TR3 nuclear orphan receptor in cardiovascular disease

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Transcription factor TR3 nuclear orphan receptor prevents cyclic stretch-induced venous smooth muscle cell proliferation

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Abstract

Bypass material applied in coronary artery bypass surgery is derived either from internal mammary arteries or from saphenous veins. The patency of arterial grafts is usually better than that of venous grafts due to 'vein-graft disease', which involves excessive proliferation of venous smooth muscle cells (SMCs) and subsequent accelerated atherosclerosis. We aim to elucidate the underlying mechanism of vein-graft disease and focus on the initiation of this pathological process that is, most likely, caused by mechanical strain on the vessel wall. We assayed expression of the transcription factor TR3 orphan receptor (TR3) and plasminogen activator inhibitor-1 (PAI-1), which is regulated by TR3, as candidate genes involved in the early response of SMCs to mechanical strain. TR3 and PAI-1 are induced in human saphenous vein segments exposed \textit{ex vivo} to whole-blood perfusion under arterial pressure. In addition, we challenged \textit{in vitro} cultured SMCs with cyclic stretch, which induced proliferation in saphenous vein SMCs, whereas mammary artery-derived SMCs remained quiescent. Only in venous SMCs, TR3 and PAI-1 expression was induced in response to mechanical strain. We have shown that TR3 inhibits SMC hyperplasia. Consequently, we hypothesize that TR3 is induced in venous SMCs upon mechanical strain to restrict excessive SMC proliferation. Indeed, adenovirus-mediated overexpression of TR3 in venous SMCs resulted in inhibition of stretch-induced proliferation. In addition, stretch-mediated proliferation was inhibited in a dose-dependent manner by 6-mercaptopurine (6-MP), an agonist for TR3. In conclusion, enhancement of the activity of TR3 may contribute to prevention of vein-graft disease.
Introduction

Smooth muscle cells (SMCs) play a key role in vascular pathologies such as atherosclerosis, (in-stent) restenosis after angioplasty and vein-graft disease following coronary artery bypass surgery. Even though the first two types of vascular disease occur in the arterial vessel wall and the latter in the venous vessel wall, SMC hyperplasia is a critical factor in the onset and progression of these large vessel diseases. Various stimuli are involved in initiation of SMC proliferation, of which inflammatory pathways are well established. Here, we study the distinct effect of mechanical strain on proliferation of venous and arterial SMCs and we try to delineate the molecular mechanisms underlying the different responses to this stimulus. Bypass surgery is an established intervention to treat coronary artery disease. Both the saphenous vein and the internal mammary artery are applied as bypass material. The arterial bypass has a better patency than the venous bypass in which vein-graft disease may develop, resulting in vein graft failure in 10-30% per year. Vein-graft disease is the result of excessive SMC proliferation that may be caused by mechanical strain, however, limited information is available on the underlying mechanism of such mechanical activation. The mammary artery is relatively short, limiting the amount of available bypass material. Therefore, it is vital to improve the function of venous bypasses in terms of enhancement of longevity, which is the ultimate goal of our studies.

TR3, also known as nerve growth factor-induced protein B (NGFI-B) or NR4A1, is a member of the superfamily of nuclear receptors. Recently, analysis of the crystal structure of the ligand-binding domain of TR3-subfamily members has revealed that these nuclear orphan receptors contain bulky hydrophobic amino-acid residues in the cavity that is normally occupied by cognate ligands. Moreover, the ligand-binding domain resembles the conformation of agonist-bound, transcriptionally active nuclear receptors, which indicates that the members of this subfamily probably function independently of traditional ligands. However, it has been shown that the transcriptional activity of TR3-like factors is regulated via non-traditional (ant)agonists such as 6-MP, which increases the activity of TR3-like factors without directly interacting with these nuclear receptors. Originally, TR3 was found to induce T cell apoptosis. Yet, in vascular endothelial cells and SMCs TR3 acts as an anti-proliferative transcription factor, which involves induction of an inhibitor of cell-cycle progression p27Kip1 and subsequent cell-cycle arrest. In the carotid artery ligation model, a murine model for restenosis, we have shown that TR3 overexpression inhibits formation of SMC-rich lesions. PAI-1 was incorporated in our studies, because at present it is the only known gene that has a functional transcriptional response element for TR3 and is related to vascular biology as well as mechanical activation of SMCs.
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To define the relative contribution of mechanical strain in initiation of vein-graft disease and to delineate the underlying mechanism of this stimulus in venous SMC hyperplasia compared to SMCs derived from the internal mammary artery, we studied the expression of the early-response gene TR3 in distinct stretch models. Finally, we assessed the function of TR3 in stretch by overexpressing the gene or by enhancing its activity with the agonist 6-MP.

