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SAUCY, F, et al.

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Ex vivo Pulsatile Perfusion of Human Saphenous Veins Induces Intimal Hyperplasia and Increased Levels of the Plasminogen Activator Inhibitor 1


Division of Thoracic and Vascular Surgery, and Division of Vascular Medicine, CHUV, Lausanne, and Division of Angiology and Hemostasis, HUG, Geneva, Switzerland

Key Words
Ex vivo perfusion · Human saphenous vein · Intimal hyperplasia · Fibrinolytic factors · Plasminogen activator inhibitor 1 · Urokinase-type plasminogen activator · Tissue-type plasminogen activator

Abstract
Vessel wall trauma induces vascular remodeling processes including the development of intimal hyperplasia (IH). To assess the development of IH in human veins, we have used an ex vivo vein support system (EVVSS) allowing the perfusion of freshly isolated segments of saphenous veins in the presence of a pulsatile flow which reproduced arterial conditions regarding shear stress, flow rate and pressure during a period of 7 and 14 days. Compared to the corresponding freshly harvested human veins, histomorphometric analysis showed a significant increase in the intimal thickness which was already maximal after 7 days of perfusion. Expression of the endothelial marker CD31 demonstrated the presence of endothelium up to 14 days of perfusion. In our EVVSS model, the activity as well as the mRNA and protein expression levels of plasminogen activator inhibitor 1, the inhibitor of urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), were increased after 7 days of perfusion, whereas the expression levels of tPA and uPA were not altered. No major change was observed between 7 and 14 days of perfusion. These data show that our newly developed EVVSS is a valuable setting to study ex vivo remodeling of human veins submitted to a pulsatile flow.

Introduction
In developed countries, the number of patients suffering from peripheral arterial disease and requiring bypass grafting is increasing in the elderly population. For those patients suffering from peripheral arterial disease and necessitating vascular bypass, the saphenous vein remains the most commonly used and suitable graft material [1]. Nevertheless, about 50% of vein grafts fail during the first 2 years after implantation; occlusion of the graft lumen due to neointimal hyperplasia (IH) remains the main complication [2, 3]. This process involves the proliferation of smooth muscle cells (SMCs) exhibiting a synthetic migratory phenotype and the production of extracellular fibrous matrix; this leads to fibrosis and stiffening of the vein graft which may limit the vein’s ability to...
properly remodel in the arterial circulation. Vascular injuries may be caused by various factors such as mechanical trauma during harvesting of the vein and anastomosis, changes in blood pressure and flow, inflammation or oxidative stress. The biomechanical changes occurring after transposition of a vein in the arterial system induce an adaptive remodeling of the venous wall. This is characterized by endothelium disruption and SMC proliferation and migration from the media into the intima. These events occur within days after bypass grafting, inducing intimal and medial thickening [1, 2]. Nevertheless, arterial conditions induce conflicting effects. Indeed, pulsatility, high pressure, and flow result in increased wall tension inducing IH. Conversely, an increase in flow is known to decrease IH [4–6]. These changes provoke alterations and dysfunction of endothelial cells, leading to alteration of the fibrinolytic potential [7]. Although specific mechanisms of vessel wall remodeling are partially understood, it has been reported that fibrinolysis activation plays a key role [8, 9]. The plasminogen activation system offers an important response mechanism to vascular injury. The conversion of plasminogen to plasmin by tissue- (tPA) and urokinase-type (uPA) plasminogen activators is the central reaction of the system. Plasminogen activator inhibitor 1 (PAI-1) regulates fibrinolysis by directly inhibiting plasmin [10]. In addition, PAI-1 plays a key role in determining proliferation response to vascular injury by inhibiting the degradation of fibrin and several extracellular matrix proteins by plasmin [11]. Our previous studies have shown that the ex vivo vein support system (EVVSS) allows to study vascular wall remodeling up to 14 days in a low continuous laminar flow setting under reproducible and standardized conditions [12, 13]. We hypothesized that the same observation could be demonstrated in our modified pulsatile ex vivo perfusion system, characterized by the presence of high shear stress conditions, pulsatile flow and high intraluminal pressure applied to the veins to create physiological arterial conditions. In the present study, we investigated the impact of high flow perfusion on the histological, biological and fibrinolytic response of human saphenous vein segments.

