Fibrillar Collagen Inhibits Cholesterol Biosynthesis in Human Aortic Smooth Muscle Cells

Nicola Ferri, Elisa Roncalli, Lorenzo Arnaboldi, Simone Fenu, Olena Andrukhova, Seyyedhossein Aharinejad, Marina Camera, Elena Tremoli, Alberto Corsini

Objective—Integrin-mediated cell adhesion to type I fibrillar collagen regulates gene and protein expression, whereas little is known of its effect on lipid metabolism. In the present study, we examined the effect of type I fibrillar collagen on cholesterol biosynthesis in human aortic smooth muscle cells (SMCs).

Methods and Results—SMCs were cultured on either fibrillar or monomer collagen for 48 hours and [14C]-acetate incorporation into cholesterol was evaluated. Fibrillar collagen reduced by 72.9±2.6% cholesterol biosynthesis without affecting cellular cholesterol levels. Fibrillar collagen also reduced 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) promoter activity (−72.6±7.3%), mRNA (−58.7±6.4%), protein levels (−35.5±8.5%), and enzyme activity (−37.7±2.2%). Intracellular levels of the active form of sterol regulatory element binding proteins (SREBP) 1a was decreased by −60.7±21.7% in SMCs cultured on fibrillar collagen, whereas SREBP2 was not significantly affected (+12.1±7.1%). The overexpression of the active form of SREBP1a rescued the downregulation of fibrillar collagen on HMG-CoA reductase levels. Blocking antibody to α2 integrin partially reversed the downregulation of HMG-CoA reductase mRNA expression. Finally, fibrillar collagen led to an intracellular accumulation of unprenylated Ras.

Conclusions—Our study demonstrated that α2β1 integrin interaction with fibrillar collagen affected the expression of HMG-CoA reductase, which led to the inhibition of cholesterol biosynthesis in human SMCs. (Arterioscler Thromb Vase Biol. 2009;29:00-00.)

Key Words: ●●●

Smooth muscle cells (SMCs) normally reside in the media of the artery surrounded by a meshwork of extracellular matrix (ECM) components, including type I collagen. The adhesion of SMCs to type I collagen is mainly mediated by the interaction with α1β1 and α2β1 integrins that play a pivotal role as a receptor for ECM. After their occupancy and clustering, integrins can activate intracellular signaling pathways,1,2 followed by the induction of transcription factors, and subsequent gene and protein expressions.3 Indeed, ECM 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.4,5 To resemble the extracellular environment surrounding the SMCs present in the arterial wall, a cell culture system based on 3 dimensional type I fibrillar collagen gel has been developed.6,7 Cells cultured on fibrillar collagen have been also compared to cells cultured on petri dishes coated with monomeric collagen, a structural distinct form of collagen that has been considered to resemble the ECM environment surrounding SMCs in atherosclerotic plaques.6,7 In this in vitro system, several changes have been described, from inhibition of cell proliferation,7 migration,8,9 dedifferentiation,4 and ECM remodeling,6,8,10 supporting the notion that cell–collagen interaction directly regulates SMC function. Although these studies have helped to understand the role of type I collagen on SMC behavior, very little is known on its effect on cholesterol biosynthesis and on mevalonate (MVA) pathway. On this matter it is important to note that cholesterol present in the plasma membrane lipid rafts has been shown to affect signal transduction events initiated by cell adhesion to the ECM through integrins.11,12

Cholesterol represents 1 critical end-product of the MVA pathway required for cell membrane formation in proliferating cells.13 Moreover, MVA is not only the precursor of cholesterol but also of a number of nonsteroidal isoprenoid
moieties, including farnesyl-pyrophosphate (F-PP) and geranylgeranyl-pyrophosphate (GG-PP). These 2 isoprenoid derivatives are indeed substrates of prenyltransferases that catalyze the prenylation of several intracellular proteins, including the small GTP-binding protein Ras and Ras-like proteins, such as Rho and Rac involved in several cellular activities. Thus, the inhibition of the MVA pathway may impair the activity of several intracellular proteins, resulting in different cellular behavior. It is for this reason that the MVA pathway and thus the cholesterol biosynthesis are tightly regulated cellular events.

