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Objective—Activation of collagen receptors expressed by smooth muscle cells induces matrix metalloproteinase (MMP) expression. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been shown to interfere with integrin signaling, but their effects on collagen receptor-mediated MMP expression have not been investigated.

Methods and Results—In the present study, we show that simvastatin (3 μmol/L) reduces MMP1 expression and secretion in human smooth muscle cells cultured on polymerized type I collagen by 39.9±11.2% and 36.0±2.3%, respectively. Reduced MMP1 protein levels correlate with a similar decrease in MMP1 promoter activity (−33.0±8.9%), MMP1 mRNA levels (−37.8±10.5%), and attenuation of smooth muscle cell collagen degradation (−34.2±6.1%). Mevalonate, and the isoprenoid derivative geranylgeraniol, precursors of geranylgeranylated proteins, completely prevent the inhibitory effect of simvastatin on MMP1. Moreover, the protein geranylgeranyltransferase inhibitor GGTI-286 significantly decreases MMP1 expression. Retroviral overexpression of dominant-negative mutants of geranylgeranylated Rac1 lead to a reduction of MMP1 protein (−50.4±5.4%) and mRNA levels (−97.9±1.0%), and knockdown of Rac1 by small interfering RNA downregulates MMP1 expression. Finally, simvastatin reduces GTP-bound Rac1 expression levels in smooth muscle cells cultured on polymerized collagen.

Conclusions—These results demonstrate that simvastatin, by inhibiting Rac1 activity, reduces MMP1 expression and collagen degradation in human smooth muscle cells. (***Arterioscler Thromb Vasc Biol*. 2007;27:1043-1049.)**

Key Words: atherosclerosis ■ integrins ■ matrix metalloproteinases ■ prenylated proteins ■ Rac1

The integrity of interstitial type I collagen in the fibrous cap that covers the atherosclerotic lesions represents a critical factor for the clinical sequelae associated with cardiovascular disease, such as myocardial infarction and stroke.[1,2] Therefore, a pharmacological intervention aimed at inhibiting extracellular matrix degradation may have beneficial effects on the development of atherosclerotic plaques and their stability. Extracellular matrix not only provides a scaffold for mechanical support and tissue organization but also directly alters cell behavior by influencing proliferation, migration, differentiation, and gene expression. For example, smooth muscle cells (SMCs) cultured on polymerized type I collagen show altered expression of a group of genes including matrix metalloproteinase (MMP) 1, type I collagen, and other extracellular matrix components.[3–6] This 3-dimensional culture system has been developed as an in vitro model that more closely mimics the in vivo extracellular matrix environment surrounding SMCs.[7] In particular, SMC transmembrane adhesion receptors for type I collagen of the integrin family and discoidin domain receptors (DDRs) have been shown to mediate MMP1 upregulation, MMP2 activation, and inhibition of collagen biosynthesis.[8,9]

The 3-hydroxy-3-methylglutaryl hydroxymethylglutaryl coenzyme A reductase inhibitors (statins), drugs commonly used to reduce plasma cholesterol levels, have been shown to directly interfere with the major processes of atherogenesis, including SMC proliferation and migration,[10–12] cholesterol accumulation in macrophages,[13] and endothelial cell activation.[14] Moreover, experimental models of atherosclerosis have clearly documented the beneficial effect of statins on fibrous plaque stability.[15] This effect primarily has been attributed to the inhibition of MMP expression,[16–19] but the precise molecular mechanisms remain unclear.