**Materials and Methods**

**Harvesting of Human Saphenous Veins**

Surplus segments of nonvaricose human saphenous veins were obtained from 8 to 10 patients (mean age 67.3 ± 0.5 years; 3 females, 6 males) undergoing lower limb bypass surgery. The greater saphenous vein was harvested and immediately stored at 4°C in a calcium-free Krebs solution (in mmol/l: NaCl 118; KCl 4.7; MgSO$_4$ 1.2; KHPO$_4$ 1.2; NaHCO$_3$ 25; EDTA sodium 0.026; glucose 11.1). Vein segments were rapidly divided into 3 segments. Two segments were perfused during a period of 7 or 14 days and the third segment was divided into 2 parts, one was kept in formal and the other one was rapidly frozen in liquid nitrogen. The Ethical Committee of the University of Lausanne approved the experiments, which are conformed to the principles outlined in the Declaration of Helsinki for use of human tissue.

**Ex vivo Vein Perfusion System**

We previously described an ex vivo vein system, in which venous segments were perfused in a continuous laminar unidirectional flow with low levels of shear stress [12, 13]. The vein system was modified in order to generate pulsatile perfusion induced by a gearing pump (Reglo-Z, Ismatec®, Zurich, Switzerland), along with a unidirectional head pump, inducing a 4- to 420-ml/min flow velocity. The pulsatile signal is a cardioid curve produced by an arbitrary waveform generator controlled by a computer (National Instruments PCI-6024 E Acquisition card). This program integrates constant acquisition and monitoring of pressures, flow velocity, pulse rate and signal (Labview®, National Instrument). Vein segments were connected to the perfusion pump by silicone peroxide-treated tubing (internal diameter 3.2 mm) (Ismatec, Switzerland) and stored at 37 ± 0.1°C inside a perfusion chamber placed in a cell culture incubator (Model 310, Forma Scientific Inc., Marietta, Ohio, USA) (fig. 1). The culture medium, used as perfusion solution, contained RPMI-1640 + Glutamax with 30% fetal calf serum (Gibco) supplemented with 2% 70-kDa dextran (Sigma) supplemented with 1% antibiotic-antimycotic solution (penicillin G: 10,000 U/ml, streptomycin sulfate: 10,000 µg/ml, amphotericin B: 25 mg/ml, and gentamycin: 0.5 µg/ml). Medium viscosity was measured at 1.35-10−2 dyn·s/cm² using a Coulter viscometer (Coulter Electronics, UK). Shear stress was calculated with the following equation: shear stress = 4 Q/π r³ [µ: viscosity (dyn·s/cm²), Q: flow rate (ml/s), r: vein radius (cm)]. Because of the limited amount of human veins, we only use one single flow condition. In this setting, we reproduced ‘arterial’ conditions regarding shear stress, flow rate and pressure as previously described by Labadie et al. [14]. The flow parameters were designed to obtain arterial conditions, comprising high levels of shear stress (9-15 dyn/cm²), a 60-pulse/min flow rate of 120 ± 15 ml/min and a systolic/diastolic pressure of 80 ± 10 mm Hg/40 ± 10 mm Hg as described by several authors [14]. The pH value was kept constant at 7.4 ± 0.01 using a CO2/PH algorithm based on the Henderson-Hasselbach equation. Vein segments were perfused during 7 and 14 days and the culture media was changed every 2 days. The length of perfusion was determined following several reports indicating that vein segments usually remain functional as assessed by histology and vasomotion during a 14-day period of culture contrary to longer duration of culture [14].

**Vasomotor Function Assessment**

Each vessel segment was assessed for viability of the different components of the vessel wall (muscle, intima) before and after perfusion and checked for changes in isometric tension induced by incubating the vein in the presence of various vasoactive drugs in an experimental system called Radnoti® as described [12]. A 3-mm-long ring was cut from the harvested venous segment before and after perfusion, respectively. Rings were suspended between 2 stainless steel hooks in 20 ml Krebs solution (in mmol/l:
NaCl 118; KCl 4.7; CaCl₂ 2.5; MgSO₄ 1.2; KHPO₄ 1.2; NaHCO₃ 25; EDTA calcium disodium 0.026; glucose 11.1) at 37.0 °C and aerated with 95% O₂ and 5% CO₂. One hook was fixed to a force transducer (Statham Universal UC2) recording changes in isometric tension. The segment was then kept for 1 h with a fixed pretension of 1.6 g. After this equilibration period, contractions to cumulative doses (10⁻⁸ to 10⁻⁵ M) of norepinephrine were measured. Relaxations were then serially determined in the presence of the endothelial-independent agonist sodium nitroprusside (SNP; 10⁻⁸ up to 10⁻⁶ M).

