Differential Regulation of Hyaluronic Acid Synthase Isoforms in Human Saphenous Vein Smooth Muscle Cells
Possible Implications for Vein Graft Stenosis


Abstract—Autologous saphenous vein bypass grafts (SVG) are frequently compromised by neointimal thickening and subsequent atherosclerosis eventually leading to graft failure. Hyaluronic acid (HA) generated by smooth muscle cells (SMC) is thought to augment the progression of atherosclerosis. The aim of the present study was (1) to investigate HA accumulation in native and explanted arterialized SVG, (2) to identify factors that regulate HA synthase (HAS) expression and HA synthesis, and (3) to study the function of the HAS2 isoform. In native SVG, expression of all 3 HAS isoforms was detected by RT-PCR. Histochemistry revealed that native and arterialized human saphenous vein segments were characterized by marked deposition of HA in association with SMC. Interestingly, in contrast to native SVG, cyclooxygenase (COX)-2 expression by SMC and macrophages was detected only in arterialized SVG. In vitro in human venous SMC HAS isoforms were found to be differentially regulated. HAS2, HAS1, and HA synthesis were strongly induced by vaso dilatory prostaglandins via G\(_\text{i}\) -coupled prostaglandin receptors. In addition, thrombin induced HAS2 via activation of PAR1 and interleukin 1\(\beta\) was the only factor that induced HAS3. By small interfering RNA against HAS2, it was shown that HAS2 mediated HA synthesis is critically involved in cell cycle progression through G\(\text{1}\)/S phase and SMC proliferation. In conclusion, the present study shows that HA-rich extracellular matrix is maintained after arterialization of vein grafts and might contribute to graft failure because of its pro proliferative function in venous SMC. Furthermore, COX-2-dependent prostaglandins may play a key role in the regulation of HA synthesis in arterialized vein grafts. (Circ Res. 2006;98:36-44.)

Key Words: hyaluronic acid \(\square\) extracellular matrix \(\square\) cyclooxygenase-2 \(\square\) vein graft stenosis

Autologous saphenous vein grafts (SVG) are frequently used for bypass grafting in patients with symptomatic occlusive disease of coronary arteries or arteries of the lower extremities. Subsequently, the grafted vein segments are exposed to arterial blood pressure and shear stress, which are thought to initiate extensive remodeling, intimal thickening, in-graft thrombosis, and superimposed atherosclerosis associated with long-term failure rates of approximately 30% to 40%.1,2 The pathophysiological mechanisms eventually resulting in graft failure include activation and dedifferentiation of vascular smooth muscle cells (SMC) from a contractile into a secretory phenotype characterized by high migratory and proliferative activity.3 In addition, the extracellular matrix (ECM) undergoes remodeling in arterialized venous grafts. This remodeling is characterized by high ECM turnover conferred by matrix metalloproteinases 1, -2, and -94,5 and increased deposition of newly synthesized ECM components including collagen and proteoglycans.6,7 ECM remodeling is thought to be required for the proliferative and migratory activation of SMC and to support intimal volume expansion.8

Recently, hyaluronic acid (HA) has been shown to be a major component of thickened neointimal, restenotic, and atherosclerotic lesions in humans and to be associated with proliferating SMC and thrombosis of eroded plaques,9,10 suggesting that HA is a critical factor during the pathophysiology of cardiovascular disease. HA is a polysaccharide composed of repeating disaccharide units (\(\alpha\)-glucuronic acid \(\beta\)-1,3-\(\alpha\)-N-acetylglucosamine-\(\beta\)1,4) that is synthesized at the plasma membrane by 3 different HA synthases (HAS1 to -3). During synthesis the growing HA-polypeptide is extruded into the extracellular environment.11 Studies in mesothelial cells, epithelial cells, and endothelial cells showed that epidermal growth factor, platelet-derived growth factor (PDGF)-BB, and transforming growth factor (TGF)-\(\beta\) all participate in transcriptional regulation of HAS isoforms in both an isoform
and cell type–specific manner. However, very little is known about the regulation of HAS isoform expression in vascular SMC. HAS2, which is the main HAS isoform in cultured vascular SMC, is induced by PDGF-BB and vasodilatory prostaglandins. In vitro studies using vascular SMC and fibroblasts showed that HA is critically involved in proliferation and migration and cell spreading. Thus, based on the evidence from various forms of arterial vessel disease and functional studies in cultured SMC, it is likely that HA plays an important role during neointimal thickening and possibly also during failure of venous bypass grafts.