Methods

Human tissue specimens

The *ex vivo* perfusion model in which human saphenous vein segments were exposed to whole-blood under arterial pressure was used as described previously. Briefly, vein segments were placed in a loop of the extracorporeal circulation during bypass surgery and were exposed to autologous blood under flow (non-pulsatile) and arterial pressure (60 mm Hg). To study the effect of overdistension on bypass veins, vein segments were perfused in the presence or absence of an external stent. After one and six hours of perfusion the vein segments were harvested, fixed in formalin and embedded in paraffin for histological examination. Patients included in this study gave their informed consent and the study was approved by the local medical ethical committee. Anesthesia and cardiopulmonary bypass surgery were performed according to routine protocols.

In situ hybridisation

*In situ* hybridizations were performed as described. TR3 and PAI-1 probes were synthesized: TR3, GenBank No. L13740, base pairs (bp) 1221 to 1905; PAI-1, GenBank No. X12701, bp 52 to 1308. The probes were labeled with [35S]-UTP (Amersham Biosciences, Buckinghamshire, U.K.). Paraffin sections (5 mm) of control and perfused saphenous vein segments were mounted on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). After hybridization and stringent washes, the *in situ* sections were covered with nuclear research emulsion (ILFORD Imaging UK Limited, Cheshire, U.K.), exposed for 2 to 9 weeks, then developed and counterstained with hematoxylin and eosin. Matching sense riboprobes were assayed for each gene and were shown to give neither background nor aspecific signal. As a control for the integrity of RNA, *in situ* hybridizations were performed with an antisense riboprobe for thrombin receptor PAR-1 (Genbank M62424 bp 3076-3472). PAR-1 was abundantly expressed in SMCs of control and perfused vein segments, indicating that the integrity of the RNA was comparable in all specimens (data not shown).

Immunohistochemistry

Paraffin sections (5 mm) were deparaffinized, rehydrated and incubated with 0.3% (v/v)
hydrogen peroxide and blocked with 10% (v/v) pre-immune goat serum (DAKO, Glostrup, Denmark) in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl (TBS). Subsequently, sections were incubated overnight at 4°C with biotinylated Ulex Europaeus Agglutinin (Vector Laboratories, Inc. Burlingame, CA) (1:50 dilution) in TBS, followed by detection with streptavidin-horseradish peroxidase conjugates (DAKO) and, subsequently, with aminoethylcarbazole and hydrogen peroxide. Cultured cells were fixed with methanol and stained for SM α-actin with monoclonal antibody 1A4 (1:200; DAKO), and biotinylated goat anti-mouse secondary antibodies (DAKO). After counterstaining with hematoxylin, sections were embedded in glycergel (Sigma, St. Louis, MO). Immunofluorescent nuclear staining was performed with Hoechst 33258 (Sigma).

**SMC culture**

Venous and arterial SMCs were cultured from explants of saphenous vein (SV) and internal mammary artery (IMA) in Medium 199 with HEPES containing 20% (v/v) fetal bovine serum (FBS) with penicillin and streptomycin (GIBCO, Invitrogen Life Technology, Breda, The Netherlands) and were used at passages 4 to 6. SMCs were characterized with monoclonal antibody 1A4, directed against SM α-actin (DAKO) and demonstrated homogenous fibrillar staining. Overnight incubation with 10 μM carbonyl cyanide chlorophenylhydrazone (CCCP) induced SMC apoptosis.