Histological, Histomorphometrical and Immunohistochemical Analysis
For each vein, a 5-mm-thick ring was harvested before and after perfusion. After fixation in 4% formalin, vein segments were paraffin embedded and 5-µm-thick sections analyzed. Hematoxylin-eosin staining was used for histological analysis, Van Gieson-elastin staining for histomorphometric assessment. For histomorphometry, digital pictures were taken and measurements performed with a specially designed software (KS 400, Zeiss®, Germany) using a standardized protocol. Twenty-four measurements of intima and media thickness were processed for each sample at a magnification of ×20 and ×1.25, respectively. Immunohistochemistry was performed with the following 3 antibodies: monoclonal antibody CD31 (1:40 Dako, Denmark), monoclonal anti-smooth muscle actin (1:10,000, Sigma, USA), and Ki-67 (1:10,000, Sigma, USA). Binding sites of the different antibodies were visualized using an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, Calif., USA), diluted 1:50 [15]. After rinsing in PBS, the product of the peroxidase reaction was visualized using 0.1% 3,3-diaminobenzidine tetrahydrochloride hydrate, dissolved in PBS containing 0.3% H₂O₂. Reactions were stopped after 5–15 min to maximize signal intensity. Antibody binding was revealed using the avidin-biotin peroxidase technique. Sections were, if needed, counterstained with hemalun to visualize the nucleus.

Analysis of PAI-1, tPA and uPA Gene Expression
Total RNA of human saphenous veins was homogenized in Tripure Isolation Reagent (Roche, Switzerland), as described [15, 16]. Quantitative real-time PCR was performed on total RNA treated for 30 min in the presence of DNase I (DNA-free kit, Ambion, Cambridge, UK). One microgram of total RNA was used for reverse transcription with the ImPromII® Reverse Transcription system (Promega, Madison, Wisc., USA). Human PAI-1, tPA and
uPA mRNA levels were determined by quantitative reverse transcription PCR using the SYBR® Premix ExTaq™ (Takara) in a Lightcycler Instrument (Roche Diagnostics). Negative controls included amplification of distilled water, and RNA samples that had not been reverse transcribed. Analysis of data was performed using the 3.5 version of the Lightcycler software (Roche Diagnostics GmbH) [17]. Results from each sample were normalized relative to the expression level of the human housekeeping gene GAPDH. cDNAs were amplified using the following primers: human PAI-1: 5'-GGCTGGTGTGGTGAATG-3' (sense) and 5'-GCTGGTGCTGGTGAAGT-3' (antisense); human uPA: 5'-ACGCAAGGGAGATGAAGT-3' (sense) and 5'-GAGGGCAGGATGCT-3' (antisense); human tPA: 5'-ACACAGCAGAACCAGCAGAATG-3' (sense) and 5'-CAGGAGGGCAGATACTACAGTA-3' (antisense); human GAPDH: 5'-AAGAATTGATGATTGATGATGT-3' (antisense) and 5'-GAAGTGGTGAAGG-3' (antisense).

Analysis of PAI-1 Activity
The measurements of PAI-1 activity were performed in the perfusion medium (1:5 dilution) after 7 and 14 days of perfusion by enzyme immunoassay using the TintElize PAI-1 kit (Biopool International) following the manufacturer protocol.

Statistical Analysis
All Western blot and quantitative PCR experiments were quantitatively analyzed and results are shown as mean ± SEM. One-way analysis of variance was performed to compare the mean values between groups, using the post hoc Bonferroni test, as provided by the Statistical Package for the Social Sciences (SPSS 17.0, Chicago, Ill., USA). For PAI-1 activity, Student t test was used. Statistical significance was set at p < 0.05.