Sterol regulatory element binding proteins (SREBPs), bound to membranes of the endoplasmic reticulum (ER) and nuclear envelope, play a pivotal role in the regulation of cellular cholesterol content. The family consists of 3 different proteins, SREBP1a, SREBP1c, and SREBP2. In cultured cells, SREBP1a is expressed at much higher levels than SREBP1c. The membrane associated proteins are transcriptionally inactive and are bound to the SREBP-cleavage-activating protein (SCAP), which functions as a sterol sensor. When cells are deprived of sterols, SCAP escorts the SREBPs from the ER to the Golgi, where a 2-step proteolytic cleavage releases the amino terminus portion of the SREBPs from cell membranes. These transcriptionally active fragments of the SREBPs translocate to the nucleus activating the transcription of genes involved in cholesterol biosynthesis and in the MVA pathway.

SREBP1a is a potent activator of all SREBP-responsive genes, including those that mediate the biosynthesis of cholesterol, fatty acids (FA), and triglycerides, but the roles of SREBP1c and SREBP2 are more restricted. It is generally assumed that genes involved in FA metabolism (eg, FA-synthase) are preferentially regulated by SREBP1c, whereas the expression of enzymes involved in cholesterol metabolism (eg, HMG-CoA synthase and reductase) and uptake (LDL-receptor) are equally modulated by the activity of both SREBP1c and SREBP2.

In the present study we have investigated the effects of type I fibrillar collagen on cholesterol metabolism in human aortic SMCs. We show that fibrillar collagen, through SCAP escort, has a profound effect on HMG-CoA reductase expression, event the altered cholesterol biosynthesis in human aortic SMCs, without changing the intracellular cholesterol content.

Experimental Procedures

Cell Culture and Collagen Matrix Preparation

Human aortic SMCs were isolated from the thoracic aorta of cardiopathic patients undergoing cardiac transplantation. SMCs between passages 5 and 9 were cultured in 0.2% BSA/DMEM on the surface of 3 dimensional fibrillar collagen gels (PureCol, Nutacon BV, Leimuden, NE, 1.0 mg/mL final concentration) and monomer collagen-coated dishes for 48 hours. For detailed descriptions of the Materials and Methods, please see supplemental materials (available online at http://atvb.ahajournals.org).

Results

Fibrillar Collagen Inhibits Cholesterol Biosynthesis in Human SMCs

To study the effect of cell–collagen interaction on cholesterol homeostasis we examined the effects of structurally distinct forms of type I collagen, monomer versus polymerized fibrils. All the experiments were performed under serum free conditions, to induce cholesterol biosynthesis and to avoid any confounding factors deriving from growth factors and cytokines present in the FCS. Compared to monomer, fibrillar collagen reduced cholesterol biosynthesis, measured by incorporation of [14C]-acetate into total cholesterol, by 72.9±2.6% (Figure 1A). In contrast, the total intracellular cholesterol content was similar under the 2 experimental conditions (Figure 1B).

HMG-CoA Reductase Activity Is Responsible for the Reduction of Cholesterol Biosynthesis in Human SMCs Cultured on Fibrillar

To investigate the molecular mechanisms underlying the inhibition of cholesterol biosynthesis by fibrillar collagen, we measured the HMG-CoA reductase activity, the rate limiting step of the MVA pathway, under the aforementioned experimental conditions. The conversion of radiolabeled HMG-CoA by isolated microsomes from human SMCs cultured on fibrillar collagen to MVA was reduced by 37.7±2.2% (Figure 2A). This effect strongly correlated with the expression of HMG-CoA reductase measured by Western blot analysis, which was decreased by 35.5±8.5% (Figure 2B).

The expression of HMG-CoA reductase is primarily regulated at the transcription levels, thus we measured the mRNA levels and the promoter activities. As shown in Figure 2C, fibrillar collagen significantly reduced the mRNA of HMG-CoA reductase by 58.7±6.4%, determined by quantitative RT-PCR reaction. The analysis of the HMG-CoA...
incubating SMCs with [14C]-acetate was strongly suppressed in Figure 2E, the evaluation of cholesterol biosynthesis by incorporation of radiolabeled MVA, the product of HMG-CoA into cellular cholesterol.13 As shown in Figure 2E, the evaluation of cholesterol biosynthesis by incubating SMCs with [14C]-acetate was strongly suppressed by fibrillar collagen, whereas the incorporation of [3H]-MVA was not affected.

