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Comparative Assessment of Intimal Hyperplasia Development after 14 days in Two Different Experimental Settings : Tissue Culture Versus *ex-vivo* Continuous Perfusion of Human Saphenous Vein

THESE

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Comparative Assessmment of Intimal Hyperplasia Develpment after 14 Days in Two Different Experimental Settings: Tissue Culture *versus* ex Vivo Continuous Perfusion of Human Saphenous Vein.

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Résumé de l'article :

L'hyperplasie intimale est un processus de remodelage vasculaire ubiquitaire après une lésion, pouvant menacer la perméabilité de tout type de reconstruction vasculaire. Les mécanismes physiopathologiques impliqués dans le développement de l'hyperplasie intimale ne sont que partiellement élucidés. Il est par conséquent nécessaire d'effectuer des recherches complémentaires afin d'en améliorer la compréhension et ainsi permettre l'élaboration de nouvelles stratégies thérapeutiques médicamenteuses.

La culture de veines en milieu statique permet le développement de l'hyperplasie intimale. Ce modèle maintient la viabilité tissulaire, comme décrit précédemment dans d'autres études, mais empêche l'analyse des paramètres hémodynamiques. La mise au point d'un modèle de perfusion in vitro permettant la perfusion de segments vasculaires représente une approche expérimentale intégrant les différents facteurs hémodynamiques.

Le système de perfusion (Ex Vivo Vein Support System) que nous avons élaboré conserve l'intégrité pariétale ainsi que les propriétés vasomotrices des veines pour une durée de 14 jours. Cette étude démontre que les deux modèles permettent le développement de l'hyperplasie intimale. Toutefois, les propriétés vasomotrices ainsi que l'influence des paramètres hémodynamiques ne peuvent être analysées que par l'utilisation du système de perfusion. Ce dernier a permis de perfuser des vaisseaux humains sans contamination bactérienne tout en maintenant l'intégrité cellulaire. Ce modèle de perfusion se rapproche plus des conditions hémodynamiques rencontrées in vivo que le modèle statique.

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Comparative Assessment of Intimal Hyperplasia Development after 14 Days in Two Different Experimental Settings: Tissue Culture *versus* *ex Vivo* Continuous Perfusion of Human Saphenous Vein

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Background. **Intimal hyperplasia (IH) is a vascular remodeling process which often leads to failure of arterial bypass or hemodialysis access. Experimental and clinical work have provided insight in IH development; however, further studies under precise controlled conditions are required to improve therapeutic strategies to inhibit IH development. *Ex vivo* perfusion of human vessel segments under standardized hemodynamic conditions may provide an adequate experimental approach for this purpose. Therefore, chronically perfused venous segments were studied and compared to traditional static culture procedures with regard to functional and histomorphologic characteristics as well as gene expression.**

Materials and methods. **Static vein culture allowing high tissue viability was performed as previously described. *Ex vivo* vein support system (EVVSS) was performed using a vein support system consisting of an incubator with a perfusion chamber and a pump. EVVSS allows vessel perfusion under continuous flow while maintaining controlled hemodynamic conditions. Each human saphenous vein was divided in two parts, one cultured in a Pyrex dish and the other part perfused in EVVSS for 14 days. Testing of vasomotion, histomorphometry, expression of CD 31, Factor VIII, MIB 1, α-actin, and PAI-l were determined before and after 14 days of either experimental conditions.**

Results. **Human venous segments cultured under traditional or perfused conditions exhibited similar IH after 14 days as shown by histomorphometry. Smooth-muscle cell (SMC) was preserved after chronic perfusion. Although integrity of both endothelial and smooth-muscle cells appears to be maintained in both culture conditions as confirmed by CD31, factor VIII, and α-actin expression, a few smooth-muscle cells in the media stained positive for factor VIII. Cell-proliferation marker MIB-1 was also detected in the two settings and PAI-1 mRNA expression and activity increased significantly after 14 days of culture and perfusion.**

Conclusion. **This study demonstrates the feasibility to chronically perfuse human vessels under sterile conditions with preservation of cellular integrity and vascular contractility. To gain insights into the mechanisms leading to IH, it will now be possible to study vascular remodeling not only under static conditions but also in hemodynamic environment mimicking as closely as possible the flow conditions encountered in reconstructive vascular surgery.**