The pleiotropic effects of statins have been proposed to be dependent on inhibition of the mevalonate (MVA) pathway that reduces the biosynthesis of cholesterol and a number of nonsteroidal isoprenoid moieties essential for normal cellular activity, including farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate.[20] These intermediates serve as important lipid attachments for the posttranslational modification of a variety of proteins, including the small GTP-binding protein Ras and Ras-like proteins, such as Rho, Rac, and Rap.[21–23] The Rho family of GTP-binding proteins include Cdc42, Rac1, and RhoA, which can regulate the adhesive function of integrins by

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From the Department of Pharmacological Sciences (N.F., G.C., C.F., A.C.), University of Milan, Milan, Italy; Department of Pathology (E.W.R.), University of Washington, Seattle, Wash; Institute of Pathophysiology (B.L.), Center of Internal Medicine, University Hospital Essen, Essen, Germany.
Correspondence to Dr Nicola Ferri, Department of Pharmacological Sciences, Via Balzaretti 9, 20133, Milano, Italy. E-mail nicola.ferri@unimi.it

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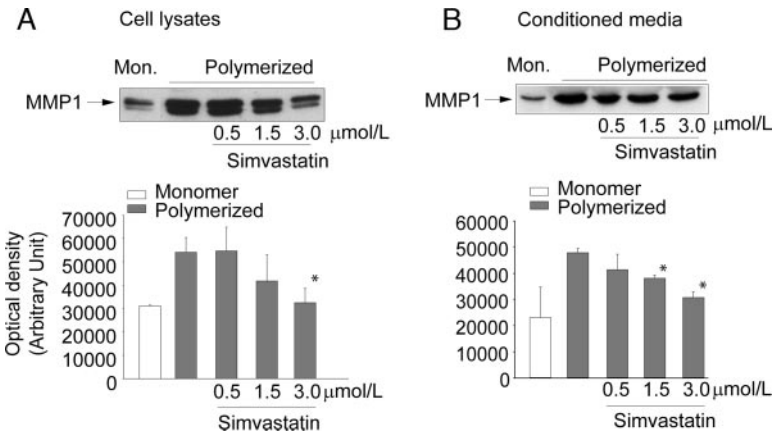

Figure 1. Simvastatin inhibits MMP1 expression and secretion by human SMCs cultured on polymerized collagen. MMP1 expression was evaluated by Western blot analysis of total cell lysates (A) and conditioned media (B). Quantitative densitometric analysis (lower panels) performed with Gel Doc acquisition system and Quantity One software (BIO-RAD). *$P<0.05$ by Student t test.

promoting avidity modulation through interaction with their effectors.[24] Thus, statins by inhibiting protein prenylation and the function of Rho GTP-binding proteins may indirectly regulate integrin clustering and function.[25] In the present study, we tested the hypothesis that simvastatin may affect MMP expression in response to polymerized collagen by interfering with protein prenylation.

Materials and Methods

For detailed descriptions of the Materials and Methods, please see supplemental material (available online at http://atvb.ahajournals.org).

Cell Culture and Collagen Matrix Preparation

Human newborn arterial SMCs were isolated from the thoracic aorta as previously described.[26] SMCs between passages 5 and 11 were cultured in 0.2% bovine serum albumin/modified Eagle medium on the surface of 3-dimensional polymerized collagen gels (Vitrogen; Nutacon BV, Leimuden, the Netherlands; 1.0 mg/mL final concentration) and monomer collagen-coated dishes.[27]

Results

Simvastatin Inhibits MMP1 Expression in Human SMCs Cultured on Polymerized Collagen

