The development of an in vitro flow model of human saphenous vein graft intimal hyperplasia

Karen E. Porter a,*, Stefan Nydahl a, Paul Dunlop a, Kevin Varty a, Abigail J. Thrush b, Nicholas J.M. London a

a Department of Surgery, University of Leicester, Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, UK
b Department of Medical Physics, University of Leicester, Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, UK

Received 25 April 1995; accepted 9 November 1995

Abstract

Objective: Although the role of blood flow has been investigated in animal models of intimal hyperplasia, there have been no detailed studies in intact human vein owing to the difficulties in designing a suitable laboratory model. The aim of this study was to develop a flow model of human vein graft intimal hyperplasia. Methods: Organ cultures of human saphenous vein were exposed to laminar flow by culturing in a closed circulatory system under predetermined conditions of venous and arterial shear stress for 14 days. Following fixation and processing, paraffin sections were immunostained and neointimal thicknesses measured. Results: It was found that arterial flow completely inhibited neointima formation, but venous flow only partly suppressed the response when compared with vein cultured under static conditions. These results are in agreement with previous in vivo studies in a primate graft model, where increased shear stress inhibited intimal proliferation. Conclusion: The endothelial cell is believed to be the key mediator of haemodynamic effects which influence smooth muscle cell proliferation, and the flow rig developed in this study offers the potential to study inter-cellular interactions within the intact vessel. Furthermore, this method provides the facility to study the effects of different flow conditions on segments of vein from the same patient. This model has scope for further development and sophistication which may ultimately lead to increasing our understanding of the aetiology of vein graft stenoses, and hence formulation of preventative strategies.

Keywords: Smooth muscle cell proliferation; Shear stress; Hyperplasia; Human, saphenous vein

1. Introduction

Although over the last decade our understanding of the molecular and cellular events associated with vein graft stenosis has increased dramatically, there is still no effective preventative therapy. The vasculature is continuously subjected to blood flow and the accompanying mechanical forces and it is now apparent that such forces play an important role in determining the biological functions and, more specifically, pathological changes within blood vessels.

In this regard, there is now a wealth of evidence to support the theory that blood flow, and more specifically the resulting shear stress acting on the endothelium, plays a central role in determining vascular structure [1,2] by a process dependent on an intact endothelium [2]. Blood flow is associated with shear stress, the tractive force acting in the direction of flow on the surface of the inner wall of the blood vessel at the endothelial cell. Wall shear stress \( \tau \) is proportional to flow velocity \( Q \) and medium viscosity \( \mu \), and inversely proportional to the third power of the internal radius \( R \) of the vessel according to the Hagen-Poiseuille formula:

\[ \tau = \frac{4\mu Q}{\pi R^3} \]

There is considerable experimental evidence that intimal hyperplasia in vein grafts is also influenced by haemodynamic factors. In 1989 Dobrin and colleagues [5] demonstrated an inverse correlation between graft flow and intimal thickness in canine vein grafts, and subsequent work has confirmed that low shear stress associated with
low flow is responsible for intimal hyperplasia (IH). Furthermore, it has been shown by Morinaga and colleagues [6] that the endothelium plays a central role in the detection of shear stress and its subsequent transformation into vessel wall responses. Intimal hyperplasia is characterised by the abnormal proliferation of smooth muscle cells (SMC) and deposition of extracellular matrix, the stimuli for which are still not fully defined. Under conditions of shear stress, endothelial cells become elongated and aligned in the direction of flow [7,8]. These morphological changes seem to be caused by reorganisation of actin microfilaments into stress fibres which play a role in endothelial cell adhesion and protection against damage imposed by shear stress [9,10]. In addition to changes in cellular shape, an increase in flow induces several rapid responses in endothelial cells including potassium channel activation [11], intracellular calcium increase [12] and cytosolic acidification [13]. The rapid responses are followed by increases in the production of various vasoactive substances such as EDRF [14], prostacyclin [15], tissue plasminogen activator [16], platelet-derived growth factor (PDGF) [17] and a decrease in endothelin production [18,19]. These substances may therefore all contribute to the shear-stress-induced changes in the vascular wall.