To study stretch-induced responses, SMCs were seeded in 6-well plates containing collagen I-coated flexible membranes (BioFlex® culture plates, Dunn Labortechnik GmbH, Asbach, Germany) and were stretched in the Flexercell FX3000 apparatus (Dunn Labortechnik) for 1, 2, 4, 6, or 24 h at 10% stretch at 0.5 Hz or served as control (without stretch). Silicone-based lubricant was applied to prevent friction between the membrane and loading post.

[^H]-Thymidine incorporation and virus infection

SMCs were seeded in 6-well stretch plates and when wells were confluent, SMCs were made quiescent for 16 h in medium containing 0.5% (v/v) FBS. The plates were transferred into the Loading Station™ and stretched for 24 h. Control plates, without stretch, were cultured under identical conditions. Thereafter, cells were labeled for 4 h with 0.5 μCi/mL [methyl-[^H]]-thymidine (Amersham Biosciences). Incorporated radioactivity was precipitated for 30 min at 4°C with 10% (wt/v) trichloroacetic acid, washed twice with 5% (wt/v) trichloroacetic acid and dissolved in 0.5N NaOH. [^H]-thymidine was measured by liquid scintillation counting. When cells were infected with mock- or TR3-containing adenovirus (3x10⁸ plaque-forming units) for 2 h, the cells were allowed to recover for 24 h in complete medium before they
were made quiescent. 6-MP-treatment (Sigma) was initiated 1 h prior to stretch with 0, 1, 10, 25 μM 6-MP (stock at 50 mM in DMSO).

**Western blotting analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with cell lysates (30 mg per lane) and concentrated culture media (equivalent of 200 ml per lane). Proteins were transferred to nitrocellulose-Protran (Schleicher and Schuell, 's Hertogenbosch, The Netherlands). Expression of p27Kip1 (BD Biosciences, Alphen a/d Rijn, The Netherlands), p21Waf1 (BD), SM α-actin (DAKO), PAI-1 (MAI-12; Biopool, Umea, Sweden), TR3 (M-210; Santa Cruz Biotechnology, Santa Cruz, CA), calponin (clone hCP; Sigma) and α-tubulin (Cedar Lane, Hornby, Ontario, Canada) was studied, using the indicated antibodies directed against these proteins. Primary antibodies were incubated overnight at 4°C in 5% Protifar plus (Nutricia, Cuijk, The Netherlands) in TBS. As secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit (for p27Kip1 and TR3 detection) or goat anti-mouse (for all others) (BioRad Laboratories Inc., Hercules, CA) in a dilution of 1:5000 in TBS were used. Proteins were visualized by enhanced chemiluminescence detection (Lumi-Light; Roche Diagnostics GmbH, Mannheim, Germany). Quantitative analysis was performed by the Lumi-Imager (Boehringer Mannheim, Mannheim, Germany). α-Tubulin staining served as a control for loading.

**Real-time RT-PCR**

Total RNA was isolated using Trizol reagent (GIBCO). cDNA was synthesized by reverse transcription (RT) from 1 μg of total RNA with Superscript II (GIBCO) and 0.5 μg (dT) primer. Real-Time polymerase chain reaction (PCR) was performed with the use of the FastStart DNA Master SYBR green I kit (Roche) in the LightCycler System (Roche). Primers for TR3 were as follows: (forward) 5'-GGTTCCTGAGGTTGTCATCCGCAAG-3' and (reverse) 5'-GCAGGGACCTTGAGAAGGCCA-3'. As a control for equal amount of first strand cDNA in different samples we determined hypoxanthine phosphoribosyl transferase (HPRT) mRNA levels with primers (forward) 5'-TAATTATGGAGTTAATGCAATCG-3' and (reverse) 5'-CAAGCAGGGACGTTGAGAAGGCCA-3'.