Taken together, fibrillar collagen significantly affected cholesterol biosynthesis by inhibiting the transcription and therefore the activity of HMG-CoA reductase, the rate-limiting step of the MVA pathway.

Figure 2. HMG-CoA reductase activity was suppressed in human SMCs cultured on fibrillar collagen. A through E, Human SMCs were cultured for 48 hours with DMEM containing 0.2% BSA on monomer and fibrillar collagen. A. After this period, the HMG-CoA reductase activity was determined by measuring the rate of conversion of radioactive HMG-CoA into MVA in detergent-solubilized cell-free extract as described in Material and Methods. Each bar represents the mean ± SD of triplicate samples. B, HMG-CoA reductase expression was evaluated by Western blotting analysis of total protein extracts. Quantitative densitometric analysis was performed with Gel Doc acquisition system and Quantity One software (BIO-RAD). C, HMG-CoA reductase mRNA expression was evaluated by quantitative real-time polymerase chain reaction from total RNA. The data are representative of 2 independent experiments. D, HMG-CoA promoter activity was evaluated in human SMCs retrovirally infected with pBM-SIN-HMG-CoApro-luciferase construct. The data are representative of 2 independent experiments. E, Human SMCs were cultured on monomer and fibrillar collagen in DMEM containing 0.2% BSA in the presence of either [14C]-Acetate or [3H]-MVA. After 48 hours [14C]-Acetate and [3H]-MVA incorporation into cellular cholesterol was evaluated. Each bar represents the mean ± SD of triplicate dishes. The data are representative of 2 independent experiments. *P<0.05; **P<0.01 by Student t test.

To directly demonstrate that the inhibition of cholesterol biosynthesis elicited by fibrillar collagen was dependent by the activity of the HMG-CoA reductase, we measured the incorporation of radiolabeled MVA, the product of HMG-CoA reductase reaction, into cellular cholesterol.13 As shown in Figure 2E, the evaluation of cholesterol biosynthesis by incubating SMCs with [14C]-acetate was strongly suppressed by fibrillar collagen, whereas the incorporation of [3H]-MVA was not affected.

Taken together, fibrillar collagen significantly affected cholesterol biosynthesis by inhibiting the transcription and therefore the activity of HMG-CoA reductase, the rate-limiting step of the MVA pathway.

Figure 3. Fibrillar collagen affected SREBP1a activation in human SMCs. A through D, Human SMCs were cultured for 48 hours with DMEM containing 0.2% BSA on monomer and fibrillar collagen. Two hours before preparation of protein extracts 25 μg/mL of the proteasome inhibitor ALLN was added to the cultured media. SREBP1a and SREBP2 expression and processing were then evaluated by Western blotting analysis of total protein extracts (A and B). As a positive control, cells were incubated with simvastatin (5 μmol/L). For SREBP1a precursor form the film was exposed for 10 minutes, whereas for SREBP1 active form for 2 minutes. C and D, mRNA expression levels of SREBP1 and SREBP2 were evaluated by quantitative real-time polymerase chain reaction from total RNA extracted from SMCs cultured for 24 hours on monomer and polymerized collagen.

Fibrillar Collagen Regulates SREBP-1a Transcriptional Activity

Several studies have firmly established the pivotal role of SREBPs in the regulation of HMG-CoA reductase transcription.13,28 To investigate whether the observed reduction of HMG-CoA reductase expression levels mediated by fibrillar collagen was dependent by the transcriptional activity of SREBPs, we evaluated the processing and the expression of SREBP1a and SREBP2. Western blotting analysis from total cell lysates demonstrated that fibrillar collagen reduced the expression levels of the active form of SREBP1a at 68 KDa (−60.7±21.7%) with a minor reduction of the pro form at 125 KDa (−29.4% the SREBP1a precursor form the film was exposed for 10 minutes, whereas for SREBP1 active form for 2 minutes. C and D, mRNA expression levels of SREBP1 and SREBP2 were evaluated by quantitative real-time polymerase chain reaction from total RNA extracted from SMCs cultured for 24 hours on monomer and polymerized collagen.

To directly demonstrate that the inhibition of cholesterol biosynthesis elicited by fibrillar collagen was dependent by the activity of the HMG-CoA reductase, we measured the incorporation of radiolabeled MVA, the product of HMG-CoA reductase reaction, into cellular cholesterol.13 As shown in Figure 2E, the evaluation of cholesterol biosynthesis by incubating SMCs with [14C]-acetate was strongly suppressed by fibrillar collagen, whereas the incorporation of [3H]-MVA was not affected.