We have previously demonstrated that polymerized type I collagen induces MMP1 expression and MMP2 activation in human SMCs by activating the collagen receptors, $\alpha2\beta1$ integrin, DDR1, and DDR2.[3,28,29] In agreement with these studies, a significant induction of MMP1 expression and processing from pro-MMP2 to MMP2 is observed in human SMCs cultured on polymerized collagen compared with monomer collagen (Figure 1; supplemental Figure I, available online at http://atvb.ahajournals.org). Because statins have been shown to interfere with integrin signaling,[25,30,31] we hypothesized that simvastatin may interfere with MMP expression in response to polymerized collagen. In a first set of experiments, we exposed SMCs cultured on polymerized collagen to simvastatin for 24 hours. Increasing concentrations of simvastatin (0.5 to 3 μmol/L) significantly reduce intracellular and secreted MMP1 levels as determined by Western blot analysis of conditioned media and total cell lysates. Simvastatin at 3 μmol/L reduces MMP1 expression levels by 39.9±11.2%, and 36.0±2.3%, respectively; Figure 1A, 1B). Moreover, both atorvastatin (10 μmol/L) and fluvastatin (3 μmol/L) significantly affected MMP1 secretion from human SMCs, by 53.9±6.2 and 57.6±16.6% (supplemental Figure II). Under the same experimental conditions, simvastatin at 3 μmol/L reduces pro MMP2 expression levels at both intracellular (−30.6±3.6%) and extracellular levels (−16.6±3.4%) (supplemental Figure II). Simvastatin 1.5 and 3 μmol/L also significantly affected MMP2 secretion by 51.75±10.7 and 50.25±10.3%, as assessed by gelatin zymography analysis. Levels of MMP9, which is not normally expressed by cultured human SMCs, were evaluated at the same time and no alterations were observed with simvastatin (supplemental Figure II). These data indicate that simvastatin significantly affected MMP1 production and secretion from humans SMCs cultured on polymerized collagen together with reduced levels of MMP2 in the conditioned media.

Simvastatin Reduces MMP1 mRNA Levels and Inhibits MMP1 Promoter Activity

To further investigate the inhibitory effect of simvastatin on MMP1 expression, we measured mRNA levels of MMP1 by quantitative real-time polymerase chain reaction analysis. In the presence of 3 μmol/L simvastatin, MMP1 mRNA levels were reduced by 37.8±10.5% (Figure 2A). Similar results were obtained with 2 other statins, atorvastatin (10 μmol/L) and fluvastatin (3 μmol/L), that significantly reduce MMP1 mRNA levels by 85.1±6.7% and 93.0±0.8%, respectively, in SMCs cultured on polymerized collagen (Figure 2B). The statin inhibitory effect is caused by decreased MMP1 transcription, as simvastatin reduces MMP1 promoter activity in luciferase gene reporter assays in a concentration-dependent manner reaching a 33.0±8.9% reduction of transcription at 3 μmol/L (Figure 2C).

Simvastatin Inhibits MMP1 Expression by Interfering With the Mevalonate Pathway

Statins have been proposed to interfere with integrin signaling by mechanisms both dependent and independent of the 3-hydroxy-3-methylglutaryl-hydroxymethylglutaryl coenzyme A reductase activity,[25,30,31] the rate-limiting step of the MVA pathway. At concentrations where a significant reduction of MMP1 expression is observed (3 μmol/L), simvastatin completely abrogates de novo cholesterol biosynthesis, as assessed by [^{14}C]-acetate incorporation into cellular sterols (Figure 3A). The coincubation of simvastatin with MVA, or GGOH, a MVA-derived isoprenoid, completely prevents the