**Results**

**TR3 and PAI-1 expression in perfused saphenous vein segments**

To study the molecular processes causing vein-graft disease, we applied an *ex vivo* perfusion model in which segments of saphenous veins were placed in the extracorporeal circulation
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during coronary artery bypass surgery. During perfusion significant distension was observed in the non-stented saphenous veins which resulted in an almost complete loss of the endothelial cell layer after already 1 h of perfusion under arterial pressure. Veins protected against excessive mechanical strain due to placement of an external stent contained intact endothelium after perfusion (as illustrated by endothelium-specific immunohistochemistry, Figure 1A). In the non-stented vein segments endothelial cell-specific staining revealed the presence of endothelial cells in capillaries at the adventitia, whereas the luminal endothelium had disappeared (Figure 1B).
The structure of saphenous veins differs in SMC organization from the arterial wall, as veins contain two SMC layers that are oriented in opposite directions. A layer of longitudinally oriented SMCs is situated close to the lumen of the vessel and a circular SMC layer (like in arterial vessels) is present adjacent to the adventitia (Figure 1, schematic drawing).
In search for genes involved in vein-graft disease we assayed mRNA expression of early response gene TR3 in ex vivo perfused vein segments by radioactive in situ hybridization. After 1 h of perfusion, TR3 expression was detected in occasional endothelial cells and SMCs in the stented vein segments (Figure 1C, E). However, extensive TR3 expression was detected predominantly in the circular SMC layer of the non-stented vein segments (Figure 1D, F). TR3 expression was virtually absent in the control vein segment (Figure 2A). Yet, after 6 h of perfusion TR3 was abundantly expressed throughout the entire vessel, in both the longitudinal and circular SMC layers, in the non-stented perfused vein (Figure 2B). In addition, PAI-1 mRNA expression was analyzed since at present PAI-1 is the only known gene that is both related to vascular biology and has a functional TR3 response element. PAI-1 was present in occasional endothelial cells and SMCs in control veins (Figure 2C) and after 1 h of perfusion (data not shown). However, PAI-1 expression was strongly increased in SMCs after 6 h of perfusion (Figure 2D). In conclusion, TR3- and PAI-1 mRNA are expressed in SMCs in saphenous vein grafts subjected to perfusion under arterial pressure.

Cyclic stretch-induced proliferation in venous SMCs
To investigate why mammary artery bypass material has a better patency than bypass material derived from saphenous vein, the intrinsic difference between SMCs derived from these different vessels was studied in response to mechanical strain. For our in vitro stretch experiments we applied an experimental stretch-device (Flexercell FX-3000 apparatus) in which all cells are exposed to the same extent of stretch. Standardization of this stretch model involved analysis of DNA synthesis. We subjected SMCs, derived from mammary artery or saphenous vein origin, to 10% cyclic stretch (0.5 Hz) for 24 h and measured [3H]-thymidine incorporation. In line with previous data, we observed that stretch induced
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Figure 1. Endothelial cell-specific immunohistochemistry and TR3 mRNA expression in perfused vein segments. Vein segments were placed in an extracorporeal bypass loop during bypass surgery and exposed to autologous whole blood flow under arterial pressure for 1 h. Upon perfusion, non-stented vein grafts (B,D,F) displayed overdistension. In vein grafts with an external stent (A,C,E) biomechanical activation was prevented. Vein segments exposed to perfusion at high pressure showed loss of endothelium, whereas capillary endothelial cells (red) were observed near the adventitia as a control for the procedure (B). Stent placement preserved endothelium integrity (A; red monolayer). TR3 mRNA expression was observed by radioactive in situ hybridization (black dots) in the circular (Ci) SMC layer in non-stented vein grafts (D 200x; F 400x). Scarce TR3 expression was seen in the stented vein grafts (C 200x; E 400x) or longitudinal (Lo) SMC layer (C-F). The schematic drawing of the venous vessel wall structure shows two distinct SMC layers; the Lo and Ci SMC layer. The dotted line indicates the border between Lo and Ci SMC layer. Nuclei were counterstained in purple (C-F).