Taken together, fibrillar collagen significantly affected cholesterol biosynthesis by inhibiting the transcription and therefore the activity of HMG-CoA reductase, the rate-limiting step of the MVA pathway.

The expression of levels SREBP1a and SREBP2 mRNA were also measured by RT-PCR from total RNA extracted from SMCs cultured for 24 hours on monomer and fibrillar collagen. Fibrillar collagen reduced by 48.3±29.4% the expression level of SREBP1 mRNA, although this difference was found not to be statistically significant (Figure 3C), whereas SREBP2 expression levels were found similar between monomer and fibrillar collagen. The incubation with simvastatin induced both genes in SMCs cultured either on monomer or fibrillar collagen (Figure 3C and 3D).
To further establish the effect of fibrillar collagen on SREBP1a, we analyzed the mRNA levels of 2 genes directly regulated by this transcription factor, such as HMG-CoA synthase and FA-synthase.28 The expression levels of HMG-CoA synthase and FA-synthase in SMCs cultured on fibrillar collagen were reduced by 46.2 ± 6.4% and 67.3 ± 16.0%, respectively, further supporting the effect of fibrillar collagen on SREBP1a activity (supplemental Figure I).

To directly demonstrate that SREBP1 transcriptional activity was responsible for the reduction of HMG-CoA reductase in SMCs cultured on fibrillar collagen, we retrovirally overexpressed in SMCs the active amino terminal portion of SREBP1a. As shown in Figure 4, the overexpression of SREBP1a in SMCs led to the upregulation of HMG-CoA reductase mRNA (Figure 4A) and protein (Figure 4B) in SMCs on either monomer or fibrillar collagen, compared to cells transduced with empty vector control. Finally, the downregulation of SREBP1 in human cultured SMCs by 2 different siRNA probes significantly reduced the expression of HMG-CoA reductase on both monomer and fibrillar collagen (supplemental Figure II).

Taken together these results demonstrate that the inhibition of HMG-CoA reductase transcription observed in human SMCs cultured on fibrillar collagen is dependent by the activity of SREBP1a.

**α2β1 Integrin Collagen Receptor Mediates the Reduction of HMG-CoA Reductase Transcription**

The adhesion of SMCs to fibrillar collagen is primarily mediated by α1β1 and α2β1 integrin receptors.30 To investigate which of these 2 collagen receptors were responsible for the observed responses, we first determine the effect of blocking antibodies toward α1 and α2 integrin subunits on SMC adhesion to fibrillar collagen. In agreement with previous studies,31 the adhesion of human SMC to collagen is largely mediated by α2β1 integrin, indeed the anti-α2 blocking antibody reduced cell adhesion by 70.0 ± 3.2%, whereas anti-α1 antibody affected only the 27.4 ± 5.1% (Figure 5A). Interestingly, the mRNA expression levels of α2 and β1 integrin subunits in SMCs cultured for 48 hours on fibrillar collagen were increased by 8.9- and 8.7-fold, respectively, whereas mRNA of α1 subunit was unaffected (Figure 5B).

Incubation of human SMCs with blocking antibody to α2 integrin significantly prevented the effect of fibrillar collagen on HMG-CoA reductase mRNA expression, whereas a blocking antibody directed to the α1 integrin subunit was ineffective (Figure 5C). These data indicate that the interaction of SMCs with fibrillar collagen through α2β1 integrin partially...
mediates the downregulation of HMG-CoA reductase and cholesterol biosynthesis.

Ras Farnesylation Is Partially Impaired in Human SMCs Cultured on Fibrillar Collagen

Because the HMG-CoA reductase activity is the rate-limiting step of the MVA pathway, the inhibition of its catalytic activity in human SMCs could affect the biosynthesis of isoprenoids involved in protein prenylation, such as F-PP and GG-PP. Thus, to study whether fibrillar collagen can alter the prenylation of intracellular proteins we determined, by Western blotting analysis, the expression of prenylated and unprenylated Ras. The slower migrating band was prenylated Ras (pren. Ras), whereas the faster migrating band was unprenylated Ras (unpren. Ras). Lower panel, Quantitative densitometric analysis of prenylated and unprenylated Ras was performed with Gel Doc acquisition system Quantity One software (BIO-RAD) from films of 3 independent experiments.

