM environment, and the present effort uncovered significant differences in that regard.

Inhibition of the HMG-CoA reductase pathway is simvastatin’s predominant mechanistic action with respect to cholesterol reduction. However, addition of mevalonate to simvastatin-treated VICS was not successful in reversing the nodule-suppressing actions of simvastatin, implying that simvastatin’s actions on nodule inhibition or regression in VICS occurred independently of simvastatin’s cholesterol-lowering function. These results are somewhat different than those documented by Wu et al., who found that addition of mevalonate to aortic valve myofibroblasts (AVMFs) did partially reverse simvastatin- and pravastatin-induced decreases in alkaline phosphatase (an indicator of calcification). However, AVMFs and VICS are slightly different cell populations, and the effects of simvastatin (and, by extension, any potential reversal by mevalonate) are likely to vary with cell type, as noted above.

In addition to inhibiting or regressing nodule formation, simvastatin also decreased VIC contractility in a dose-dependent manner. The increase in VIC contractility in conditions associated with increased nodule number is consistent with previous characterizations of diseased valves and calcified VIC cultures. Our results showed an inverse relationship between simvastatin concentration and ROCK concentration; decreased ROCK is consistent with the decreased contractility achieved with simvastatin administration. These results come together to suggest a possible mechanism for regulation of VIC nodule formation by simvastatin: simvastatin either directly or indirectly reduces the amount of ROCK, which consequently leads to decreased cell contraction, ultimately resulting in decreased levels of nodule formation in VIC cultures. Supplemental Figure II underscores the importance of ROCK in regulating nodule formation in VIC cultures, as this experiment demonstrated that targeted inhibition of ROCK via H1152 drastically reduced nodule formation in VIC cultures.
a more direct link between ROCK activity and nodule formation. These reductions in VIC contractility were not accompanied by a decrease in the total amount of α-SMA in VIC cultures (supplemental Figure I), although these data do not account for possible changes in the presentation of α-SMA.

The effects of simvastatin with respect to regressing 2-D nodule formation were also somewhat mimicked in 3-D contraction assays. Application of simvastatin was able to partially reverse TGF-β1-induced cell contraction, but only when the simvastatin was applied very early in the contraction process—contraction reversal was no longer possible when simvastatin was applied at t=24 hours after collagen gel release. Interestingly, no such reversal was possible in the converse situation, where simvastatin-treated gels were switched to procontractile conditions. This result may imply that some long-term cell relaxation is imparted by simvastatin (even after delivery of simvastatin has been replaced with delivery of a procontractile factor), or that disruption of early contractile events in this assay is sufficient to prohibit any contraction from occurring.

This work clearly demonstrates that, in several 2-D and 3-D in vitro environments, simvastatin exerts a potent antinodule effect on cultures of valvular interstitial cells. Moreover, the efficacy of this treatment is highly dependent on multiple factors, including the nature of the disease stimulus, the composition of the culture environment, and the timing of the statin treatment regimen. There are several implications of this work with respect to studying and treating calcific valvular disease. First, these findings stress the importance of investigating pharmaceutical interventions in in vitro environments that are more complex or biologically-inspired than traditional TCPS. Furthermore, our data suggest that a reduction in VIC contractility is a likely mechanism by which simvastatin reduces VIC nodule formation, and this finding may be further exploited to better elucidate the etiology of valvular disease as well as identify potential therapeutic agents. Statin-induced decreases in apoptosis may also play a role in nodule inhibition (supplemental Figure I), but neither apoptotic nor proliferation events were responsible for nodule regression.

The results obtained in this work on combination of TGF-β1 with simvastatin also highlight an intriguing avenue for further studies. Ultimately, these results, combined with continued investigation of interactions between HMG-CoA reductase inhibitors and VICs, may help to shed more light on the current controversy related to treatment of valvular disease with HMG-CoA reductase inhibitors and lead to the identification of appropriate treatment regimens to reduce valvular disease.

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Disclosures
None.

References


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Supplemental Figure 1. (A) Simvastatin-induced reduction in VIC apoptosis may be one mechanism by which simvastatin inhibited nodule formation in VIC cultures. (B) In a nodule regression experiment, VIC cultures were treated with 5 ng/mL TGF-β1 for 5 days, followed by an additional 5 days of either untreated medium or 1 µmol/L simvastatin. Although significant nodule reduction was achieved with simvastatin treatment, no change in apoptosis was observed during nodule regression events. * p<0.01 vs. untreated control.
Supplemental Figure 2. VICs were treated for 5 days with 5 μM H1152, a Rho kinase inhibitor, or left untreated. Treatment with the ROCK inhibitor resulted in the formation of fewer nodules on Day 5 on each surface (TCPS, fibrin, and laminin). By demonstrating a relationship between direct inhibition of ROCK and reduction in nodule formation, these data provide a link between our separate observations of decreased ROCK activity and decreased nodule formation obtained with simvastatin treatment. *p<0.0001 compared to untreated control.
Supplemental Figure 3. α-smooth muscle actin levels for VICs that were treated for 5 days with 0.1-1 μmol/L simvastatin. * p<0.04 vs. untreated control.
Supplemental Methods

Apoptosis

VICs cultured on TCPS, fibrin, or laminin were treated with 0.1-1 μmol/L simvastatin for five days, then analyzed for Caspase 3/7 activity via a luminescence-based apoptosis kit, Caspase-Glo (Promega, Madison, WI) per manufacturer’s instructions. In a separate experiment, VICs were treated for five days with 5 ng/mL TGF-β1, followed by an additional 5 days of either 1 μmol/L simvastatin or untreated medium, and then assayed for apoptosis via the aforementioned kit.

ROCK Inhibition

VICs were treated with H1152 (S-(+)
-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-homopiperazine), a Rho kinase (ROCK) inhibitor \(^1\) (Calbiochem, San Diego, CA) during culture on TCPS, laminin-coated, and fibrin-coated surfaces. Twenty-four hours after cell seeding, media in all plates was changed and supplemented with 5 μM H1152 or left untreated. The inhibitor was applied every other day until Day 5, at which point nodules were counted as described earlier.

α-SMA

An ELISA assay was used to assess α-smooth muscle actin (α-SMA) in VIC cultures that were treated for 5 days with a range of simvastatin dosages (0.1-1 μmol/L). For the α-SMA quantification, a primary mouse anti-α-SMA antibody (monoclonal, clone 1A4) was used at 10 μg/mL, with an HRP-conjugated goat anti-mouse secondary antibody used at a 1:3,000 dilution (Chemicon, Billerica, MA). 1-Step Turbo TMB-ELISA (Thermo Scientific, Waltham, MA) was used for colorimetric development, and the samples were read at a wavelength of 450 nm.
Supplemental Reference