***Key Words: ex vivo* perfusion; vein culture; saphenous vein; intimal hyperplasia; histomorphometry; immunohistochemistry; fibrinolytic factor; PAI-1; flow.**

INTRODUCTION

Intimal hyperplasia (IH) development is one of the leading causes of failure after vascular interventions, irrespective of the procedure performed (bypass surgery, percutaneous dilation, stent deployment) [1–5]. IH is characterized by proliferation of smooth-muscle cells (SMC) and their migration through the internal elastic lamina into the sub-intimal layer with production of extra-cellular matrix (ECM) proteins. IH typically occurs between 1 and 12 months after interven-

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tion. The pathogenesis of IH is not fully understood. Several authors have suggested that a combination of physical, hemodynamic, cellular, and humoral factors may contribute to IH development [6–9]. Animal models have markedly contributed to the understanding of these mechanisms, although it was demonstrated that IH in humans was different [10, 11]. A number of investigators have studied IH development in organ culture of human saphenous veins [8]. In this model integrity of vessel wall is maintained up to 14 days and allows us to study the interactions between endothelium and SMC. However, a major limitation of this experimental model is the absence of flow through the vessel segment. IH development has been associated with the presence of peculiar local hemodynamic conditions such as turbulent flow, low shear stress, flow separations [12, 13]. Indeed, Dobrin *et al.* described in an animal model a correlation between the blood flow through a vessel or a graft and the IH development with low shear stress being associated with increased IH in their model [14]. The endothelium plays a key role in the detection of shear stress and its translation to the vascular wall [15, 16].

The *Ex Vivo* Vein Support System (EVVSS) allows us to assess the biological response of the low-flow perfused segments under standardized and reproducible conditions. Low shear stress conditions were applied to the veins to produce maximal intimal proliferation [14]. Previous studies have shown that non-perfused human saphenous vein cultures allow us to study the development of IH up to 14 days. We hypothesized that the same observation could be demonstrated in our *ex vivo* perfusion system. In the present study we investigated the impact of low shear stress perfusion on the biological response of human venous segments (endothelial and muscular layers) and compared it to non-perfused organ culture of the same veins.

MATERIALS AND METHODS

Harvesting of human saphenous vein. All procedures were approved by the Ethical Committee of the University of Lausanne. Surplus segments of non-varicose human saphenous vein were obtained from nine patients (mean age, 56.5 ± 0.5; three females, six 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 three portions. One portion was used for the static culture; the second portion was used for the perfusion experiments and the third portion was snap-frozen in liquid nitrogen.

Static vein culture. A 2-cm-long segment of the harvested vein was opened longitudinally with luminal surface uppermost. The venous segment was pinned onto a Mersilene mesh (60 × 20 mm; Ethicon®, USA) in a Petri dish containing a layer of Sylgard 184 resin (Dow Corning, Seneffe, Belgium). The vein was then covered with 5 ml of the culture medium (M 199, 10% newborn calf serum, 1% antibiotic-antimycotic solution: penicillin G, 10,000 U/ml; streptomycin sulfate, 10,000 μg/ml; amphotericin B, 25 μg/ml; gentamicin, 5 μg/ml; Life Technologies, Inc., Invitrogen Corp., USA). Medium was changed every 2 days until the end of the experiments [17, 18].

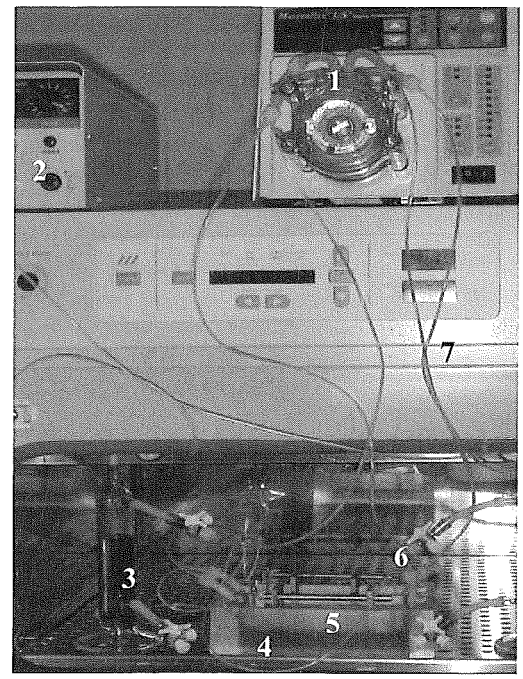

FIG. 1. Picture of flow model. (1) Perfusion lamp; (2) pressure control; (3) fluid reservoir; (4) perfusion chamber; (5) vein segment; (6) ultrasound flow meter; (7) silicone peroxide treated tubing. (Color version of figure is available online.)