Figure 2. TR3 and PAI-1 expression in perfused vein segments. Vein segments were exposed for 6 h to autologous whole blood under arterial pressure (B,D) or instantly fixed to serve as controls (A,C). TR3 mRNA and PAI-1 mRNA expression was detected by radioactive in situ hybridization (black dots) throughout the vein grafts after 6 h of perfusion (B,D), whereas TR3 and PAI-1 expression was only scarcely present in control vein segments (A,C).
DNA synthesis in venous SMCs (2 to 3.5 fold, dependent on donor A or B), whereas arterial SMCs derived from the same individuals remained quiescent (Figure 3A). To further substantiate changes in cell-cycle progression, the expression level of cell-cycle proteins was analyzed in cell lysates of stretched SMCs of venous and arterial origin. Cyclin-dependent kinase inhibitor p27$\textsuperscript{kip1}$ was found to be decreased upon stretch in venous SMCs (Figure 3B). In contrast, stretch did not alter the expression of p21$\textsuperscript{cip1}$ in arterial SMCs. The expression of another cell-cycle inhibitor, p21$\textsuperscript{cip1}$, was not affected by stretch in both venous and arterial SMCs. SM $\alpha$-actin expression was assayed as a marker for quiescent SMCs and was moderately decreased in venous SMCs after stretch (Figure 3B). In conclusion, cyclic mechanical stretching induced the proliferative phenotype in saphenous vein SMCs, while mammary artery SMCs remained quiescent.

**Cyclic stretch-induced TR3 and PAI-1 expression in venous SMCs**

To establish whether TR3 mRNA was also expressed in SMCs upon mechanical strain *in vitro*, analogous to what we observed in our *ex vivo* experiments, TR3 mRNA expression was determined by real-time RT-PCR. Saphenous vein and mammary artery SMCs were stretched
for 1, 2, 4 and 6 h, while non-stretched cells served as a control. TR3 was up-regulated in arterial SMCs (Figure 4A). However, in venous SMCs, TR3 mRNA expression was 4.6-fold higher than in arterial cells, reaching an optimum at 1 to 2 h after initiation of stretch.

PAI-1 protein levels were already relatively high in arterial SMCs and did not alter notably in response to stretch, whereas in venous SMCs induction in PAI-1 protein level was observed in cell-lysates as well as in culture media after stretch (Figure 4B). Analogous to the data of Gruber and colleagues using endothelial cells, TR3 may also be involved in enhanced transcripational activation of PAI-1 in SMCs.

**Adenoviral expression of TR3 decreased proliferation in venous SMCs**

To evaluate functional involvement of TR3 in the response of venous SMCs to mechanical strain, TR3 was overexpressed applying adenoviral infection. TR3 protein expression in stretched SMCs, was confirmed by Western blotting analysis (Figure 5A). Even after stretch, TR3 virus-infected SMCs showed a more differentiated (contractile) SMC phenotype reflected by increased synthesis of SM α-actin, calponin and p27Kip1 protein when compared to mock virus-infected cells (Figure 5A).

After 24 h of stretch, the virus-infected cells were assayed for DNA synthesis by [3H]-thymidine incorporation. Mock virus-infected cells showed a similar response as the non-
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infected venous SMCs (compare with Figure 3A) \(^{[1]}\)-thymidine incorporation was induced 2.7-fold upon stretch (Figure 5B). TR3-infected SMCs did not proliferate under conditions of cyclic stretch as revealed by an equal amount of \(^{[1]}\)-thymidine incorporation in control and stretched TR3-infected SMCs. The fact that TR3 overexpression prevents the differentiation to a proliferative phenotype corresponds with our previous findings, showing that TR3 inhibits SMC hyperplasia.\(^{13}\)