The aims of the present study were (1) to analyze native saphenous vein segments and arterialized SVG with respect to HA-accumulation, SMC proliferation, and macrophage accumulation; (2) to study the transcriptional regulation of the 3 HAS isoforms in human venous SMC; and (3) to investigate the functional significance of HAS2-mediated HA synthesis by small interfering RNA (siRNA) targeting HAS2 in vitro.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture
Venous SMC were isolated by the explant technique from the media of human saphenous veins. Leftover segments were obtained from patients undergoing coronary bypass surgery according to the guidelines of the local Medical Ethical Board. SMC of passages 4 to 10 were used. Four different venous cell lines were studied. The SMC were grown in DMEM containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. Cells were seeded at 10 000 cells/cm², growth arrested by serum withdrawal for 24 hours, and subsequently treated with the compounds to be studied.

Analysis of Human Veins
Native Vein Segments
Leftover fragments of surgical specimens from saphenous veins prepared for bypass grafting (n=12; 4 females, 8 males; median age, 64 years [range, 37 to 82 years]) were collected prospectively from the files of the Institute of Vascular surgery (Universitätsklinikum Düsseldorf). The veins were cut transversally, fixed in 10% formalin, and embedded in paraffin. Four-micrometer sections from the paraffin blocks were stained with hematoxylin/eosin and elastic–van Gieson stain. The staining patterns were evaluated by a senior pathologist (M.S.).

Veins Exposed to Arterial Blood Pressure
Six human veins that had been used to bypass arterial stenosis were obtained from the files of the Institute of Pathology (Universitätsklinikum Düsseldorf). Five veins had been implanted in the lower extremity and 1 in the upper extremity. Explantation had been performed in the year 2004 for various reasons: in 3 cases, the lower extremity had been amputated for ischemic soft tissue necrosis; in 1 case, the venous bypass was selectively resected because of thrombotic occlusion; in 1 case, for obliteration of the Arteria brachialis; and in 1 case, for unknown reasons. Two of the 6 patients were...
female, the age ranged between 41 and 79 years (median, 57 years). Veins were processed as described above.

Results

Native Saphenous Veins

Figure 1A shows a representative section of a native saphenous vein derived from a 62-year-old woman, which was prepared for bypass surgery. The thickened intima and the tunica media contained muscle actin (M-actin)–positive cells (Figure 1B). In the intima, media, and adventitia, abundant accumulation of HA was detected, which colocalized intimately with M-actin–positive cells in the intima and in the media (Figure 1B). In addition, mRNA expression of all 3 HAS isoforms was detected by RT-PCR (not shown) and real-time RT-PCR (Figure 1C). Double immunohistochemical staining for HA and the Ki-67 antigen, which is only expressed in proliferating cells, revealed only a few proliferating cells in the wall of native veins (Figure 1D). Rare CD68-positive macrophages were detected in both the intima and/or the media of human veins (Figure 1E). COX-2 expression was not detectable in SMC of native SVG, whereas COX-2 reactivity was detected in endothelial cells as an internal control (Figure 1F).

Veins Exposed to Arterial Blood Pressure

Conventional histological examination based on hematoxylin/eosin stains and on elastic–van Gieson stain revealed various degrees of intimal thickening resulting in moderate to subtotal stenosis of the lumen (Figure 2A). All specimens showed fibrosis of the media, which was very pronounced in 4 cases. The thickened intimas were characterized by accumulation of SMC, which were intimately associated with HA (Figure 2B). SMC showed a very low proliferative activity as indicated by Ki-67 staining (Figure 2C). In 2 of the explanted SVG, thrombosis was observed. In the 2 thrombosed SVG and in 1 of the cases without thrombosis, strong expression of COX-2 in intimal and medial SMC was observed (Figure 2D). In the cases with thrombosis, marked infiltration of the intima and subintimal media with CD68-positive macrophages was observed (Figure 2E and 2F), which strongly expressed COX-2 (Figure 2F). In contrast, in the cases without thrombosis, low numbers of CD68-positive macrophages were present in both the intima and media.

