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The use of external mesh reinforcement to reduce intimal hyperplasia and preserve the structure of human saphenous veins

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ABSTRACT

The saphenous vein is the conduit of choice in bypass graft procedures. Haemodynamic factors play a major role in the development of intimal hyperplasia (IH), and subsequent bypass failure. To evaluate the potential protective effect of external reinforcement on such a failure, we developed an *ex vivo* model for the perfusion of segments of human saphenous veins under arterial shear stress. In veins submitted to pulsatile high pressure (mean pressure at 100 mmHg) for 3 or 7 days, the use of an external macroporous polyester mesh 1) prevented the dilatation of the vessel, 2) decreased the development of IH, 3) reduced the apoptosis of smooth muscle cells, and the subsequent fibrosis of the media layer, 4) prevented the remodelling of extracellular matrix through the up-regulation of matrix metalloproteinases (MMP-2, MMP-9) and plasminogen activator type I. The data show that, in an experimental *ex vivo* setting, an external scaffold decreases IH and maintains the integrity of veins exposed to arterial pressure, via increase in shear stress and decrease wall tension, that likely contribute to trigger selective molecular and cellular changes.

1. Introduction

About one million vascular reconstructions are performed annually worldwide, and the great saphenous vein remains the most widely used conduit [1]. Still, 30–50% of the saphenous grafts fail 1–18 months after the implantation [2]. The main reason for this failure is intimal hyperplasia (IH), which reflects the adaptation of a vein to the injuries caused by an arterial environment [3]. Insertion into the arterial circulation is associated with proliferation and migration of smooth muscle cells (SMCs) into the intima layer [4,5], leading to wall thickening, vessel stenosis and, eventually, graft failure. Among the proteins involved in the development of IH, selected matrix metalloproteinases (MMP-2 and MMP-9) [6], tissue inhibitors of these enzymes (TIMP) [7], and possibly the plasminogen activator inhibitor-1 (PAI-1) [8], play a major role, due to their ability to selectively degrade components of the extracellular matrix, to release SMCs and to promote SMCs migration [3]. Mechanical forces, and particularly low shear stress and high wall tension, contribute to the development of these changes [9]. Following implantation, the vein graft is immediately subjected to arterial pressure as well as to circumferential, radial and pulsatile deformations [10]. Further haemodynamic disturbances occur at the site of anastomoses, because of compliance mismatches [11–13].

Mammalian studies suggest the utility of an external support to prevent the over-distension of grafts, increasing shear stress and reducing wall tension [14–19]. However, the molecular and cellular events affected by such a reinforcement have not yet been investigated under rigorous experimental conditions. We hypothesised that a mesh reinforcement could preserve the architecture of veins exposed to arterial haemodynamic conditions and limit the development of IH through selective molecular modulations. To compare the behaviour of human saphenous veins in the absence and presence of an external macroporous

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polyester tubular mesh, we have developed an *ex vivo* vein perfusion system (EVPS), which allows for the independent control of the major haemodynamic forces affecting a venous graft, thus permitting to differentiate their selective effect on specific molecular and cellular parameters [8,20].