**Decreased proliferation in venous SMCs by 6-MP treatment**

To provide additional evidence that endogenous TR3 directly affects stretch-induced SMC proliferation, we assayed the effect of a TR3 agonist. 6-MP is the active metabolite of the immunosuppressive drug azathioprine (Imuran) that induces apoptosis of T cells and is an agonist of TR3-like factors.\(^{9,19}\) To determine whether 6-MP influences stretch-induced proliferation, venous SMCs were treated with 6-MP at various concentrations. To exclude that 6-MP induces apoptosis in SMCs at the applied concentrations, venous SMCs were cultured with 25 \(\mu\)M 6-MP or 10 \(\mu\)M CCCP, which is known to induce apoptosis. The CCCP-treated cells demonstrated less cell-spreading (data not shown) and shrunken nuclei.

![Figure 5. Decreased proliferation in venous SMCs with TR3 adenovirus. The expression of TR3 protein after infection of venous SMCs with TR3-encoding adenovirus was demonstrated by Western Blotting (A). TR3 was expressed in TR3-infected stretched SMCs, whereas mock infected cells did not express measurable endogenous TR3 protein. In the TR3-infected cells SM \(\alpha\)-actin, calponin and \(p27^{kip1}\) synthesis was more pronounced than in mock-infected cells. \(\alpha\)-Tubulin served as control for equal loading. \(^{[1]}\)-Thymidine incorporation was increased after 24 h of cyclic stretch in mock virus-infected SMCs, whereas TR3 virus-infected SMCs were indifferent to stretch (B). \(^{[1]}\)-Thymidine incorporation was expressed as percentage of the mock control value.](#)
(a mild apoptotic phenotype), while cells treated with 6-MP and control cells spread well (morphology as revealed by SM α-actin staining) and had large round nuclei, indicating that these cells were not apoptotic (Figure 6A). Untreated venous SMCs, subjected to 24 h of stretch, showed a 2.5 fold induction in proliferation, whereas the effect on DNA synthesis was reduced in a dose-dependent manner with 6-MP treatment (Figure 6B). At 25 μM 6-MP, stretch-induced DNA synthesis was completely inhibited. In conclusion, the 6-MP response is in line with the assumption that TR3 is (further) activated by 6-MP to prevent excessive SMC proliferation.

**Figure 6.** Decreased SMC proliferation in response to mechanical strain after 6-MP treatment. 6-MP did not induce apoptosis as determined by healthy nuclear morphology in control and 6-MP treated SMCs using Hoechst staining (A). Only the CCCP treated cells showed small apoptotic nuclei. SM α-actin staining demonstrated actin fibers in control venous SMCs. [3H]-Thymidine incorporation was increased in response to 24 h of cyclic stretch, whereas 6-MP reduced stretch-mediated proliferation in a dose-dependent manner (B). [3H]-Thymidine incorporation was expressed as percentage of the control value.

**Discussion**

Coronary bypass surgery relieves atherosclerosis patients from angina and may prevent myocardial infarction. However, when saphenous veins are applied as bypass material excessive SMC hyperplasia causes vein-graft disease, an important disadvantage for a significant number of patients. In contrast, mammary artery bypass grafts display a better patency than saphenous vein grafts, a difference which we believe needs to be unraveled to identify the targets to eventually enhance the performance of bypasses from venous origin.
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In the ex vivo perfusion model applied in this study, the early-response gene TR3 was not expressed in control veins, whereas TR3 mRNA was up-regulated in the circular SMC layer after 1 h of perfusion and throughout the vessel wall after 6 h. The circular SMC layer is the outer part of the venous vessel wall indicating that TR3 mRNA expression was presumably not induced by a circulating factor or in response to endothelial cell damage, but rather that the key stimulus was mechanical strain. Anatomical differences between the internal mammary artery and saphenous vein, i.e. the lack of elastic laminae in veins, may in part explain the observed graft damage by overdistension of vein segments under arterial pressure. Indeed, vein segments that were protected for overdistension by an external stent displayed normal morphology, as illustrated by preserved luminal endothelium and no induction of TR3 mRNA expression.