Differential Regulation of HAS1 and HAS3 Isoforms in Human Venous SMC

The mRNA of HAS1, which is the HAS isoform expressed at the lowest level in cultured human SMC, was induced only by the prostacyclin (PGL₂) analog iloprost and prostaglandin E₂ (PGE₂) (Figure 3). To identify the responsible prostaglandin receptors, selective receptor agonists were used. Both the specific PGI₂ (IP-receptor) agonist cicaprost and the specific E-type prostaglandin receptor subtype 2 (EP₂-receptor) agonist butaprost mimicked the effects of iloprost and
PGE₂, suggesting that the Gs-coupled IP- and EP₂ receptors are both capable of inducing HAS1 mRNA expression (Figure 3) in venous SMC. In line with the hypothesis that cAMP-dependent signaling of the Gs-coupled IP and EP₂ receptors is responsible for HAS1 induction, forskolin, an activator of adenylate cyclase, induced HAS1 as well (Figure 3C). PDGF-BB, TGF-β₁, angiotensin-II, thrombin, and interleukin (IL)-1β had no effect on HAS1 expression (Figure 3). Angiotensin II (10⁻⁷ mol/L) was used in the presence and absence of the AT₁ receptor antagonist losartan (10⁻⁵ mol/L), because blocking of the AT₁ receptor can be used to unmask effects of the AT₂ receptor that could otherwise be missed. However, angiotensin II did not have any effects on HAS isoform expression under the current experimental conditions.

HAS3 expression was analyzed in response to the same factors as mentioned above. Interestingly, HAS3 was markedly induced only by IL-1β (Figure 4).

**Differential Regulation of HAS2 in Cultured Venous SMC**

The mRNA of HAS2, which is the main isoform in venous SMC, was induced by 100 nmol/L iloprost and 10 U/mL thrombin, respectively (Figure 5A and 5B). The HAS2 induction in response to thrombin was concentration dependent starting at 1 U/mL (data not shown) and could be mimicked by the PAR1 activating peptide (AP)-1 (100 μmol/L) as shown in Figure 5C. In contrast, the PAR2, -3, and -4 activating peptides had no significant effect on HAS2 expression. Furthermore, HAS2 was strongly induced by PDGF-BB (20 ng/mL), as described previously, and was not changed by TGF-β₁ (10 ng/mL).

The regulation of HAS2 by prostaglandins was investigated in more detail, because prostaglandin-mediated HAS2 mRNA upregulation was strongest among the stimuli investigated and dramatic upregulation of COX-2 was detected in half of the cases of explanted, arterialized venous bypass grafts (Figure 2). The mRNA induction of HAS2 was maximal at 3 and 6 hours after stimulation and occurred in a concentration-dependent manner (data not shown). HAS2 mRNA was also strongly upregulated by a selective EP₂ agonist (butaprost), forskolin, and dibutyryl-cAMP (db-cAMP), suggesting that Gs-coupled IP and EP₂ receptors were involved (Figure 6A). Consequently stimulation of venous SMC with iloprost, cicaprost, and PGE₂ resulted in increased secretion and accumulation of HA in the conditioned cell culture medium (Figure 6B). Furthermore, prostaglandin-induced HA secretion was mimicked by forskolin and db-

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**Figure 3.** Characterization of HAS1 expression in human venous SMC in vitro. A, After 24 hours of serum withdrawal, cells were stimulated for 3 hours with iloprost (100 nmol/L), angiotensin II (10⁻⁷ mol/L), angiotensin II (10⁻⁷ mol/L) plus losartan (10⁻⁵ mol/L), IL-1β (10 ng/mL), and thrombin (10 U/mL). Subsequently semi-quantitative RT-PCR for HAS1 was performed. B, Densitometric quantification of the iloprost effect, which was expressed as fold over control after calculation of the ratio of HAS1 and GAPDH bands; n=3 independent experiments (mean±SEM). *P<0.05 vs control. C, HAS1 expression after incubation of venous SMC with butaprost (1 μmol/L), cicaprost (100 nmol/L), forskolin (10 μmol/L), iloprost (100 nmol/L), and PGE₂ (100 nmol/L). D, HAS1 expression in response to PDGF-BB and TGF-β₁. In A, C, and D, original gels representative of n=3 experiments are shown.
cAMP (not shown) and was inhibited by the protein kinase A (PKA) inhibitor H89 (Figure 6C). Prostaglandin-induced HA synthesis was in the same order of magnitude as after stimulation with 20 ng/mL PDGF-BB (not shown).