2. Material and methods

2.1. Human saphenous veins

Twenty seven surplus segments of non varicose human saphenous veins, hereafter referred to as veins, were obtained from of 27 randomly selected patients (15 men and 12 women) with a median age of 72 years (interquartile range 51–82), who underwent lower limb bypass surgery for critical ischaemia. A 9 cm long segment of the greater saphenous vein, considered to be suitable for bypass surgery, with an external diameter of 2.5–4 mm, was harvested and stored at 4 °C in a RPMI-1640 Glutamax medium, supplemented with 12.5% fetal calf serum (Gibco). All sampled veins were used, whichever the native thickness of their intima layer. Within 1 h after the surgery, the segment was divided in 3 equal parts. One part was fixed in either formalin for immunohistochemistry or rapidly frozen in liquid nitrogen for molecular analyses. A second part was reinforced with an external macroporous polyester tubular mesh (ProVena, B.Braun Medical SA), hereafter referred to as mesh, which covered the entire vein segment (Fig. 1), and whose diameter (4 mm) was chosen to obtain a cross-sectional area quotient Qc > 0.45, where $Qc = a_h/a_g$ (a_h being the cross-sectional area of the host vessel, and a_g that of the interposition graft) (Fig. 1B and C). This part and the third segment of the very same vein, which had not been reinforced by a mesh (control), were perfused in parallel in the EVPS (Fig. 1A and B) for 3 or 7 days, at either low or high pressure. The Ethical Committee of the University of Lausanne approved the experiments, which are in accordance with the principles outlined in the Declaration of Helsinki of 1975, as revised in 1983 for the use of human tissues.

2.2. Ex vivo perfusion system

The EVPS [8,20–22] was modified for the simultaneous perfusion of two vein segments, one of which was reinforced with the mesh (Fig. 1). The 2 segments were connected to the perfusion pump by a peroxide-treated silicone tubing (internal diameter 3.2 mm; Ismatec®, Switzerland), and maintained at 37 ± 0.1 °C in two distinct perfusion chambers, which were placed inside a cell culture incubator (Fig. 1A). In all the experiments, the medium of both the chambers (250 ml each) and the perfusate (150 ml) was RPMI-1640, supplemented with Glutamax, 12.5% fetal calf serum (Gibco), 8% 70 kDa dextran (Sigma), and 1% antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin sulphate, 25 mg/ml amphotericin B, and 0.5 μg/ml gentamycin). This medium was changed every 2 days. The conditions of the perfusion were set to obtain a shear stress (SS) of 9–15 dyn/cm^2, as expected in the femoral artery [8], given by $SS = 4\mu Q/\pi r^3$, where μ is the viscosity of the perfusion medium set to $3.73 \cdot 10^{-2}$ dyn s/cm^2, as measured in a Coulter viscometer (Coulter Electronics, High Wycombe, UK), Q the flow rate (mL/s), and r the radius (cm) of the vein segment.

Veins were exposed for 3 or 7 days to a pulsatile biphasic flow of 60 pulses/min under either low (MP = 7 mmHg; systolic/diastolic pressure = 8 ± 1/6 ± 1 mmHg) or high perfusion pressure (MP = 100 mmHg; systolic/diastolic pressure = 120 ± 5/90 ± 5 mmHg). Upon completion of the perfusion, the 5 mm proximal and distal ends, which attached the vein to the equipment, were discarded. A central, 5 mm-thick ring was cut from the remaining segment and fixed in formalin for morphometry. The remaining fragments were frozen and reduced into powder for RT-PCR and Western blot analysis.

2.3. Morphometry

Morphometric analysis was performed on vein sections processed for Van Gieson-elastin (VGEL) staining, using the Leica Qwin® software (Leica, Switzerland). Twenty-four measurements of the thickness of the intima and media layers, as well as of the perimeter of the internal (IEL) and external elastic laminae (EEL) were made in each sample at a magnification of ×100 and ×2, respectively.

2.4. Image processing and cleaved-caspase-3 staining quantification

For the assessment of caspase-3 cleavage, images of human veins were converted using the Fiji software (http://fiji.sc/Fiji), to provide a binary image with a consistent threshold method. After application of the "open" binary operation, particles with an area >50 pixels were summed, and related to the area of the region of interest (one region per vein). At least 5 veins from different patients were analysed in the 5 experimental groups which we compared.

2.5. Quantitative real-time PCR

Quantitative real-time PCR analysis was, performed on vein RNA as previously described [23,24], using the primers given in Table 1. All experiments included negative controls (amplification of distilled water or RNA samples that had not been reversed transcribed). Levels of expression were determined relative to those of GAPDH.