It has become increasingly clear that animal IH is very different from IH in humans [20,21], and that experiments on arteries may not be relevant to veins [22-24]. Organ culture of the saphenous vein to model human vein graft IH was described in 1990 by Soyombo and colleagues [25] and Angelini and colleagues in 1991 [26]. Using this in vitro model in our laboratory we have shown that the endothelium produces a paracrine mediator of SMC proliferation [27]. The major disadvantage of organ culture is that it represents the response of a vein segment to culture in the absence of flow. In view of the findings of other workers described above, it is possible that the responses observed in static culture might be exaggerated in a no-flow situation. This study therefore describes the development of an experimental model (hereafter referred to as the "flow rig") to simulate flow in arterial bypass grafts, in an attempt to mimic the in vivo situation in man.

2. Methods

An important consideration in the design of a flow model was to develop a method which was capable of examining the effect of differing flow conditions on multiple segments of vein from any one patient, and that valid comparisons could be made with vein cultured under static conditions. This was essential because the macroscopically normal saphenous vein frequently shows some degree of pre-existing intimal thickening [28] and we have previously shown that the response of a vein to culture is variable between individuals, hence emphasizing the need for paired analysis [27,29,30].

2.1. Flow rig development

The method was evolved using modified glass conical flasks as reservoirs containing 500 ml of complete culture medium (RPMI 1640, Northumbria Biologicals Ltd, Cramlington, UK; 30% v/v Foetal Calf Serum, Seralab, Crawley Down, Sussex, UK; penicillin 50 U/ml; streptomycin 50 μg/ml; L-glutamine 2 mmol/l, all Northumbria Biologicals), such that medium outflow and return could be effected in a closed circulatory loop by the use of silicone...
rubber tubing of 1 cm diameter. The viscosity of the medium was determined as 0.0098 N·m⁻²·s⁻¹, using a Coulter viscometer II (Coulter Electronics, Luton, Beds., UK). A narrow inlet tube was linked to a cylinder of 5% carbon dioxide which was bubbled gently through the medium at a rate which maintained the pH at 7.4 (+0.05) throughout all the studies performed.

The flow rig was designed to provide two ‘channels’: one at venous flow, pressure and shear stress (70 ml/min; 15 mmHg; 1 dyn/cm²), and the other at arterial flow, pressure and shear stress (500 ml/min; 85 mmHg; 9 dyn/cm²), by adjusting the flow rate of the medium and the height of the reservoir. These shear stresses are those commonly thought to be representative of the required conditions [31-33]. Flow rates were measured by collection of the medium over timed intervals. Circulation of culture medium in each circuit was provided by a Watson-Marlow 505-S pump (Watson-Marlow Ltd, Falmouth, Cornwall, UK) and the temperature of the circulating medium was maintained by placing the vein tubing in an incubator (Pickstone Equipment Ltd, Thetstone, Norfolk, UK) at 40°C and wrapping the reservoirs and exposed tubing in a thick foam insulating material. Using this method, it was possible to maintain the temperature of the flowing medium between 35°C and 38°C. The medium temperature was monitored by the use of an Ellab CTD 87 digital thermometer (Ellab Instruments, Roedovre, Denmark) with the probe inserted into the reservoir of culture medium.

In order to confirm that the flow circuits were delivering laminar flow, a preliminary observational study was performed. The reservoirs were filled with water, flow was established in both circuits and any air bubbles eliminated. A quantity of trypan blue dye was injected into each circuit, and as it travelled along the tubing, a parabolic profile characteristic of laminar flow was observed [34] and maintained along each circuit (Fig. 1).

2.2. Experimental design

Segments of the human long saphenous vein were obtained from 5 patients undergoing arterial bypass grafting, assessed for endothelial coverage and prepared for culture as previously described [27]. For each experiment, six 0.5 cm segments were prepared; two segments were established in static culture and four prepared for flow; two at arterial and two at venous flow. A 90 cm length of sterile silicone tubing was used to accommodate each pair of vein segments. Briefly, vein segments were cleaned of excess fat and adventitia, opened up longitudinally and pinned out, luminal surface uppermost, to approximately their in situ length onto the wall of the tubing using fine Al minuten pins (Watkins and Doncaster, Cranbrook, Kent). This was achieved by making a longitudinal slit in the tube of approximately 10 cm in order to gain access to the inner surface of the tubing. The veins were fully immersed in culture medium whilst the slit was resealed using a proprietary silicone sealant (approx. 45 min).