Because the present data revealed that prostaglandins induce both HAS1 and HAS2 mRNA, siRNA targeting HAS2 was used to roughly estimate the relative contribution of HAS1 and HAS2 to prostaglandin-induced HA synthesis. HAS2 siRNA significantly inhibited the iloprost-induced expression of HAS2 mRNA by 47.6\% (n=4, P<0.05). Furthermore, HAS2 siRNA inhibited the induction of HA secretion by iloprost by 39.3\% (mean of 2 independent experiments), which suggests that HAS2 synthesized the majority of HA in response to iloprost.

Induction of Pericellular HA in Human Venous SMC In Vitro

To investigate whether the factors that were shown above to increase the expression of HAS isoforms were also able to induce cell-associated HA, cultured venous SMC were stimulated with vasodilatory prostaglandins (iloprost [100 nmol/L], PGE2 [100 nmol/L]), with PDGF-BB (20 ng/mL), thrombin (10 U/mL), and IL-1\(\beta\) (10 ng/mL). After 24 hours of serum withdrawal, cells were incubated with streptomyces hyaluronidase to remove any preformed extracellular HA. Subsequently, SMC were stimulated with the factors indicated above for 48 hours and pericellular HA was visualized by HA-binding protein staining. All stimuli induced deposition of pericellular HA. However, deposition of cell-associated HA was stronger in response to PDGF-BB, IL-1\(\beta\), and thrombin as compared with vasodilatory prostaglandins (online data supplement). In contrast, the stimulated secretion of free soluble HA into the cell culture medium and induction of HAS2 in response to iloprost was at least as high as in response to PDGF-BB and thrombin. This finding suggests that the effects of vasodilatory prostaglandins differ from PDGF-BB, thrombin, and IL-1\(\beta\) with respect to the ratio of secreted and pericellular HA, which might be attributable to differential regulation of additional proteins required for the formation of pericellular HA coats.

Functional Significance of HAS2-Mediated HA Synthesis in Venous SMC

The stimuli used to characterize the differential regulation of HAS isoform expression in venous SMC were selected because of their potential relevance during vein graft stenosis and failure. HAS2 was the HAS isoform that was found to be regulated by most of the stimuli namely the PGI2 analogue (iloprost), PGE2, PDGF-BB, and thrombin and contributed most of the HA in response to iloprost. Therefore, the functional significance of HAS2 was investigated, applying HAS2 siRNA to inhibit expression of HAS2 in cultured venous SMC. Cell cycle analysis of SMC after siRNA targeting of HAS2 revealed a partial (\sim 20\%) suppression of the progression through the G1/S phase 18 hours after PDGF-BB stimulation compared with control siRNA (Figure 7A). Western blot analysis of cell cycle proteins showed marked downregulation of cyclins A and E as well as upregulation of the cyclin-dependent kinase inhibitor p27 after PDGF-BB in cells transfected with HAS2 siRNA compared with control siRNA (Figure 7B). No differences were observed in cyclin D1, p21, and cdk2 (data not shown). Furthermore, HAS2 siRNA caused decreased DNA synthesis in response to PDGF-BB as determined by \(^{3}H\)-thymidine.
incorporation (Figure 8A). HAS2 siRNA caused decreased mitogenesis as determined by cell counting in comparison to venous SMC transfected with nonsilencing control siRNA (Figure 8B). As described previously,15 cells undergoing mitosis and cytokinesis after PDGF-BB stimulation had a pronounced pericellular HA coat (data not shown). Proliferating SMC of native and arterialized saphenous vein segments as detected by positive Ki-67 staining appeared to be in direct contact with HA-rich pericellular matrix as well (Figure 8C). A schematic diagram illustrating regulation of HAS1 and HAS2 by prostaglandins and the proproliferative function of HAS2-mediated HA synthesis is depicted in Figure 8D.

Discussion

The current investigation characterizes the ECM of saphenous vein segments used for bypass surgery in patients with ischemic artery disease with respect to HA accumulation. All native veins showed intimal thickening, which is in line with previous reports of pronounced intimal thickening of primary vein graft tissue.6,24 Strong accumulation of HA in association with SMC was observed in the thickened intima of primary as well as in arterialized saphenous vein segments. Because extracellular HA has a relatively short half-life,25 the presence of HA in arterialized SVG suggests that HA synthesis is induced after bypass grafting. However, the factors that are responsible for induction and maintenance of the HA-rich ECM in saphenous vein grafts after arterialization are unknown. Therefore, the effects of various factors that are known to be involved in control of SMC proliferation, ECM synthesis, and vascular inflammation were analyzed with respect to HA synthesis and HAS isoform expression in cultured SMC from human saphenous vein. The factors investigated in the present study included vasodilatory prostaglandins (iloprost and PGE2), thrombin, IL-1β, PDGF-BB, and TGF-β1.