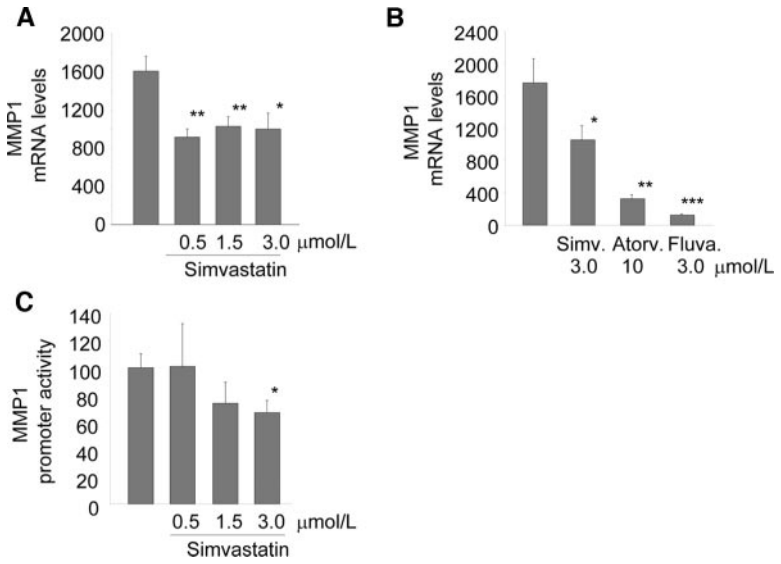

Figure 2. MMP1 mRNA expression and promoter activity is inhibited by simvastatin in human SMCs cultured on polymerized collagen. A and B, MMP1 mRNA levels were determined by quantitative real-time polymerase chain reaction analysis. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by Student *t* test. C, MMP1 promoter activity was evaluated in human SMCs retrovirally infected with pBM-SIN-proMMP1-luciferase constructs. *$P<0.05$ by Student *t* test. The data are representative of 2 independent experiments.

simvastatin block of MMP1 secretion from human SMCs, as evaluated by Western blot analysis of conditioned media (Figure 3B), and by quantitative real-time polymerase chain reaction (Figure 3C). Similar results were also observed with fluvastatin by real-time polymerase chain reaction quantification (supplemental Figure III). Furthermore, the incubation of SMCs with a specific protein geranylgeranyl transferase-I inhibitor, GGTI-286, reduces the amount of secreted MMP1 in human SMCs cultured on polymerized collagen by 53.1±7.6% at 10 μmol/L concentration (Figure 3D). These results suggest that statins reduce MMP1 expression levels by interfering with protein geranylgeranylation processes.

Rac1 Regulates MMP1 Secretion From Human SMCs Cultured on Polymerized Collagen

To further establish the role of geranylgeranylated proteins in the regulation of MMP1 expression, dominant-negative forms of RhoA and Rac1 were overexpressed in human SMCs with HA epitope-tags. Western blot analysis of total cell lysates shows that N19RhoA and N17Rac1 are efficiently expressed in human SMCs (data not shown). Whereas N19RhoA expression in human SMCs cultured on polymerized collagen has no effect on MMP1 secretion, N17Rac1 significantly reduced MMP1 expression levels by 50.4±5.4% (Figure 4A). MMP1 mRNA levels are also significantly reduced in human SMCs cultured on polymerized collagen overexpressing N17Rac1 (−97.9±1.0%) (Figure 4B). Overexpression of either dominant negative mutants does not affect MMP2 expression and activation in SMCs, as assessed by gelatin zymography analysis (Figure 4C).

To confirm the role of Rac1 in MMP1 expression, SMCs were transfected with siRNA against Rac1 and control siRNA, and MMP1 expression was evaluated by Western blot analysis of conditioned media. A marked reduction of Rac1