Results
Characteristics of Vascular Remodeling in Human Saphenous Veins Submitted to Pulsatile Flow
The pattern of reactivity of the venous segment in the presence of norepinephrine or SNP was assessed in the experimental setting by Radmoti as previously published [12]. Vasomotor function measurements showed a dose-dependent contraction to norepinephrine and relaxation to SNP before and after 7 and 14 days of perfusion in each segment. There was no significant difference regarding the contraction and relaxation response between the freshly isolated vein and the perfused veins after 7 and 14 days (data not shown). These results confirmed the preserved contractility of SMCs after the 14-day perfusion period. Immunostaining for the endothelial marker CD31 demonstrated the presence of endothelial cells all along the vessel segment during the entire period of per-
fusion (fig. 2, upper panel). The presence of SMCs was confirmed by α-actin staining in all control and perfused segments (fig. 2, lower panel). Analyses of antibodies against KI-67, a marker of cell proliferation, were performed on the same sections stained with α-actin antibodies; no nuclear staining was generally found in the control veins, whereas a strong staining in the intimal region was observed after 7 and 14 days of perfusion. A proliferation index of about 8.2% was observed in the perfused veins. The mean diameter of the human saphenous vein segments was 3.1 ± 0.1 mm at the beginning of perfusion, and 3.8 ± 0.1 mm after 7 days and 4.3 ± 0.1 mm after 14 days of perfusion with a mean estimated shear stress of 9.7, 5 and 3.4, respectively.

Histological assessment of the vessel wall integrity was determined using hematoxylin-eosin staining (fig. 3, upper panel). Veins showed the absence of morphological evidence for the presence of cellular degeneration, cytoplasmic alteration or apoptosis. Morphometric analysis performed by measuring the individual layers and total wall thickness showed that compared with the freshly isolated control veins (D0), the perfused vein segments (D7 and D14) undergo a significant decrease in the media thickness (fig. 3, lower panel). The neointima development (fig. 4, upper panel) was similarly increased in veins submitted to a pulsatile flow during a period of 7 and 14 days (fig. 4, middle panel). The intima/media ratio was also similarly increased between 7 and 14 days of perfusion (fig. 4, lower panel).

**PAI-1 Is Increased in the Media of Perfused Veins**

Quantitative analysis demonstrated that, after 7 or 14 days of perfusion, PAI-1 expression is markedly increased due to a transcriptional regulation (fig. 5a). Accordingly, immunostaining showed that PAI-1 was increased in the SMCs of the media (fig. 5b) after perfusion compared to control veins.
the PAI-1 expression observed in the control veins (D0). Moreover, compared to baseline values, a significant increase in PAI-1 activity was detected after 7 and 14 days of perfusion (fig. 5c).

Quantitative reverse transcription PCR evaluation of tPA and uPA mRNA expression (fig. 6a) demonstrated no change in these transcripts after 7 or 14 days of perfusion. Immunolabeling further demonstrated no major change in the expression of tPA and uPA, which were expressed by the SMCs of the human veins (fig. 6b).

Discussion

The present work characterizes a new model to study the development of IH in human saphenous veins submitted to a pulsatile flow in the presence of high pressure conditions. Human saphenous veins showed vessel wall integrity, significant development of IH, and an increase in PAI-1 activity and expression after 7 and 14 days of perfusion. Vein segments retained their reactivity to vasoactive drug infusion as demonstrated by preserved responses following chronic perfusion. Therefore, our EVVSS associated with a pulsatile flow appears to be a well-suited system to conduct chronic perfusion studies in order to elucidate the mechanisms associated with the arterialization of the vein and the development of IH. Histomorphometry demonstrated significant changes in intimal thickness and media thinning after 7 and 14 days of pulsatile perfusion. The enlargement of the venous wall in response to increased flow is generally correlated with tissue proliferation [18, 19]. Concerning the media, the role of pulsatility, flow, pulse pressure, and medium viscosity in medial hypertrophy has not been clearly de-
Surgical vein harvesting and ex vivo perfusion imply the disruption of the vasa vasorum and denervation of the vein thus altering vessel wall function. As a consequence, medial thinning may be secondary to high stretch pressure and to the inability of the vein to respond by hypertrophy of the muscular layer. High stretch pressure induces vein diameter increase and distension of all the vessel wall components. Shear stress appears to be one of the major stimuli influencing the development of IH even if its exact role is still controversial [20, 22, 23]. In our perfusion system, the shear stress values are within the high range (9–15 dyn/cm²) and