Fig. 2. A diagrammatic representation of the flow rig, showing arterial and venous channels.
Meanwhile the reservoirs were filled with medium, mounted at appropriate heights on a clamp stand and the tubing connected. The tubes containing the pinned vein segments were positioned in the circuit using 1 cm connectors, flow was established and any air was expelled by gentle manipulation of the tubing. The system was allowed to equilibrate under slow flow for 12 h to identify and rectify any leakage, followed by the application of venous and arterial conditions for a further 13 days. A diagrammatic representation of the flow rig is shown in Fig. 2, and the complete functioning assembly is shown in Figs. 3 and 4.
At the end of the culture period the medium was drained from the tubing and veins were immediately removed from the flow circuits with a scalpel blade such that they remained pinned into a portion of the tube. One segment from each of the experimental conditions was fixed by immersion in 10% formalin overnight, and the remaining segment was used to assess contractile responses to vasoactive substances in an organ bath as a measure of viability [35]. The formalin-fixed segments were processed, paraffin-embedded and sections of 4 μm thickness were cut. Immunostaining was performed with a combined monoclonal anti-smooth-muscle α-actin antibody and Millers elastin stain (SMA/Millers) [27] and endothelial integrity was assessed using the monoclonal antibody CD 31 [36] staining on 3 consecutive sections of each vein segment for every experiment. Measurements of neointimal thickness were made on each vein section using a computerised image analysis system as described previously [27]. Results are expressed as median and range, differences between groups were analysed using a Wilcoxon paired rank test with 95% confidence intervals.

2.3. Control of infection

It was necessary to terminate several of the early attempts to establish the flow model due to bacterial infections, which, upon microbiological investigation, were all found to be due to gram-negative organisms. At this stage it was considered necessary to include an appropriate antibiotic in the culture medium, therefore gentamicin was investigated. Gentamicin is known to have toxic effects in some preparations—e.g., keratinocytes [37] and fibroblasts [38]; therefore an initial study was performed to investigate the effects of gentamicin on neointimal development in cultured saphenous vein.

Saphenous vein was obtained from 5 patients, each divided into 3 segments and one segment cultured in each of the following: culture medium only, culture medium containing 50 μg/ml gentamicin sulphate (Roussel Ltd, Dublin, Ireland), culture medium containing 5 μg/ml gentamicin sulphate. After 14 days, veins were fixed overnight in 10% formalin, processed and 4 μm sections stained with SMA/Millers to facilitate measurements of neointimal thickness. The results are expressed as median and range, differences between groups were assessed using a Wilcoxon paired rank test with 95% confidence intervals (Fig. 5).

As a result of this analysis, gentamicin was routinely added to the culture media in all subsequent flow experiments at 5 μg/ml, a concentration found to control infection without toxic effects on neointimal development.

3. Results

3.1. Light microscopic appearance

Histological examination of vein segments cultured conventionally under static conditions revealed the development of a cellular neointima beneath the endothelium. The venous veins all showed reduced neointimal thickening, with a total absence of neointima in the arterial veins. Histological sections of veins cultured under the various conditions are represented in Fig. 6. A point of interest to note is that the endothelium-lined channels observed by ourselves [27] and others [25,26] were not a feature of veins cultured under flow conditions. Positively stained endothelium was observed as a flattened monolayer of cells covering 70–80% of the luminal surface in all cases.

3.2. Neointimal thickness

The median neointimal thickness of the static veins was significantly greater than that of venous veins: 20 μm (range 12–33) versus 8 μm (range 0–11), median difference = 15 μm, 95% confidence interval = 4.0, 24.0. The median neointimal thickness of the arterial segments was 0 μm (range 0–0), significantly less than both venous and arterial segments. Median difference static versus arterial = 22.5 μm, 95% confidence interval = 12.0, 33.0; median difference venous versus arterial = 8 μm, 95% confidence interval = 0.0, 11.0 (Fig. 7).