Ex vivo vein perfusion. Harvested venous segments of 3 cm (mean internal diameter: 3.03 ± 0.5 mm) were perfused in the EVVSS as previously described [19–23]. Briefly, vein segments were connected to a perfusion pump (L/S, Masterflex, Cole-Parmer Instrument Co., USA) by silicone peroxide treated tubing (internal diameter, 3.2 mm) (Ismatec, Switzerland) and stored at 37°C (±0.1°C) inside a perfusion chamber placed in a cell culture incubator (Model 310, Forma Scientific Inc., Marietta, OH, USA) (Fig. 1). The culture medium was used as perfusion solution. Because of the limited amount of human vein, we were constrained to use only one flow condition. The pH was kept constant at 7.4 (±0.01) using a CO_2/pH algorithm based on the Henderson–Hasselbach equation [20]. Vein segments were perfused for 14 days and the culture media were changed every 2 days.

Vasomotion assessment. Vasomotion of saphenous vein wall (intima-media) was performed according to Radnoti® for each vessel segment before and after perfusion. Changes in isometric tension, induced by exposure of the vein to increasing concentrations of Noradrenaline and sodium nitroprussiate (SNP), were measured. Briefly, a 3-mm-long ring was cut from the harvested venous segment and suspended between two stainless hooks in 20 ml Krebs solution at 37.0°C and gassed with 95% O_2 and 5% CO_2. One hook was fixed to a force transducer (Statham Universal, USA) recording changes in isometric tension. The segment was kept for 1 h with a fixed pretension of 1.6 g defined as the precontracted state [24]. As the vein segments were longitudinally opened for organ culture, no vasomotion testing could be assessed after a 14-day culture. We measured contractions to cumulative doses (10^{-8} to 10^{-4} M) of NA. After submaximal precontracted (with 10^{-6} M NA), relaxations were serially determined to an endothelial-dependent agonist acetylcholine (Ach) and to an endothelial-independent agonist SNP.