Discussion

In the present study we showed, for the first time, that adhesion of human aortic SMCs on fibrillar collagen strongly impaired cholesterol biosynthesis, the major end-point product of the MVA pathway. Because the HMG-CoA reductase is the rate limiting step of the MVA pathway, we focused our attention on this enzyme by determining the effect of fibrillar collagen on the HMG-CoA reductase expression at 4 different levels: the promoter activity, the mRNA and protein expression, and the enzyme activity. All these parameters were significantly inhibited by fibrillar collagen, indicating that the observed reduction of cholesterol biosynthesis was indeed the result of reduced HMG-CoA reductase activity. A direct demonstration that the activity of this enzyme was affected by fibrillar collagen was then provided by the fact that the incorporation of radiolabeled MVA, the product of HMG-CoA reductase reaction, into cellular cholesterol was not affected. Together with the HMG-CoA reductase we also found a significant reduction of the expression of the HMG-CoA synthase mRNA level, indicating that both enzymes contributed to the inhibition of the cholesterol biosynthesis in SMCs cultured on fibrillar collagen.

Many of the mRNAs encoding enzymes involved in cholesterol and FA synthesis are coordinately regulated by the SREBP family of membrane-bound transcription factors. We therefore determined whether SREBPs were involved in the regulation of HMG-CoA reductase expression by fibrillar collagen. We observed that SREBP1a, but not SREBP2, was differentially processed in cells cultured on fibrillar versus monomer collagen. In particular, we observed a consistent reduction of the active SREBP1a cleaved product in human SMCs cultured on fibrillar collagen and a minor reduction of the pro form of SREBP1a. In association with these changes the inhibition of SREBP1a transcriptional activity was demonstrated by the fact that the HMG-CoA reductase promoter activity and the mRNA expression levels of 2 genes directly regulated by SREBP1a (ie, FA-synthase and HMG-CoA synthase) were reduced in SMCs cultured on fibrillar collagen.

The direct relationship between SREBP1a transcriptional activity and the expression of HMG-CoA reductase was also demonstrated by the fact that the exogenous overexpression of the active form of SREBP1a almost completely prevented the effect of fibrillar collagen on HMG-CoA reductase levels at the protein and mRNA levels. Moreover, a partial reduction of SREBP1a levels by siRNA significantly reduced the expression of HMG-CoA reductase mRNA.

Although we clearly demonstrated a reduction of SREBP1a transcriptional activity in SMCs cultured on fibrillar collagen, the molecular mechanism governing this event still needs to be determined. The fact that the mRNA levels of SREBP1a was reduced, but not in a statistically significant manner, in response to fibrillar collagen indicates that the regulation of SREBP1a may partially occur at the transcriptional levels, but additional posttranscriptional regulation mechanisms cannot be excluded. Further experiments will be carried out to determine the effect of fibrillar collagen on SREBP transport from ER to the Golgi, a very complex event regulated by several factors, such as SCAP, Site-1, and Site-2 protease, and Insig1 and Insig2.

The activation of SREBPs is a tightly regulated process that responds primarily by the intracellular concentrations of sterols. In the present study all the experiments were performed in serum-free media, a condition that induces the cholesterol neosynthesis by the activation of SREBP1a and SREBP2. Under these conditions we did not observe any significant change of total intracellular cholesterol content, suggesting a specific role of fibrillar collagen in the regulation of SREBP1a-dependent HMG-CoA reductase expression. The lack of reduction in total cholesterol content was expected for at least 3 reasons: (1) the incubation time used was relatively short (48 hours),
(2) the cultured media used was free of cholesterol acceptors (ie, HDL), and (3) the cells were in a quiescent state under both cultured conditions.\(^6^,7\) Moreover, our observation is in agreement with pioneer experiments conducted by Maltese et al\(^3\) in which the suppression of cholesterol biosynthesis by 25-hydroxycholesterol in C-6 glioma cells incubated with serum free media for 48 hours did not affect the sterol/protein ratio.