PGI2 and PGE2 are vasodilatory prostaglandins that are synthesized by PGI2 synthase and PGE synthase from PGH2, which is generated by COX-2. Both, HAS1 and HAS2 were induced by iloprost and PGE2 via the Gs-coupled IP and EP2 receptors in a cAMP- and PKA-dependent manner. A similar response to vasodilatory prostaglandins with respect to HAS2 expression was recently demonstrated in arterial SMC.16

Figure 5. Characterization of HAS2 expression in human venous SMC in vitro. A, After 24 hours of serum withdrawal, cells were stimulated for 3 hours with iloprost (100 nmol/L), angiotensin II (10⁻⁷ mol/L), angiotensin II (10⁻⁷ mol/L) plus losartan (10⁻⁵ mol/L), IL-1β (10 ng/mL), and thrombin (10 U/mL) and semiquantitative RT-PCR for HAS2 was performed. B, HAS2 expression is expressed as fold over control after calculation of the ratio of HAS2 and GAPDH bands after densitometric quantification; n=5 independent experiments (mean±SEM). *P<0.05 vs control. C, HAS2 expression after incubation (3 hours) of venous SMC with AP-1 (100 μmol/L), AP-2 (100 μmol/L), AP-3 (100 μmol/L), and AP-4 (100 μmol/L); n=3 independent experiments (mean±SEM). *P<0.05 vs control. D, HAS2 expression in response to PDGF-BB and TGF-β1. In A and D, original gels representative of (n=5 and n=3) independent experiments are shown.

Figure 6. Induction of HAS2 mRNA and HA synthesis by vasodilatory prostaglandins. A, HAS2 expression in venous SMC in response to iloprost (100 nmol/L), butaprost (1 μmol/L), forskolin (10 μmol/L), and db-cAMP (1 mmol/L). HAS2 mRNA expression was determined by semiquantitative RT-PCR after 3 hours and the ratio of the signals of HAS2 and GAPDH were normalized to control. B, Levels of HA in the cell culture medium were determined 24 hours after treatment of SMC with iloprost (100 μmol/L), cicaprost (100 nmol/L), and PGE2 (100 nmol/L). C, Levels of HA in the cell culture medium 24 hours after treatment of cells with iloprost (100 nmol/L), iloprost+H89 (100 nmol/L). HA levels are presented as percentage of the concentration measured in response to iloprost; n=3, mean±SEM. *P<0.05 vs iloprost.
However, this is the first report showing that HAS1 is induced by vasodilatory prostaglandins as well. This finding might be relevant, because strong COX-2 expression was detected in 3 of 6 arterialized veins in the current study, whereas COX-2 was absent from native vein segments. COX-2 is frequently found to be upregulated after vessel injury and during atherosclerosis.26,27 However, the role of COX-2–dependent prostaglandins during atherosclerosis is currently discussed controversially.28 PGI2 mediates vasoprotective and antithrombotic functions by inhibiting platelet aggregation and vasoconstriction, whereas induction of matrix metalloproteinase expression and inflammatory functions have been attributed to PGE2 in atherosclerotic lesions.27 Recently, it was shown that transgenic mice overexpressing HAS2 in SMC in apolipoprotein E–deficient mice showed increased atherosclerosis29 suggesting that HAS2 induction by prostaglandins is proatherogenic. Thrombin is generated in vivo at the lipid surface of activated platelets during thrombus formation which is frequently induced in venous bypass grafts.30,31 Thrombosis was also detected in 2 of 6 cases in the current study. Thrombin which is known to increase the propensity of SMC to proliferate and migrate32 activates PAR1, -3, and -4 receptors.33 Thrombin and the PAR1 activating peptide induced HAS2 expression, suggesting that thrombin via the PAR1 receptor participates in the regulation of HA synthesis in venous SMC.