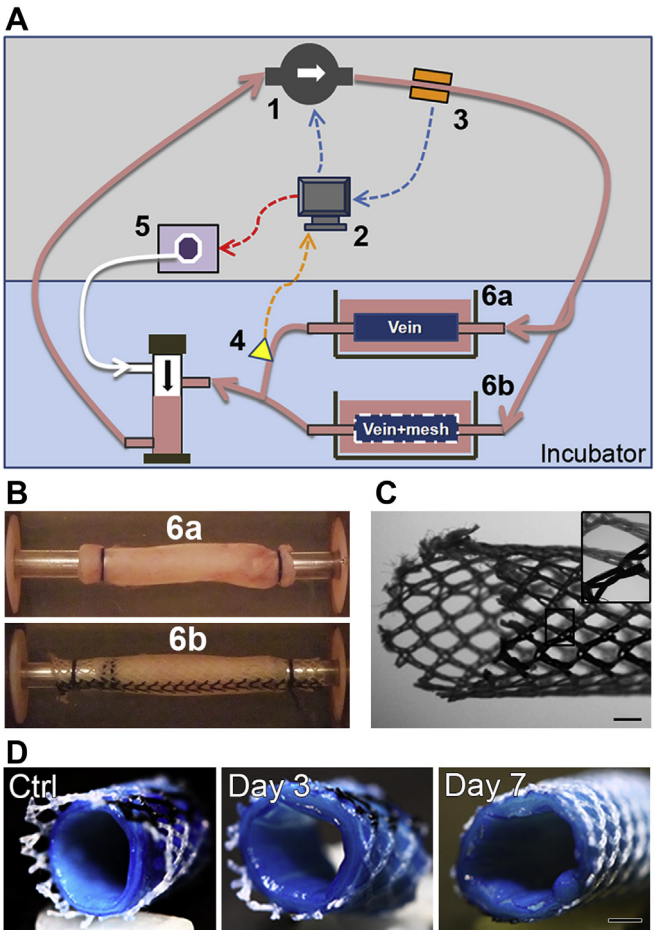

Fig. 1. The *ex vivo* perfusion system, A: A gearing pump (1) generates a pulsatile signal controlled by a computer (2), which monitors the pressure (3) and flow velocity (4), and controls the minimal diastolic pressure (5). B: Two segments of a very same saphenous vein were connected in parallel to the perfusion pump inside separate perfusion chambers (6a and 6b in A), which were placed in a cell culture incubator. C: View of the tubular mesh of 4 mm diameter, made of a polyester wire defining pores of 750 μm, and which was tested as external reinforcement. The insets show enlargements of the boxed area, that highlight the honeycomb structure. Bar, 500 μm. D: Views of a same vein (stained with 2% Evan's blue) inside the mesh reinforcement, immediately before the perfusion (Ctrl) and after 3 and 7 days of perfusion at a mean pressure of 100 mmHg. Bar, 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Western blots

Veins were reduced to powder and processed for Western blotting as previously reported [23,24] using the following primary antibodies: rabbit polyclonal against MMP2 (Abcam); rabbit polyclonal against MMP-9 (Abcam); mouse monoclonal against TGFβ$_2$ (Abcam); rabbit polyclonal against PAI-1 (Novus Biologicals); mouse monoclonal against eNOS (BD Biosciences); mouse monoclonal against HO-1 (Abcam); goat polyclonal against TIMP-1 (R&D systems), at dilutions 1:400–1000. To evaluate total protein levels, membranes were probed with a mouse monoclonal against α-Tubulin (Sigma), diluted 1:1000.

2.7. Immunostaining

Paraffin sections of veins were immunostained using the primary antibodies mentioned above and an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit, USA), and counterstained with hemalun.

Frozen sections of unfixed veins were immunostained as previously described [20,21], using the same antibodies at a dilution of 1:100–1:3000, a relevant secondary Alexa Fluor 488-labelled antibody (Dako), diluted 1:500 and counterstained with 0.02% Evans Blue.