The interaction of human SMCs to collagen is primarily mediated by \(\alpha_\beta1\) and \(\alpha_\beta1\) integrin receptors. By gene array analysis we confirmed a previous observation that fibrillar collagen specifically induces \(\alpha_2\) and \(\beta1\) integrin subunit in human SMCs.\(^3,18\) By using blocking antibodies to \(\alpha_1\) and \(\alpha_2\) integrin subunits we then demonstrated that the \(\alpha_\beta1\) integrin receptor is at least partially involved in the suppression of HMG-CoA reductase mediated by fibrillar collagen, whereas \(\alpha_\beta1\) had no effect. These data indicate that other collagen receptors may be involved in this cellular response, including the recently discovered Discoidin domain receptors (DDRs).\(^20,36\)

Importantly, cell signal transduction events initiated by cell adhesion to the ECM through integrins are profoundly regulated by the presence of cholesterol within the plasma membrane lipid rafts.\(^11,12\) Indeed, disruption of lipid rafts by cholesterol depletion mimicked loss of cell adhesion, preventing targeting of prenylated protein Rac to the plasma membrane and its coupling with downstream effector molecules.\(^1,12\) Reports from Pozo et al and Palazzo at al also indicate that lipid rafts are commandeered by integrins to target Rho and Rac to the cell membrane.\(^11,12\) Thus, integrin-mediated adhesion to ECM appears to be directly involved in lipid raft assembly. Moreover, an elegant study conducted in \(\beta1\) null fibroblast cell line demonstrated that cell adhesion through \(\beta1\) integrin receptor regulates the amount of cholesterol present in the lipid raft without altering the total intracellular cholesterol levels.\(^28\) Our study further implements these observations demonstrating, for the first time, that signaling from integrin receptors regulates cholesterol biosynthesis by altering the activity of HMG-CoA reductase. Moreover, we demonstrated that the inhibition of HMG-CoA reductase and therefore the MVA pathway affected protein prenylation of Ras. On this matter it is tempting to speculate that the inhibition of Ras prenylation, and therefore its function, may contribute to the G1 arrest of the cell cycle described in human SMCs cultured on fibrillar collagen.\(^7\)

The effect of fibrillar collagen on SMC proliferation has been previously interpreted to being relevant in maintaining SMCs in a low proliferative index in normal media, whereas in response to proteolytic enzymes, the degradation of collagen may alter the cellular response to growth factors facilitating the accumulation of SMCs within the atherosclerotic plaque.\(^6,7\) Our findings further implement this hypothesis demonstrating that the suppression of the MVA pathway and protein prenylation may be one of the basic molecular events implicated in the maintenance of SMCs in a quiescent state and preventing their accumulation in the neointimal during atherogenesis.

In association with cell cycle arrest Koyama et al have described a reduction of focal adhesion sites and actin stress fibers formation in SMCs cultured on fibrillar collagen.\(^7\) These effects may be indicative of a deregulation of the prenylated proteins Rho, Rac, and Cdc42, potentially related to the reduced activity of HMG-CoA reductase and therefore protein prenylation.\(^39\) Further studies will be aimed at investigating the effect of fibrillar collagen on Rho protein functions as related to cell morphology and plasma lipid rafts formation.

In conclusion, our study adds new insights in the complexity of the regulation of cholesterol homeostasis, revealing the \(\alpha_\beta1\) integrin-dependent effect of fibrillar collagen on HMG-CoA reductase expression and synthesis of MVA-derived isoprenoids involved in protein prenylation processes. Type I fibrillar collagen-dependent regulation of HMG-CoA reductase expression reported here could perhaps be informative in understanding the basic molecular mechanisms that regulate SMC accumulation in atherosclerotic plague but also for the development of dyslipidemia in patients affected by liver fibrosis where deposition of fibrillar collagen is observed.\(^40,41\)

Acknowledgments
The authors thank Dr T.F. Osborne (University of California, Irvine) for generously providing the pSynSRE plasmid, expressing partial hamster HMG-CoA reductase promoter, and Dr Garry Nolan (Stanford University, Stanford, Calif) for generously providing the pBM-series retroviral vectors and Phoenix-A retroviral packaging cells.

Sources of Funding
This work was partially support by the EU grant # 018671, Drug Design for Cardiovascular Diseases: Integration of in Silico and in Vitro Analysis (CARDIOWORKBENCH) and by the grant from “Ministero dell’Istruzione, dell’Università e della Ricerca” (First 2006).

Disclosures
None.