Monocyte invasion and release of cytokines are early events in vein graft stenosis.34 HAS3 was induced in response to IL-1β which is a major cytokine released from macrophages. IL-1β was the only stimulus that upregulated HAS3 in human venous SMC. Therefore, it could be hypothesized that HAS3 expression is involved in the inflammatory response induced by macrophages. Notably, it has recently been demonstrated that macrophages adhere to HA-rich structures during inflammatory bowel disease and to intestinal SMC in vitro.35

PDGF and TGF-β1 are considered key mediators during medial and intimal thickening of autologous vein grafts36 and have been shown to be upregulated in stenotic vein grafts.6,24 PDGF-BB strongly induced HAS2 mRNA and HA synthesis in venous SMC, as has been shown before in arterial SMC15 and mesothelial cells. TGF-β1 slightly reduces HAS2 expression in mesothelial cells13 and induces HAS2 in corneal endothelial cells.14 However, in human venous SMC no effect of TGF-β1 on HAS2 was observed. Furthermore, PDGF-BB and TGF-β1 had no significant effects on HAS1 or HAS3.

It can be concluded that the HAS isoforms are differentially regulated by the stimuli investigated so far. HAS3 was induced only by IL-1β, HAS2 by prostaglandins, PDGF-BB, and thrombin and HAS1 was upregulated by prostaglandins only. In addition, IL-1β, PDGF-BB, and thrombin are known to induce the expression of COX-2 and the generation of endogenous prostaglandins in vascular SMC.37,38 which might represent a mechanism to sustain HAS1 and HAS2 upregulation in SVG (Figure 8D).

Among the HAS isoforms, HAS2 was subject of the most complex regulation. Therefore, the functional significance of HAS2 induction was analyzed by siRNA targeting HAS2. Synthesis of HA and/or RHAMM signaling have been suggested to be required for G2/M transition and cytokinesis in fibroblasts.18,39,40 In contrast, G1/S transition was delayed by antisen to HAS2 in epidermal keratinocytes derived from rats.41 However, the specific role of HAS2-mediated HA synthesis during cell cycle progression in vascular SMC has not been investigated yet. In the current study, inhibition of
HAS2 expression caused a 20% decrease of cells in S phase, as determined by fluorescence-activated cell sorting analysis, indicating inhibition of G1/S transition. Consistent with inhibition of G1/S transition, the levels of cyclin A and E were dramatically reduced and the cdk inhibitor p27 was increased. Consequently, DNA synthesis was inhibited by ~20% and cell proliferation in response to PDGF-BB was reduced as well. These findings demonstrate for the first time that HAS2-dependent HA synthesis is required for PDGF-BB–induced cell cycle progression and mitosis in vascular SMC. These data extend the observation made by others that pericellular HA coats and intracellular HA are essential for mitosis in response to PDGF-BB.15,17,18

Taken together, the present study reveals that saphenous veins of elderly patients prepared for bypass grafting contain HA-rich subendothelial ECM. It is conceivable that the HA-rich ECM supports subsequent neointimal thickening because HA-rich ECM is thought to support SMC proliferation. Further evidence for this hypothesis is presented in the present study by knock down of HAS2 expression, which inhibited PDGF-BB–induced proliferation in vitro. Because the ECM of the thickened neointima of arterialized SVG is still HA rich, it is likely that the HA matrix is actively maintained via induction of HAS isoform expression in SMC. The present data show that HAS isoforms are differentially regulated by a variety of factors that are generated during pathogenesis of vein graft failure such as PDGF-BB, IL-1β, thrombin. In addition, these factors are known to induce COX-2 expression in SMC, which could subsequently cause prostaglandin release and sustained induction of HAS1 and HAS2. Therefore, endogenous prostaglandins might play a key role in the maintenance of a proproliferative HA-matrix in saphenous vein grafts.
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References
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Expanded Material and Methods