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

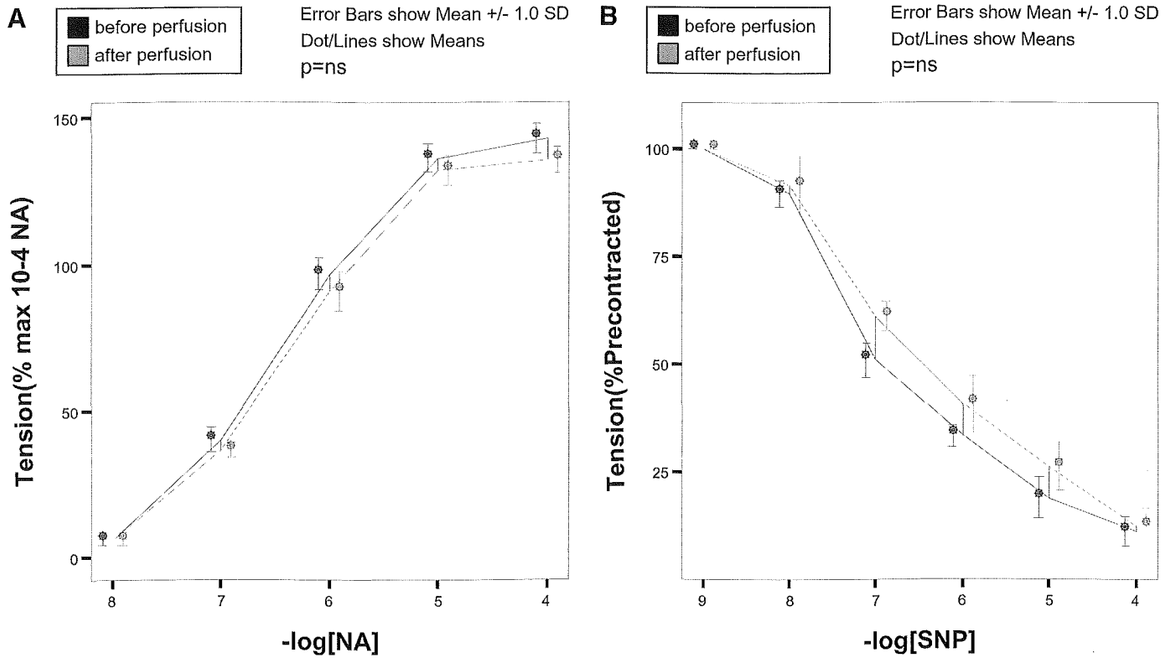

FIG. 2. (A) Dose-dependent vasoconstriction of vein segments after administration of NA. (B) Dose-dependent relaxation of vein segments after administration of SNP. (C) Dose-dependent relaxation of vein segments after administration of Ach.

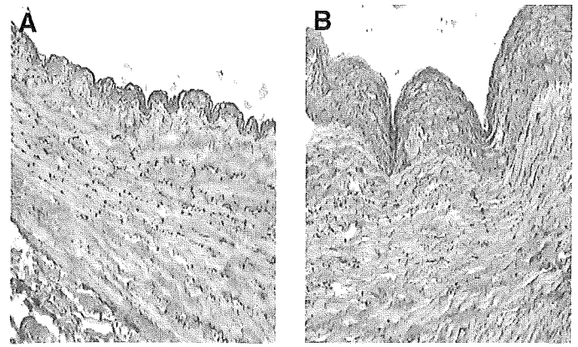

FIG. 3. Venous wall before (A) and after (B) a 14-day perfusion showing IH development. (Color version of figure is available online.)

were paraffin embedded and 5-μm-thick sections were analyzed. Hematoxylin–eosin staining was used for histological analysis and Miller staining was used for histomorphometric assessment. For histomorphometry, digital pictures were taken and histomorphometric measurements performed with a specially designed software (KS 400, Zeiss®, Germany) using a standardized protocol. Twenty-four measurements of the intima and media were processed for each sample at magnifications of $\times 20$ and $\times 1.25$, respectively. Immunohistochemistry was performed with the following five antibodies: monoclonal antibody CD31 (1:40; Dako, Denmark), monoclonal antibody CD34 (1:800; Immunotech, France), and polyclonal or monoclonal anti-human Von Willebrand factor (1:1000; Dako), monoclonal anti-smooth muscle actin (1:10,000; Sigma, USA), and monoclonal anti-human Ki 67 antigen (MIB-1, 1:50; Dako). Antibody binding was revealed using the avidin–biotin peroxidase technique. Proliferation cell-marker Ki 67 was quantified by cell-counting technique in a calibrated micrometer grid at a magnification of $\times 40$. In each field, all cells were counted, and the number of positively stained cells was expressed as a percentage of total cells, to obtain the MIB-1 index. A minimum of seven fields was quantified per section.

Analysis of PAI-1 gene expression. This analysis was performed by Northern blot. A ring of 10 mm was cut of the harvested vein segment, before and after perfusion, snap frozen in liquid nitrogen, and stored at −70°C. Total RNA was prepared as follows: frozen segments were homogenized using a Polytron (Kinematica, USA) and total RNA was extracted using the RNeasy kit (Qiagen, USA). Five micrograms of total RNA was then separated on a 1.2% agarose–formaldehyde gel in morpholine propanesulfonic acid running buffer, transferred on Biodyne A membrane (Pall, USA), UV crosslinked (Stratagene, USA), and prehybridized at 65°C in a solution containing 1.5× SSPE, 7% SDS, 10% polyethylene glycol 8000, 50 mg/ml heparin, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was carried out at 65°C in the same solution in the presence of randomly radiolabeled human PAI-1 cDNA probes. The blots were subsequently stripped and reprobed for human GAPDH. Signals were then quantified using an Instant Imager apparatus (Packard, USA) and signal intensities were normalized for the GAPDH signals. Normalized results were expressed as folds increase compared to results obtained from the same vein prior to perfusion to which an arbitrary value of 1 was given. Experiments were run in triplicate.