References
Fibrillar Collagen Inhibits Cholesterol Biosynthesis in Human Aortic Smooth Muscle Cells
Nicola Ferri, Elisa Roncalli, Lorenzo Arnaboldi, Simone Fenu, Olena Andrukhova, Seyedhossein Aharinejad, Marina Camera, Elena Tremoli and Alberto Corsini

Arterioscler Thromb Vasc Biol. published online July 16, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2009/07/16/ATVBAHA.109.187807.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/07/21/ATVBAHA.109.187807.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Experimental Procedures

Reagents and antibodies- DMEM, trypsin ethylenediaminetetraacetate, penicillin (10,000U/ml), streptomycin (10mg/ml), tricine buffer (1M, pH7.4) and nonessential amino acid solution (100X), fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA, USA). Disposable culture flasks and petri dishes were from Corning Glassworks (Oneonta, New York), and filters were from Millipore (Billerica, MA, USA). [3H]-Mevalonate and [14C]-Acetate were from Amersham (Cologno Monzese, Milan, Italy), and molecular weight protein standards from Fermentas Life Sciences (Burlington, Ontario, CA). SDS, TEMED, ammonium persulfate, glycine, and acrylamide solution (30% T, 2.6% C) were obtained from BIO-RAD Laboratories (Hercules, CA, USA). Collagenase type I and stigmasterol were purchased from SIGMA (Milan, Italy). Simvastatin in its lactone form (Merck, Sharp & Dohme Research Laboratories) was dissolved in 0.1M NaOH to give the active form, and the pH was adjusted to 7.4 by adding 0.1 M HCl.

For western blot analysis the following antibodies were utilized: rabbit polyclonal anti-HMG-CoA reductase (Upstate, Lake Placid, NY, USA); SREBP-1 (K-10, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA.), and SREBP-2 (Cayman chemical, Ann Arbor, MI, USA), mouse monoclonal anti-Ras (clone RAS10, Millipore, Billerica, MA, USA), anti-mouse and anti-rabbit peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Lab, West Grove, PA, USA). For blocking experiments the following antibodies were used: anti human α1I domain (MAB1973, Millipore, Billerica, MA, USA) and anti human α2 (Clone P1E6, Millipore, Billerica, MA, USA).

Western blot analysis- Cells were washed twice with PBS and lysed by incubation with a solution of 50mM Tris pH 7.5, 150mM NaCl, 0.5% Nonidet-P40, containing a protease and phosphatase inhibitor cocktails (SIGMA, Milan, Italy) for 30 min. on ice. For SREBP determination cells were treated for 2h at 37°C with 25μg/ml of the proteasome inhibitor N-acetyl-leucine-leucine-norleucinal (ALLN) (SIGMA) 1. Cell lysates were then cleared by centrifugation at 14.000 g for 10
min., and protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL, USA). Equal amount of total protein per sample were separated by SDS-PAGE under reducing conditions, transferred to Immobilon PVDF (Millipore, Billerica, MA, USA) and subsequently immunoblotted with primary antibody following appropriate secondary antibody, prior to visualization by enhanced chemiluminescence (ECL, GE Healthcare, Munich, Germany). Quantitative densitometric analyses were performed using Gel Doc acquisition system and Quantity One software (BIO-RAD Laboratories, Hercules, CA, USA).

Synthesis of total cholesterol- Cholesterol biosynthesis was estimated by measuring the incorporation of $[^{14}\text{C}]$-Acetate and $[^3\text{H}]$-Mevalonate into cellular cholesterol, as previously described$^2$.

Determination of total cellular cholesterol levels- The amount of total cholesterol has been quantified by Gas Liquid Chromatography GLC (DANI GC 1000, Milan, Italy) analysis on a fused silica capillary column (MEGALAP, 0.1 µm, 0.32 mm, 25 m; Mega Columns, Legnano, Italy) using the Clarity software (Data Apex, Prague).

HMG-CoA Reductase Assay- HMG-CoA reductase activity was determined by measuring the rate of conversion of radioactive HMG-CoA into mevalonate in detergent-solubilized cell-free extract, as previously described$^2$.