Reagents

Iloprost and cicaprost were kindly provided by Schering AG (Berlin, Germany), forskolin, prostaglandin E₂ (PGE₂), dibutyryl-cAMP(db-cAMP), PDGF-BB, Interleukin 1 beta (IL-1β), H89 was purchased from Sigma (Taufkirchen, Germany). Butaprost was kindly provided by Dr. P. Gardiner, Bayer (Middlesex, UK). All cell culture reagents were from Invitrogen (Karlsruhe, Germany). α-Thrombin was kindly provided by Dr. J. Stürzebecher (Zentrum für Vaskuläre Biologie und Medizin Jena, Germany). Activating peptides (AP) for PARs were: AP-1, TFFLRN (PAR-1); AP-2, SLIGKV (PAR-2), from Biosyntan (Berlin, Germany); AP-3, TFRGAP (PAR-3), AP-4, AYPGQV (PAR-4), from GL Biochem (Shanghai, China). APs were used at 100 µM. Angiotensin II was from Bachem Biochemica (Heidelberg, Germany) and Losartan was kindly provided by Dr. R. Smith (DuPont Merck Pharmaceutical, Wilmington, USA). Biotinylated hyaluronic acid binding protein (HABP) was purchased from Calbiochem (Merck, Germany) and hyaluronidase from streptomyces hyaluronolyticus from ICN (MP-Biomedicals, Germany). The antibodies used for Western blot analysis of Cyclin E, Cyclin A and p21 were from Santa Cruz Biotechnology Inc. Anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories Inc.
Quantitation of HA

The amount of HA in the cell culture supernatant was measured by a radiometric assay (Pharmacia HA Test, Pharmacia-Diagnostics, Freiburg, Germany) as described previously 1.

RNA Isolation. Total RNA from arterial SMC was isolated by using peqGOLD Trifast™ (PeqLab, Erlangen, Germany) following the manufacturer’s instructions. The RNA was quantitated by spectroscopic analysis at 260 nm.

Real-Time RT-PCR

Total RNA extracted from leftover fragments of surgical specimens from saphenous veins prepared for bypass grafting was transcribed into cDNA by using High-Capacity cDNA Archive Kit (Applied Biosystems). The expression levels of HAS 1, -2 and -3 were determined by real-time RT-PCR analysis using the ABI PRISM 5700 (Applied Biosystems). Continuous quantitative measurement of the PCR product was achieved by incorporation of SYBR Green fluorescent dye (Invitrogen) into the double-stranded DNA. PCR oligonucleotides, purchased from Invitrogen, specific for HAS1 (forward: 5’-CCTAAGCAGCCTGCAGATAC-3’; reverse: 5’-CAGGATACACAGTGGAAGTAG-3’), HAS2 (forward: 5’-GTGTGGATTATGTCAGGTTTGTG-3’; reverse: 5’-GAGATCCAGGAATCGTGACTTG-3’), and HAS3 (forward: 5’-GCCTGCACCTGCTCATTCAGAG-3’; reverse: 5’-CCTGGTATGCGGCAATGCAC-3’) messages were designed on published database sequences.

Determination of HAS-Expression in cultured SMC. The expression levels of HAS1, -2, -3 were analyzed by semiquantitative RT-PCR (One-Step RT-PCR kit;
Qiagen, Hilden, Germany) using 250 ng of total mRNA for each RT-PCR reaction. The primers were designed based on published base sequences of the respective human genes. GAPDH was co-amplified as reference gene to allow semiquantitative evaluation of the expression levels. This method has been described in detail previously for HAS2 . The following oligonucleotides (Invitrogen, Karlsruhe, Germany) were used: HAS1 (forward: 5´CAGACCCACTGCGATGAGACAG 3´; reverse: 5´GAATCTCCGAGGCGCGTCTGAA 3´), product size: 821 bp., 40 cycles; HAS3 (forward: 5´CCAGATCCTCAACAAGTACGACTC 3´; reverse: 5´CACCACAATGGTTTTGCG3´), product size: 694 bp., 35 cycles; HAS2 (forward: 5´GTCTCAAATTCATCTGATCTC 3´; reverse: 5´ACATTTCCTTAAGTAGTCTGG 3´), product size: 419 bp., 28 cycles; glycerol-aldehyde-3-phosphate-dehydrogenase (GAPDH) (forward: 5´TGATGACATCAAGAAGGTGGTTGAA 3´; reverse: 5´TCCTTGGAGGCCATGTAGGCCAT 3´); product size: 219 bp..

Transfection of vascular SMC with siRNA. Single-stranded siRNAs, with 19-nt duplex RNA and 2-nt 3´dTdT overhangs were synthesized by Qiagen-Xeragon (Hilden, Germany). The siRNA sequences targeting HAS2 and the details of the transfection procedure have been described previously.