In addition, intrinsic differences between mammary artery and saphenous vein SMCs play a crucial role in the response of SMCs to external stimuli. It has been established that saphenous vein SMCs are more responsive to mitogenic stimuli such as platelet derived growth factor, thrombin or mechanical strain than internal mammary artery SMCs. In the present study, we have investigated intrinsic differences in stretch-related responses of these two types of SMCs in an in vitro stretch model, with regard to the role of transcription factor TR3 and its downstream gene PAI-1. Both for TR3 mRNA and PAI-1 protein expression, we observed robust up-regulation in venous SMCs in contrast to internal mammary artery SMCs. The abundant stretch-mediated expression of TR3 and PAI-1 mRNA in vein grafts in the ex vivo model confirmed the relevance of the data found in vitro. In addition, stretch-induced PAI-1 mRNA expression has also been described in cultured aortic SMCs. These data demonstrate that mammary artery-derived SMCs are intrinsically different from aortic SMCs and that the latter type of arterial SMCs show a stretch response that relates more to venous SMCs. The role of PAI-1 in SMC hyperplasia remains controversial, however in our hands, in the carotid artery ligation model that induces SMC-rich lesions in mice, both TR3 and PAI-1 protect against intima formation. Recently, a molecular pathway to explain mechano-sensitive cell-cycle progression in SMCs has been revealed. Stretch-induced integrin-dependent activation of phosphoinositide 3-kinase/protein kinase B leads to inactivation of a forkhead transcription factor, which results in transcriptional down-regulation of p27Kip1. To further delineate the processes involved in initiation of proliferation and de-differentiation of venous SMC in response to cyclic stretch, we also analyzed the expression of p27Kip1. We demonstrated decreased p27Kip1 protein expression levels in venous SMCs in contrast to unchanged levels in mammary artery SMCs in response to stretch. Similar as for PAI-1, p27Kip1 was stretch-regulated in
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aortic SMCs again indicating the resemblance between venous and aortic SMCs in response to stretch, which is dissimilar to internal mammary artery SMCs. We reported previously that TR3 increases p27\textsuperscript{Kip1} expression levels and decreases DNA synthesis.\textsuperscript{13} Therefore we studied the functional involvement of TR3 in stretched-induced proliferation. Adenovirus-mediated TR3 overexpression in venous SMCs resulted in full inhibition of stretch-induced DNA synthesis. Consequently, we propose that TR3 protects venous SMCs from excessive proliferation by maintaining high p27\textsuperscript{Kip1} levels. In addition, SM α-actin and calponin, SMC-specific differentiation markers, were up-regulated in TR3-infected stretched SMCs. These data implicate that TR3 not only prevents excessive proliferation of venous SMCs, but also enhances the differentiated, contractile phenotype of these cells. Our data support a concept that TR3 may act as a target for intervention in vein-graft disease. 6-MP, the bioactive metabolite of the commonly used immunosuppressive drug Azathioprine (also named Imuran), is a recently identified agonist for TR3 that modulates TR3 activity.\textsuperscript{9} Here, we show that 6-MP decreased stretch-induced SMC proliferation in a dose-dependent way.

In conclusion, vein-graft disease is the result of excessive SMC proliferation in response to biomechanical stimulation of venous bypass grafts. Application of the mammary artery as graft material is preferred due to its high degree of patency. However, there is only limited material available. Consequently, vein grafts will remain an essential, alternative source of bypass material, which necessitates improvement. Venous SMCs respond to mechanical strain by initiation of proliferation, while at the same time also cell-cycle inhibitory feedback systems are activated, such as the recently described IEX-1 pathway\textsuperscript{24} and the TR3 transcription factor pathway as identified in this study. The activity of TR3 is enhanced by 6-MP and we hypothesize that 6-MP modulates biomechanical intimal thickening after bypass surgery as a means to prevent excessive SMC proliferation and subsequent vein-graft disease.

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