Statistical analysis. The Student's t test for unrelated samples was used where appropriate. The Wilcoxon signed rank test was used for statistical analysis of histomorphometrical measurements of media and intima alterations. A two-tailed hypothesis was used and significance was accepted at $P < 0.05$.

RESULTS

Vasomotion testing showed a dose-dependent contraction to NA and a relaxation to SNP before and after experimental exposure in each segment (Fig. 2A, B). There was no significant difference regarding the contraction and relaxation response between the control and the perfused veins after 14 days. These results confirmed the preserved contractility of SMC after the 14-day perfusion period. No venous segment before and after perfusion showed relaxation to Ach (Fig. 2C). Vessel wall integrity was determined by analysis of hematoxylin–eosin-stained vein sections (Fig. 3). No evidence of cellular degeneration was found in the control, the static vein segments, and the perfusion setting.

Histomorphometrical Analysis

Measurement of intimal and sub-intimal layers, corresponding to IH, revealed a significant thickening of perfused vein segments as compared to baseline values observed prior to perfusion and culture ($P < 0.05$). However, there was no statistical difference in intimal thickening between culture and perfusion ($P > 0.05$) (Fig. 4). Significant thinning of the media was observed in the culture in comparison with the perfusion system.

Immunohistochemical Assessment

Expression of anti-CD31 revealed 19.7 and 8% of endothelial cover in the culture and the perfused setting, respectively. This difference was not significant. No anti-CD34 positivity was observed in either control or treated vessels. However, anti-factor VIII staining demonstrated 94% of control endothelial cover, and 86 and 83% in the culture and the perfused models, respectively. Presence of SMC was confirmed by α-actin staining in all segments in the sub-intimal space and the media. The adjunction of Miller elastin stain with anti-α-actin allows us to localize SMC in the sub-intimal layer and to differentiate these cells from mesenchymal cells.

No MIB-1 antibody staining was found in the control veins, whereas 2.7 and 6.4% of the SMC stained positive for this proliferation marker in static culture and perfusion setting, respectively (Fig. 5).

Northern Blot Analysis

A significant increase of PAI-1 mRNA expression was detected after vein static culture ($P = 0.002$) and perfusion ($P = 0.002$) as compared to baseline values. However, there was no significant difference of PAI-1 mRNA expression between the two settings (Fig. 6).

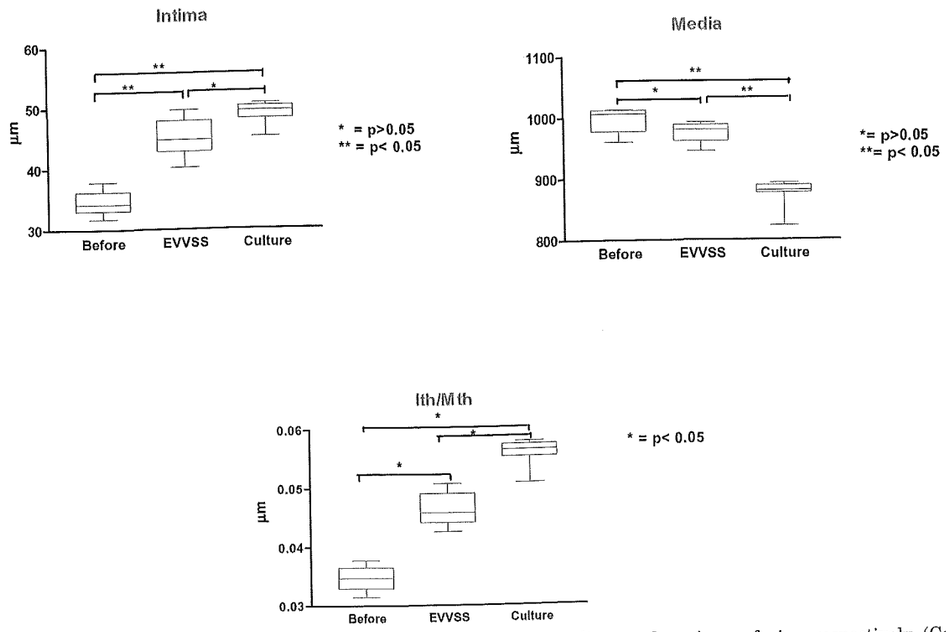

FIG. 4. Intimal and medial thickness measurements before and after vein culture and *ex vivo* perfusion, respectively. (Color version of figure is available online.)