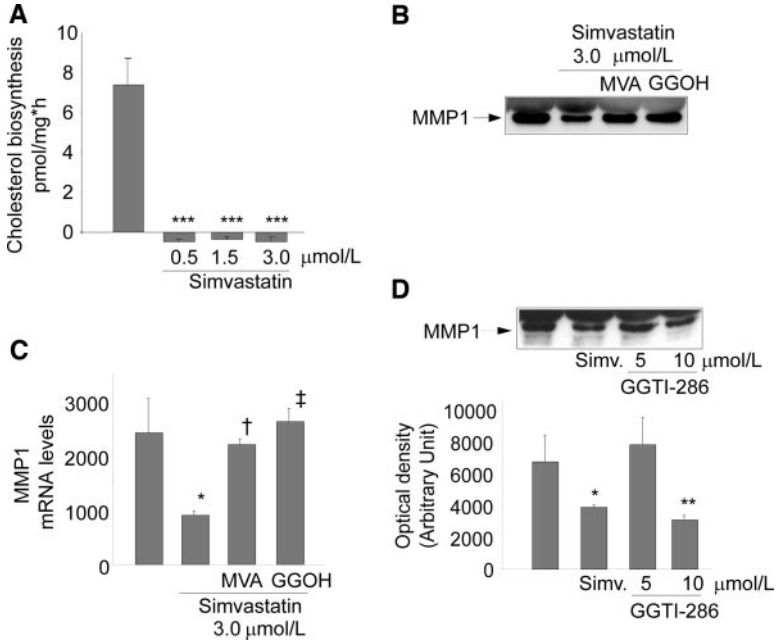

Figure 3. MMP1 expression in human SMCs cultured on polymerized collagen is dependent on protein geranylgeranylation. A, Cholesterol biosynthesis was determined by measuring the incorporation of [^{14}C]-Acetate into cellular sterols. ***$P<0.001$, simvastatin vs control by Student *t* test. B and C, Human SMCs were cultured on polymerized collagen in the presence or absence of 3 μmol/L simvastatin coincubated with 100 μmol/L mevalonate (MVA) and 2.5 μmol/L geranylgeraniol (GGOH). MMP1 protein (B) and mRNA levels (C) were evaluated by Western blot analysis of conditioned media, and quantitative real-time polymerase chain reaction of total RNA, respectively. *$P<0.05$ simvastatin vs control; †$P<0.001$ simvastatin plus MVA vs simvastatin; ‡$P<0.001$ simvastatin plus GGOH vs simvastatin by Student *t* test. D, Effect of GGTI-286 was tested on human SMCs cultured on polymerized collagen. MMP1 expression was determined by Western blot analysis of conditioned media (D). Quantitative densitometric analysis of (D). *$P<0.05$; **$P<0.01$ by Student *t* test (lower panel).

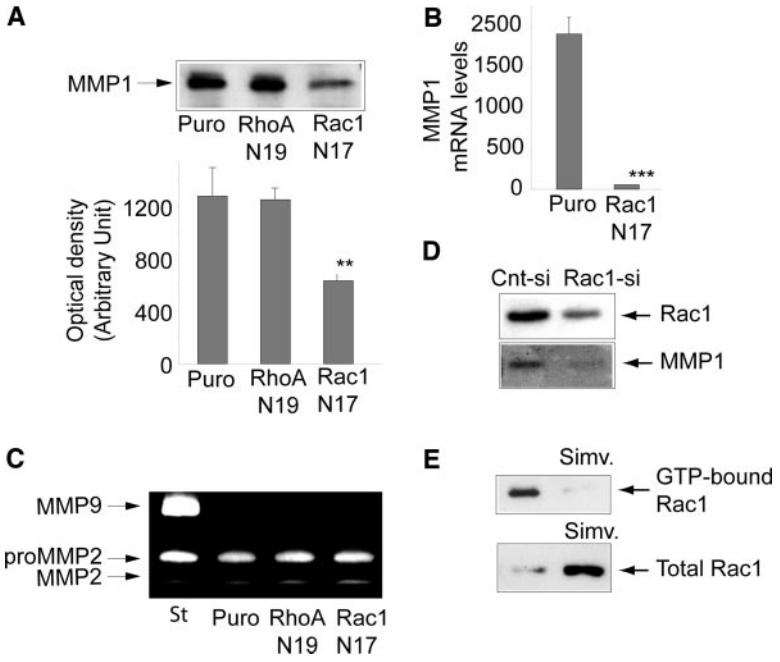

Figure 4. Simvastatin inhibits Rac1 activity that regulates MMP1 expression in human SMCs cultured on polymerized collagen. MMP1 (A) and MMP2 (C) protein expression were determined from conditioned media and mRNA (B) from cell lysates of human SMCs transduced with pBM-IRES-PURO retrovirus encoding control vector (PURO), 19NRhoA, or 17NRac1. Quantitative densitometric analysis is shown in lower panel (A). $^{**}P<0.01$ by Student *t* test. B, MMP1 mRNA levels were determined by quantitative real-time polymerase chain reaction analysis. $^{***}P<0.001$ by Student *t* test. C, Conditioned medium containing MMP9, pro-MMP2, and MMP2 was used as standard control (St). All the data are representative of 2 independent experiments. D, Human SMCs were transfected with specific siRNA against Rac1 or nonsilencing control siRNA, and Rac1 expression was evaluated by Western blot analysis after 24 hours of cultured on polymerized collagen. Conditioned media was collected and MMP1 expression evaluated by Western blot analysis. E, GTP-bound and total Rac1 expression was determined by Western blot analysis of total cell lysates of human SMCs cultured on polymerized collagen for 24 housr in the presence or absence of 3 μmol/L simvastatin.

protein expression is observed after Rac1 siRNA treatment that is associated with reduced secretion of MMP1 from the cells in response to polymerized collagen, as compared with SMCs transfected with control siRNA (Figure 4D).