RNA preparation, quantitative real time PCR and gene array analysis- Total RNA was prepared from SMC after collagen digestion by incubation with collagenase type I for 15 min at 37°C (final concentration 1.25 mg/dl) with NucleoSpin® RNA II Kit (Macherey-Nagel, Postfach, Germany) according to manufacture’s instructions. Reverse transcription-polymerase first-strand cDNA synthesis was performed with 1 µg of total RNA and oligo-dT primer, using High-Capacity cDNA Archive Kit (Applied Biosystems, Monza, Milan, Italy). Real time PCR was then performed using the Applied Biosystem assay on demand for human HMG-CoA reductase (ID Hs00168352_m1), HMG-CoA synthase (ID Hs00266810_m1), FA synthase (ID Hs00188012_m1), SREBP1 (ID Hs01088691_m1), and SREBP2 (ID Hs00190237_m1) and ABI Prism® 7000 Sequence Detection
For reverse-strand priming, first-strand cDNA was used to generate Biotin-dUTP-labeled second-strand cDNA for the cDNA arrays (GEArray Q series, SABioscience, Frederick, MD). cDNA hybridization signals were quantified through the use of ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Transfection of siRNA- The Stealth™ Select RNAi Oligo siRNAs for human SREBP1a were purchased from Invitrogen (ID HSS110187) and transfection was performed using siLentFect™ Lipid Reagent (BIO-RAD laboratories, Hercules, CA, USA) according to the manufacturer’s protocol.

Generation of HMG-CoA reductase promoter activity retroviral vector- All constructs were generated using standard molecular biology techniques. All retroviral expression plasmids were constructed using the pBM-IRES-PURO, expressing the puromycin resistance gene as a selectable second cistron gene, generated from the original pBM-IRES-EGFP, generously provided by G.P. Nolan (Stanford University, Stanford, CA, USA). SREBP1a amino terminal active form (1-1309 bp) was subcloned into retroviral expression plasmid by blunt-end ligation. The hamster HMG-CoA reductase promoter sequence from -277 +20 (kindly provided by T.F. Osborne) was subcloned into a multiple cloning site of the retroviral vector pBM-SIN (kindly provided by G.P. Nolan) to yield the pBM-SIN-HMG-CoApro-luciferase reporter retroviral vector. Retroviral infections of human SMC were performed as previously described.

Luciferase reporter assay- SMCs were infected with pBM-SIN-HMG-CoApro-luciferase, cultured on monomer and fibrillar collagen and resuspended by either trypsination or collagenase treatment, respectively. Luciferase activity was then determined as previously described.

Cell adhesion assay

Cells were preincubated with antibody in suspension for 30 min at 37°C, loaded into the micro-Boyden chamber apparatus, and allowed to adhere to the filter coated with fibrillar collagen for 30 minutes. The filters were then fixed in 3% formaldehyde and stained using 0.5% toluidine blue in 3.7% formaldehyde.
Subsequently, they were washed, and each well was cut out, solubilized in 2% SDS, and quantified by absorbance at 650 nm.

Statistical analysis - All data shown are representative of at least three replicate experiments unless otherwise noted. Data are expressed as mean ± SD. Student's t test was used to evaluate differences between groups, and statistical significance was assigned when p< 0.05.
**Figure legends**

Figure I. HMG-CoA synthase and FA-synthase are downregulated by fibrillar collagen. Human SMCs were cultured for 48 h with DMEM containing 0.2% BSA on monomer and fibrillar collagen. HMG-CoA synthase and FA-synthase mRNA expressions were evaluated by quantitative real time polymerase chain reaction from total RNA. The data are representative of two independent experiments. *p<0.05; **p<0.01 by Student t test.

Figure II. SREBP1a directly regulate HMG-CoA reductase expression. A and B Human SMCs were transfected with two specific siRNA against SREBP1 or nonsilencing control siRNA (Cnt), and SREBP1 active form expression evaluated by Western blot analysis after 72 h of transfection (A). HMG-CoA reductase mRNA was evaluated by quantitative real time polymerase chain reaction from total RNA from cells transfected with two specific siRNA against SREBP1 or nonsilencing control siRNA (Cnt) and cultured on monomer and fibrillar collagen for 48h (B).
References


Figure I

- mRNA levels / 18S RNA
- Monomer
- Fibrillar

HMG-CoA synthase

FA synthase

**" sign indicates statistical significance.
FIGURE II

A

SREBP1a

active

Optical density (% vs control)

0 20 40 60 80 100 120

Cnt siRNA#1 siRNA#2

B

HMG-CoA red mRNA/18S RNA

Monomer

Fibrillar

Cnt siRNA#1 siRNA#2 Cnt siRNA#1 siRNA#2

FIGURE II