HA staining of cultured venous SMC.
After seeding of human venous SMC into Lab-Tek chamber slides at a density of 12,000 / cm² cells were kept serum-free for 24 hours. Subsequently, HA was digested with streptomycetes hyaluronidase (2 U/ml) in serum-free medium for 1 hour at 37°C. After washing with serum-free medium the cells were stimulated with the indicated factors. After 48 hours cells were fixed with 2% paraformaldehyde / 0.5% glutaraldehyde in phosphate buffered saline and HA was detected using biotinylated
HABP as described previously\(^2\) without permeabilization of the cells to stain specifically extracellular HA.

**Determination of SMC proliferation.**

Proliferative activity of venous SMC after HAS2 siRNA transfection was assessed by \[^{3}H\]-thymidine incorporation and cell counting. \[^{3}H\]-thymidine incorporation was determined after incubation of SMC with 1 µCi/mL \[^{3}H\]-thymidine for 8 hours as described previously\(^3\). Cell number was determined in SMC transfected with non-silencing siRNA (control-siRNA) and HAS2-siRNA 1, 2, 3 and 4 days after transfection and stimulation with PDGF-BB (20 ng/mL). For this purpose cells were suspended in PBS by trypsin and counted with a hemocytometer.

**Analysis of cell cycle regulatory proteins**

Cells were seeded at 10000 cells / cm\(^2\), transfected as described above, serum starved for 24 hours and stimulated with PDGF-BB (20 ng/ml) for 18 hours. Subsequently cells washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 100 µl of buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl\(_2\), 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 10% glycerol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO\(_4\), 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 15 min on ice. Cell lysates were cleared by centrifugation at 15,000xg for 5 min, and protein concentrations were determined using the BCA protein assay (Pierce). Lysates were separated by SDS-PAGE under reducing conditions, transferred to an Immobilon polyvinylidene difluoride membrane (Millipore) and subsequently immunoblotted with specific antibodies prior to visualization by enhanced chemiluminescence (ECL, Amersham Biosciences).
Cell cycle analysis by FACS

Cells were resuspended in 500 µl PBS and then fixed with 500 µl ice-cold 100% ethanol. For DNA staining cells were incubated in 500 units/mL Ribonuclease A for 15 min at 37°C followed by addition of propidium iodide at a final concentration of 1.5 µg/mL. The samples were incubated for 30 minutes at 37°C in the dark. Flow cytometric analysis was performed by using FACScalibur (Becton Dickinson, San Jose, CA), and analysis of cell cycle histograms was carried out using CellQuestPro (Becton Dickinson, San Jose, CA) and/or FlowJo (Tree Star Inc, Ashland, OR).

Analysis of saphenous veins. HA was detected using hyaluronic acid binding protein (HABP, Calbiochem, Germany, 1:100) as described previously4,5. In addition antibodies against COX2 (polyclonal, Cayman Chemical, USA, 1:150), the macrophage antigen CD68 (monoclonal, PG-M1, 1:500, DAKO, Germany), M-actin (monoclonal, HHF 35, 1:100, DAKO, Germany) and the proliferation marker Ki-67 (monoclonal, MIB-1, DAKO, Germany) were applied. Double staining was performed detecting first HABP with streptavidin/horseradish peroxidase-coupled secondary antibody and diaminobenzidine (DAKO, Germany) as a chromogen. Subsequently, the slides were incubated with one of the above mentioned primary antibodies (one hour; room temperature). The primary antibodies were then detected with an alkaline phosphatase-conjugated secondary antibody and the reaction was developed using Fast Red (DAKO, Germany) as chromogen. Finally, the slides were counterstained with haemalaun. The staining patterns were evaluated by a senior pathologist (M. Sarbia.).

Statistical analysis. Data are the mean ± SEM of n independent experiments. If not stated otherwise statistical analysis was performed by one-way ANOVA followed by
Dunnett's multiple comparison test or comparison of selected pairs (Bonferroni); p < 0.05 was considered significant.

References


Online figure 1: Cell-associated HA-formation in human venous SMC in vitro.
Pericellular HA was stained with HABP (brown). Unstimulated cells were processed without addition of HABP as negative controls for the HA-staining (neg. control). Controls represent unstimulated cells after 72 hours of serum deprivation. After 24 hours of serum withdrawal cells were stimulated with Iloprost (100 nmol/L), PGE$_2$ (100 nmol/L), thrombin (10 U/mL), IL-1β (10 ng/mL) or PDGF-BB (10 ng/L) for 48 hours.
PDGF-BB
iloprost
thrombin
PGE$_2$
IL-1$\beta$
PDGF-BB