These results indicate that activation of Rac1 is necessary to induce MMP1 expression in human SMCs in response to polymerized collagen, and support the possibility that simvastatin may inhibit MMP1 expression through the blockade of protein geranylgeranylation. To directly address this question we investigated the effect of simvastatin on the activation state of Rac1. Total cell lysates were prepared from SMCs cultured on polymerized collagen in the presence or absence of 3 μmol/L simvastatin. Active Rac1 was precipitated with the PBD domain of human PAK1 fused to glutathione S-transferase, and total Rac1 were determined by Western blot analysis. As shown in Figure 4E, simvastatin strongly reduces Rac1 activity in SMCs cultured on polymerized collagen, in spite of simvastatin upregulation of Rac1 expression in SMCs cultured on polymerized collagen.

Simvastatin Reduces the Collagen Degrading Activity of Human SMCs

To evaluate whether the simvastatin-mediated reduction of MMP1 expression is sufficient to affect the degradative and remodeling activities of human SMCs, we measured levels of MMP inhibitors and the release of collagen fragments into the media of SMCs cultured on fluorescein isothiocyanate-labeled polymerized collagen. Simvastatin does not effect the expression of endogenously expressed inhibitors TIMP1 and reversion-inducing cysteine-rich protein with Kazal motifs,[32] and only slightly reduces TIMP2 expression levels (Figure 5B).[33] Simvastatin at 3 μmol/L inhibited SMC collagen degradation by 34.2±6.1%, whereas the broad MMP inhibitor GM-6001 led to 57.6±1.5% reduction (Figure 5A). This effect strongly correlated with MMP1 expression levels (Figure 5C).

Discussion

Rac1 Is the Primary Intracellular Target of Simvastatin-Mediated Reduction of MMP1 Expression in Response to Polymerized Collagen

We have previously shown that integrin and DDR-mediated adhesion of SMCs to polymerized collagen induces MMP1 expression and collagen degradation.[3,28,29] In the present study, we investigated the effect of simvastatin on this cellular response and show that the inhibition of the MVA pathway with a subsequent inactivation of Rac1 leads to the reduction of MMP1 expression induced by polymerized collagen (supplemental Figure IV). The demonstration that the effect of simvastatin on MMP1 expression is dependent on the inhibition of protein geranylgeranylation is supported by the evidence that geranylgeraniol, a substrate of protein

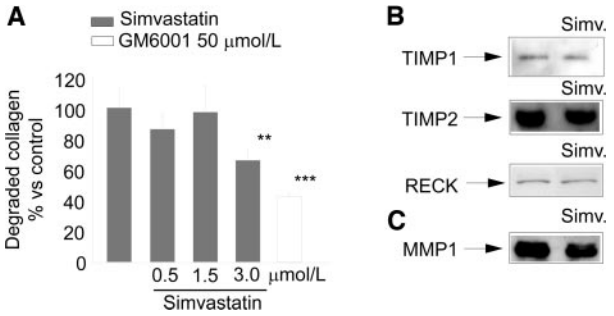

Figure 5. MMP-mediated collagen degradation is inhibited by simvastatin. A, Human SMCs were cultured on fluorescein isothiocyanate-polymerized collagen in the presence or absence of the MMP inhibitor GM6001 and indicated concentrations of simvastatin for 24 hours. Release of fluorescein isothiocyanate-collagen fragments was measured by fluorimetric analysis. $^{**}P<0.01$ and $^{**}P<0.001$ by Student *t* test. B, TIMP1, TIMP2, and (C) MMP1 expression were evaluated by Western blot analysis of conditioned media of SMCs cultured in the presence or absence of 3 μmol/L simvastatin, and reversion-inducing cysteine-rich protein with Kazal motifs (B) from total cell lysates.

geranylgeranyltransferases, completely restores protein and mRNA levels blocked by simvastatin, and that the protein geranylgeranyl transferase-I inhibitor GGTI-286 significantly inhibits MMP1 secretion. We therefore hypothesized that one or more geranylgeranylated protein(s) may be involved in this process.