![Graph](image)

**Fig. 5.** Increased PAI-1 in human saphenous veins submitted to a pulsatile perfusion flow. Upper panel: expression of PAI-1 analyzed by quantitative reverse transcription PCR is increased in veins submitted to pulsatile flow and developing IH. Middle panel: immunocytochemistry further demonstrated an increase in PAI-1 expression throughout the media and the intimal layers of human veins after 7 and 14 days of perfusion. Original magnifications, ×200. Lower panel: PAI-1 activity was also increased after 7 (D7) and 14 days (D14) of ex vivo vein pulsatile perfusion compared to control veins (D0). Data represent mean ± SEM of 6 experiments. M = Media; L = lumen. **p < 0.01, versus respective untreated control veins.
should be sufficient for proper endothelial activation and inhibition of SMC differentiation thus avoiding IH development. However, in our setting, high pressure at a constant flow rate significantly increased inner diameter and decreased shear stress from 9.7 to 3.7 dyn/cm². The endothelium trauma due to harvesting and the change in hemodynamic conditions during the perfusion may explain the significant development of IH, even after 7 days of perfusion. Porter et al. [4] described that mean arterial shear stress (6–9 dyn/cm²) totally suppresses IH and that venous conditions (1–3 dyn/cm²) only partly suppress the response.

PAI-1 acts as the primary physiological inhibitor of the two main mammalian plasminogen activators, tPA and uPA [24]. The system is regulated by PAIs, of which PAI-1 is thought to be the most important. Different studies suggested that PAI-1 prevents the formation of neointima upon vascular injury, and that decreased levels of PAI-1 allow neointimal growth and vascular remodeling [25–29]; however, other findings demonstrated that PAI-1...
may promote the formation of neointima and vascular remodeling in response to vascular injury [30, 31]. Alterations of the vessel wall after mechanical injury result in neointima development which is often associated with restenosis. An association between vascular remodeling and increased levels of PAI-1 [9] has been initially described in the vascular wall adjacent to an arterial thrombus induced by mechanical injury in the rat carotid artery [32]. The fibrinolytic system has been reported to be stimulated in case of vein grafts used as arterial bypasses; therefore, PAI-1 appears to be a key factor at the intersection between thrombosis, fibrin deposition, extracellular matrix degradation and the balance between proliferation and cell death [9].

Late occlusion of vein graft by thrombosis is a major risk after revascularization. Kauhanen et al. [7] showed that increased PAI-1 and uPA and decreased tPA were associated with occluded venous grafts. Inhibition of fibrinolysis by PAI-1 may induce fibrin deposition, which serves as a provisional matrix for migration of SMCs [9]. PAI-1 may therefore promote vascular SMC proliferation and inhibits apoptosis [33]. In our setting, we clearly observed an increased activity of PAI-1, which was correlated to PAI-1 mRNA expression in our previous studies [12, 13]. As a standard tissue culture medium is used in place of blood in an ex vivo setting, the complex interaction of blood elements and vascular wall is affected. Under these conditions, PAI-1 activity is enhanced, supporting the idea that PAI-1 promotes IH development in conditions of vascular injury, by activating SMC proliferation and inhibiting apoptosis.

As we studied vein surplus for bypass being directly harvested from the patient’s leg, we therefore dealt with a vein injury model with altered endothelial functions which might directly impair the vein graft patency [34]. In this study, we demonstrated that we can transfer this bedside situation to our EVVSS bench and assess whether hemodynamic forces may alter endothelial functions or provoke IH in a vein graft. Our study demonstrated that the newly generated EVVSS with pulsatile flow is a valuable tool to study remodeling of the human vascular wall. This setting reproduces ex vivo physiological pulsatile perfusion conditions during 14 days. We showed that under standardized pulsatile conditions, human veins develop significant IH associated with a marked increase in PAI-1 expression and activity after 7 and 14 days of perfusion. Future studies will characterize the importance of hemodynamics forces involved in the vascular wall remodeling, and the importance of the fibrinolytic system as well as the modulation of the different partners of this system and their role in the development of IH will be characterized in the presence of various levels of perfusion flow. The recent development of orally active PAI-1 inhibitors [24] will help to decipher in our newly generated model the mechanisms associated with increased PAI-1 in the development of IH.

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