DISCUSSION

Several reports indicated that vein segments remain usually viable as assessed by histology and vasomotion during a 14-day period of culture contrary to longer duration of culture [8]. Preservation of cellular integrity and vascular contractility was also demonstrated in our previous study comparing human venous segments after 7 and 14 days of perfusion. Histomorphometry indicated that IH development reached statistical significance after 14 days. However, longer duration of perfusion led to vessel wall necrosis and infection [24].

The present study demonstrates that vein exposure to EVVSS undergoes IH of the same magnitude as does the vein culture, with the advantage of maintaining flow conditions. Porter *et al.* described that mean arterial shear stress (6–9 dyn/cm^2) totally suppressed IH and that venous conditions (1–3 dyn/cm^2) only partly suppressed the response [7]. Despite our interest to compare various levels of perfusion, limitations were due to the lack of human vascular material. In this preliminary and novel experiment, venous conditions were selected to obtain maximal IH development in a chronic flow system and to compare it to the traditional static culture model. A significant increase of intimal and sub-intimal thickening was observed as compared to control vein segments after both perfusion and static vein culture ($P < 0.05$). The degree of IH development was similar in both settings, although the mechanisms are different. Indeed, the longitudinal opening of the vessel in the culture is certainly the main trigger of the neointima formation [25]. The absence of flow and shear stress in the culture may promote IH [24]. Shear stress appears to be one of the major stimuli influencing the development of IH even if its exact role is still controversial [26–29]. In our perfusion system, the shear stress is in the low range (2–6 dyn/cm^2) and may not be sufficient for a proper endothelial activation to inhibit IH development. Indeed chronic reduction of blood flow has been shown to decrease receptor-mediated release of endothelium-derived relaxing factors and to favor neointimal hyperplasia [14]. The absence of pulsatile flow and high stretch-inhibiting SMC differentiation may also explain IH development in both settings. Even though the degree of IH was more pronounced in the culture Petri dish, the changes did not reach statistical difference when compared to the perfusion conditions. The medial thinning in the culture may be explained by cell redistribution and phenotypic modifications of SMC resulting in a rapid migration of the dedifferentiated SMC into the sub-intimal space. This is clearly demonstrated by the increase in the intima/media ratio observed in the culture condition when compared to the perfusion system. True viability of the venous segments may be difficult to assess. Potential methods might include vasomotion, histology (nuclear density, relative amount of

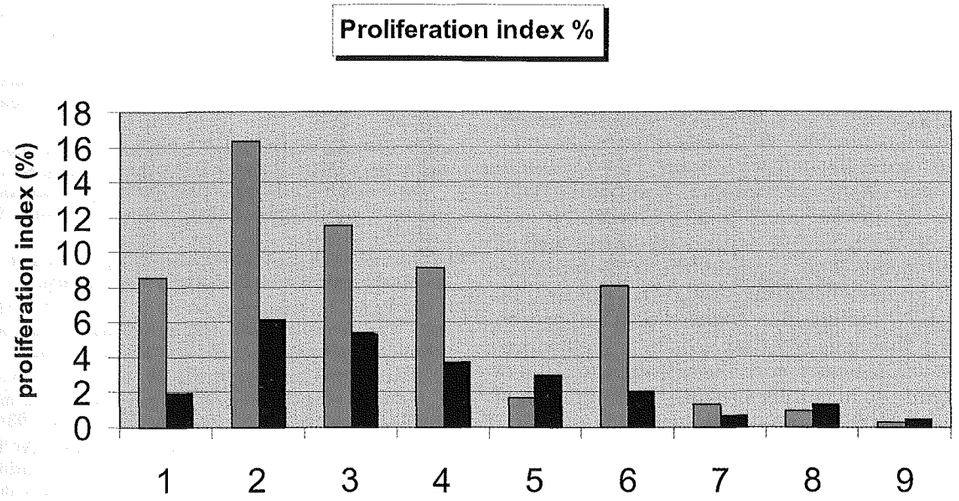

FIG. 5. Perfusion index in each venous segment after culture (black columns) and perfusion (red columns).

acellular, apoptotic, or necrotic tissue), and metabolism (LDH levels, ADP/ATP) assessment. We have relied on cellular integrity (histology and preservation of contractility and relaxation) in this study, as a surrogate marker of vascular viability.