Among several prenylated proteins, the Rho family of GTPases is the best characterized, and several studies have implicated the Rho family in the regulation of MMP1 expression. For example, it has been reported that Rac1 activity is required for the induction of MMP1 mediated by inactivation of the $\alpha 5\beta 1$ integrin.[34] Moreover, downregulation of Cdc42 by siRNA led to upregulation of MMP1 in human skin fibroblasts, an effect that required the presence of Rac1.[35] Our studies extend Rac1 involvement to normal human SMCs with the demonstration that overexpression of the dominant-negative mutant of Rac1, or suppression of Rac1 expression with siRNA, is sufficient to prevent the upregulation of MMP1 on polymerized collagen.

The dependence of simvastatin's action on Rac1 inhibition is also supported by the levels of active Rac1 in cells cultured on polymerized collagen. Rac1 activity is strongly inhibited by simvastatin, an effect most likely caused by a reduction in the intracellular availability of geranylgeranyl-PP. Thus, we conclude that Rac1 represents one of the simvastatin's intracellular targets, and its inhibition suppresses MMP1 expression (supplemental Figure IV). Moreover, we showed that simvastatin reduces the collagenolytic activity of human SMCs, by shifting the MMP/MMP inhibitors balance. Indeed, simvastatin strongly reduces MMP1 and MMP2 extracellular levels without affecting the expression of tissue inhibitor of metalloproteinse (TIMP)-1 reversion-inducing cysteine-rich protein with Kazal motifs,[32] and only slightly reduces TIMP-2 levels.[33]

Statins have been previously shown to reduce the expression of MMP1, MMP2, and MMP9 in human saphenous vein SMCs stimulated with a combination of PDGF-BB and IL1-α.[17,36] The effect on MMP9 was probably caused by the inhibition of the RhoA/ROCK pathway because the Rho-kinase (ROCK) inhibitor Y27632 also decreased MMP9 secretion,[36] while the inhibitory effect of lovastatin on MMP1 and MMP2 expression was prevented by the addition of geranylgeranyl-PP suggesting the involvement of prenylated proteins.[17] Moreover, the inhibition of Rho proteins by C3 exoenzyme has been shown to decrease the basal secretion of MMP1 from human umbilical endothelial cells,[18] indicating the involvement of a small GTP-binding protein(s). The present study specifically identifies Rac1 as the primary Rho family GTPase responsible for MMP1 induction after the SMC collagen receptor engagement on polymerized collagen. Further studies will be required to determine the role of Rac1 and/or other RhoA in MMP1 expression in response to platelet-derived growth factor (PDGF)-BB and IL-1α from human SMCs. Moreover, simvastatin significantly reduced pro MMP2 levels and MMP2 secretion, but the involvement and the identification of intracellular prenylated proteins responsible for this action still needs to be determined. In fact, the expression of both RhoA and Rac1 dominant-negative mutants did not affect MMP2 expression levels, as assessed by gelatin zymography analysis.

Simvastatin Inhibits MMP1 Transcription by Interfering With Collagen Receptor Signaling