4. Discussion

This study uses an experimental model of venous neointimal formation which simulates many of the changes...
occurring in lower limb vein bypass grafts, that is, intimal smooth muscle cell proliferation and phenotypic change accompanied by production of extracellular matrix [39]. The absence of endothelium-lined channels in the veins subjected to flow conditions suggests that in the absence of flow across the endothelium in the static conditions, the cells form channels which may be purely an artefact of the no-flow conditions. One of the benefits of this model is that the in vitro changes occur over a 14-day period compared with similar qualitative changes in the in vivo vein graft over several months [39]. The median neointimal thickness achieved in the cultured veins in this study was 20 μm after 14 days, but it is inappropriate to compare this figure with graft neointimal thicknesses measured at the time of revision, a number of months post-grafting.

This method of static culture has been further developed as a novel in vitro flow model of vein graft IH which is capable of examining the effect of different flow conditions on segments of vein from the same patient. This represents an important advantage of our experimental design since the absence of shear stress in the static situation may result in altered responses in the vein wall. It was observed that physiological levels of shear stress representing arterial magnitude (∼9 dyn/cm²) totally abolished intimal proliferation, but a physiological shear stress of venous magnitude (∼1 dyn/cm²) only partially suppressed the response when compared with vein cultured under static conditions. These levels are commonly accepted shear values [31] which have been used in other in vitro studies on human vascular cells [32,33]. The observations made in this study are comparable with those of Kohler and colleagues [30], who demonstrated that in a baboon PTFE graft model, an increase in flow reduced neointimal thickening, whereas a reduction in flow led to increased proliferation. The observation that increased blood flow results in a decreased neointimal thickness suggests a mechanism for control of SMC growth in response to changes in shear. Later studies by Geary (1993) [41], which showed that the endothelium remains present and confluent during manipulation of flow conditions, and that SMC's prolifer-
ate in the layer immediately below the endothelium, support the hypothesis that the endothelium senses the changes in shear and responds by releasing factors that stimulate or inhibit SMC proliferation. Indeed, the endothelial cell is now believed to be the key mediator of haemodynamic effects and, as such, clues as to the prevention of vein graft IH may reside in acquiring a greater understanding of how altered haemodynamics perceived by the endothelium may influence intimal SMC proliferation.

The direct contact of the endothelium with the blood means that it is uniquely placed to serve as a mediator of haemodynamic stresses. It is, however, only one of the participants in the process of intimal proliferation. During the last decade a number of laboratories have used in vitro techniques to investigate the effect of flow on the biology of endothelial cells [42,43] and SMC's [33] in isolated cultures. Although the role of flow in the development of IH has been investigated in vitro and in vivo in animal models, there have not been any detailed studies using intact human vein because of the difficulties in designing a suitable and reliable flow rig. Our findings that IH does not develop in the arterial veins in the flow rig model is not surprising in the light of current literature showing that PDGF [17,44] and endothelin [40,42,44,45] gene expression is decreased by high shear and increased by low shear conditions.

Although these results represent only 3 preliminary experiments, they demonstrate that in this model, laminar arterial flow prevents the development of IH, whereas venous flow only partly suppresses the response. Because the neointima of cultured veins is a very friable structure, it is possible that the high flow conditions may have washed it off. This was, however, not the case because it was shown that the endothelium was present at the end of the flow culture periods, despite the absence of neointima in the arterial veins. By necessity, we used a combination of high flow/low viscosity to achieve the stated shear stresses compared with the human in vivo situation where a lower flow/higher viscosity combination exists. It cannot be dismissed that the high flow on the experimental vein segments may have been more effective in preventing neointimal formation than a lower flow coupled with higher viscosity which occurs in vivo, despite achieving similar shear stresses. This question could potentially be addressed in future experiments by increasing the viscosity of the culture medium by the addition of high molecular weight dextran [46], thereby allowing a reduction of the flow rates in the experimental system without affecting shear stresses.

The in vitro flow model developed in this study is unique in such a way that it allows segments of vein from any one patient to be cultured under a range of flow conditions. Consequently, it offers future potential as an in vitro system which retains the cellular architecture of the vein wall and hence the ability to study cellular interactions. It is further envisaged that the effects of injury and turbulent flow conditions may be studied in this system, and together with in-situ hybridisation techniques, the mediators of the effects of shear stress identified at a molecular level.

Acknowledgements

The authors gratefully acknowledge the financial support of The British Heart Foundation.

References