EVVSS appears to be well-suited to conduct chronic perfusion study. Indeed, vein segments retained their reactivity to vasoactive drug infusion such as NA and SNP as demonstrated by preserved responses following chronic perfusion. In our hands, Ach did not induce any vasomotor response in any of the experimental conditions. Histological and immunohistochemical analysis revealed preservation of EC integrity in perfused and cultured vein segments. We observed different expression patterns between the endothelial markers CD 31 and Factor VIII. These results suggest that EC maintain cellular integrity. The positive staining of sub-intimal and medial spaces for Factor VIII suggest that EC may release Factor VIII in the ab-luminal space and diffuse into the vascular wall [30]. Culture media have been shown to be a major source of factor VIII; however, we did not find Factor VIII in our media. EC damaging/loss cannot be formally excluded as suggested by the absence of vasomotor responsiveness following Ach testing. Furthermore, the exposition of the sub-intimal layer after EC loss may partly be responsible for IH development in the perfusion setting. A great variability in proliferation index of the venous segments was observed based on MIB-1 expression. Medial fibrosis and pre-existing IH in the vein at the time of harvesting may partly explain this variability [31].

The veins which were exposed to flow show a greater proliferation index than those in static culture. Considering that no significant difference in IH was observed between the two settings, one may conclude that different mechanisms are involved in the development of IH. It will be very interesting to submit these venous segments to pulsatile and different shear rates in future studies.

The fibrinolytic system has recently been reported to play a major role in IH proliferation. Several authors demonstrated the involvement of plasminogen activator system in SMC migration [32]. The inactive proenzyme plasminogen is activated to the proteolytic enzyme plasmin by two plasminogen activators, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The system is regulated by plasminogen-activator inhibitors (PAI), of which PAI-1 is thought to be the most important. PAI-1 has been shown to inhibit SMC *in vitro* and inactivation of the PAI-1 gene results in exaggerated neointimal thickening [33, 34]. Kahanen *et al.* described an up-regulation of PAI-1 mRNA expression in failing vein grafts after the development of IH in a clinical study [35]. Carmeliet *et al.* suggested an inhibitory role of PAI-1 on cellular migration by observing an exaggerated intimal

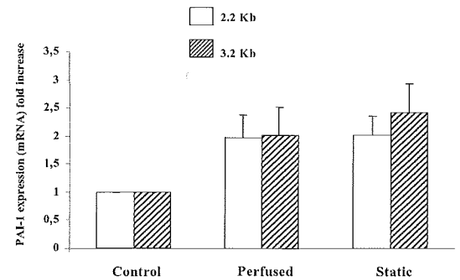

FIG. 6. PAI-1 mRNA expression before, at 7 and 14 days of vein culture and *ex vivo* vein perfusion, respectively.

proliferation secondary to injury in PAI-1-deficient mice [36]. Others demonstrated that PAI-1 inhibits SMC migration by limiting the binding of vitronectin to its receptor [37]. Moreover, Arnman *et al.* observed an enhanced expression of PAI mRNA around the thrombus in occluded saphenous veins [38]. We focused on a single factor of the fibrinolytic system (PAI-1), due to the limited amount of vein segments. Our results demonstrated a significant increase of PAI-1 mRNA expression in veins exposed to continuous flow as well as in the static model. This confirms the observation by Redmond *et al.* demonstrating that PAI-1 released by flow-induced EC inhibits flow-induced SMC migration *in vitro* [39]. This suggests that despite a higher proliferation index in continuous flow setting, the IH is of greater magnitude in culture because the migration of SMC is not inhibited in a the static condition by PAI-1 and so the process of IH by ECM secretion by SMC is possible.

In conclusion, we report the feasibility to chronically perfuse human venous segments, under sterile conditions with preservation of cellular integrity and vascular contractility. The *ex vivo* perfusion system allows the development of IH under standardized conditions for up to 14 days. This model gives insights to study vascular wall remodeling and mechanisms leading to IH development not only under no-flow conditions but also in hemodynamic environment.

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