The adhesion of SMCs to polymerized collagen is primarily mediated by $\alpha 2\beta 1$ integrin and DDRs,[8,9] and both receptor families have been shown to upregulate MMP1.[3,5,29] The hypothesis that simvastatin may inhibit this cellular response was based on previous findings that integrin signaling can be affected by statins in different ways.[25,30,31] The best-understood molecular mechanisms of integrin blockade by statins are the alteration of integrin clustering and activation through the inhibition of the prenylation and functional modulation of Rho family of GTPases.[25] However, statins have also been shown to upregulate $\alpha 2\beta 1$ integrin expression in human SMCs, and to increase cell adhesion to collagen, effects reversed by MVA and geranylgeraniol.[30] In our study, we did not observe any significant change in either SMC adhesion to polymerized collagen or cell-surface expression of $\alpha 2\beta 1$ integrin in response to simvastatin (data not shown). These data suggest that simvastatin is unlikely to alter cell–collagen interactions by directly binding the I domain of $\alpha 2$ integrin subunit, as previously described for the leukocyte function antigen-1 in T-cells.[31] Furthermore, the interaction of statins with integrin I domain was shown to be independent of its action on 3-hydroxy-3-methylglutaryl-hydroxymethylglutaryl coenzyme A reductase, whereas our study demonstrates that MVA completely rescues simvastatin-mediated suppression of MMP1. Thus, our data indicate that simvastatin may affect $\alpha 2\beta 1$ integrin and/or DDR signaling by interfering with small GTP-binding protein activity and receptor and/or DDR clustering.

MMP1 expression in response to collagen-receptor engagement is regulated at the transcriptional level, and nuclear factor κ-B (NF-κB) plays a major role in MMP1 transcription.[3,5] Although the presence of a putative responsive NF-κB elements on human MMP1 promoter is still unclear,[3,37] we demonstrate that simvastatin inhibits MMP1 promoter activity. The inhibition of MMP1 transcription by simvastatin appears to be mediated by Rac1 blockade. Expression of MMP1 mRNA is significantly reduced by the expression of a dominant-negative Rac1, and MMP1 expression is completely restored by co-incubation with geranylgeraniol. The effect of simvastatin on NF-κB activation in our cell culture model still needs to be determined, but statins have been previously shown to inhibit NF-κB activation in endothelial cells.[38] Because Rac1 is a potent inducer of NF-κB transcriptional activity,[39] it is conceivable that simvastatin may decrease MMP1 expression by affecting NF-κB activity.

Potential Implications of Statin-Mediated Inhibition of Smooth Muscle Integrin-Dependent Collagen Remodeling

Culture systems using collagen gels have been developed to more closely resemble the cell–matrix interactions observed in vivo in fibroproliferative disorders, including atherosclerosis.[4] One limitation of this cell–culture system is that SMCs change their integrin expression from the $\alpha 1$ to $\alpha 2$ subunit

when isolated from vessels and placed in culture.[40] In fact, vascular SMCs in the media of normal arteries only express $\alpha1\beta1$ integrin with no detectable $\alpha2\beta1$.[40,41] However, both $\alpha1\beta1$ and $\alpha2\beta1$ integrins have been implicated in collagen remodeling,[4,41] and pharmacological interference with both receptors may alter extracellular matrix degradation.

The casual contribution of $\alpha1\beta1$ integrin to the development of lesions of atherosclerosis in vivo have been recently shown: genetic deletion of $\alpha1\beta1$ integrin in apolipoprotein E-deficient mice led to a reduction of atherosclerotic plaque formation associated with an increase in collagen deposition.[42] Further, atherosclerotic lesions of apolipoprotein E-deficient mice treated with anti $\alpha1$ integrin antibody showed increased collagen content.[42] Thus, inhibition of $\alpha1\beta1$ integrin shifted plaque composition to a more stable phenotype, with increased collagen and SMC content. Our study shows that simvastatin strongly suppresses the induction of MMP1 and SMC collagenolytic activity dependent on collagen receptors. We propose that this mechanism may contribute to the ability of statins to stabilize lesions of atherosclerosis.

In conclusion, we show that the adhesion of human SMCs to polymerized collagen, through $\alpha2\beta1$ integrin, and the DDRs upregulate MMP1 expression and collagen degradation by a mechanism dependent on Rac1 activity. Simvastatin and the protein geranylgeranyltransferase-I inhibitor GGTI-286 interfere with this signaling pathway, thus leading to a reduction of MMP1 expression and collagen degradation in human SMCs (supplemental Figure IV).

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Disclosures

None.

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