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Development of an *in vitro* Model to Study the Response of Saphenous Vein Endothelium to Pulsatile Arterial Flow and Circumferential Deformation

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Objectives: *To develop an* in vitro *model of human saphenous vein bypass to facilitate study of the early adaptive responses of venous endothelium to arterial flow conditions.*

Design, material and methods: *Segments of human saphenous vein (with or without external polytetrafluoroethylene (PTFE) stents to limit circumferential and radial deformation) were mounted in a bypass circuit and subjected to pulsatile flow with oxygenated Krebs solution to simulate arterial or venous flow conditions for a period of 90 min. The viability of the vein was assessed by the tissue ATP concentration and vasomotor responses to phenylephrine, sodium nitroprusside and bradykinin (endothelium-dependent). Immunohistochemistry was used to assess both endothelial preservation (CD31) and the expression of proteins involved in leukocyte adhesion: E-selectin, P-selectin and ICAM-1. Freshly excised veins were used as controls.*

Results: *The concentration of ATP was* 320 ± 11 *nmol/g in freshly excised vein* ($n=8$) *and following exposure to the arterial flow circuit increased to* 566 ± 60 *nmol/g* ($n=8$, *paired t-test*, $p=0.003$) *in unstented veins and to* 421 ± 49 *nmol/g* ($n=8$, *paired t-test*, $p=0.002$) *in externally stented veins (with PTFE). Both endothelium-dependent and sodium nitroprusside-induced vasodilatation responses were preserved after veins were exposed to the arterial flow circuit, but the sensitivity to phenylephrine was increased:* EC_{50} *decreasing from* $9\mu M$ *to* $1\mu M$, $p=0.008$. *There was a 5–10% decrease in staining area for CD31 after veins, stented or unstented, were exposed to the arterial flow circuit. However, after exposure to the arterial flow circuit, the staining area ratio for ICAM-1/CD31, which remained unchanged in externally stented veins, increased two-fold in unstented veins,* $p>0.01$: *there were no changes in the staining area ratio P-selectin/CD31 and no staining for E-selectin was observed.*

Conclusion: *Vasomotor responses and tissue ATP concentrations indicate that the viability of saphenous vein can be maintained for up to 90 min in an* ex vivo *flow circuit and the CD31 staining indicated endothelial preservation. This opens up the possibility of investigating the early changes in saphenous vein endothelium following exposure to arterial pressure, as at bypass surgery. First results suggest that there is rapid upregulation of the leukocyte adhesion molecule ICAM-1, which can be prevented by limiting the circumferential deformation of the vein with an external PTFE stent.*

Key Words: Saphenous vein; Haemodynamics; ICAM-1; PTFE; Circumferential stress.

Introduction

Rapid alterations in blood flow are a consequence of revascularisation procedures such as angioplasty and bypass surgery. Some of the most extreme haemodynamic changes are imposed on saphenous vein, when this vein is used as an arterial bypass conduit for either cardiac or lower limb revascularisation. Recently there has been considerable focus on how shear stress and stretch influence the metabolism of vascular cells through the activation of stretch-activated ion channels or shear stress response elements in 5′ regulatory regions of specific genes.[1,2] The techniques for imposing shear stress and stretch on monolayers of vascular cells (endothelial and smooth muscle) are well

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developed and have revealed the wide range of endothelial genes which are regulated by shear stress. Examples of shear stress responsive endothelial genes include PDGF, the chemoattractant MCP-1, ICAM-1 and other proteins involved in regulating leukocyte adhesion and proteins controlling coagulation at the vessel wall, e.g. thrombomodulin and tissue plasminogen activator.[3-6] The expression of all these proteins increases within 4–6 h of exposing cultured endothelial cells to shear stress.

The responses in the vessel wall also are governed by complex cell–cell and cell–matrix interactions which are not present in cultured cell monolayers. Recognition that responses may be different in the intact vessel has stimulated the development of techniques to study the response of excised vessels to variable flow and shear conditions. During the time period in which we were developing a technique to study the "arterialisation" of excised saphenous vein, there have been at least three other groups who have published techniques for imposing variable flow conditions on excised vessels.[7-9] Schwartz *et al.* designed a special plexiglass chamber in which saphenous vein was mounted, internally perfused with tissue culture medium and externally perfused with oxygenated tissue culture medium for up to 48 h: endothelium-dependent and endothelium-independent relaxation responses were unchanged after this period of time, although there was increased sensitivity of veins to norepinephrine.[7] Labadie *et al.* used computer-controlled opening and closing of an in-line gate valve to generate arterial pressure waveforms in excised vessels perfused with oxygenated tissue culture medium.[8] The system was developed using canine carotid artery and preservation of vasomotor responses, and endothelial cell morphology was demonstrated after perfusion periods of up to 48 h. These same authors also described the preservation of cobblestone morphology in saphenous vein endothelium after 24 h of perfusion.[8] Porter *et al.* developed a flow rig where segments of saphenous vein were pinned out in silicone rubber tubing, which was perfused by serum containing oxygenated tissue culture medium at either arterial pressure and shear stress or venous pressure and shear stress for up to 14 days.[9] In this study[9] the only outcome measure was vein evaluation by light microscopy.

Early changes observed after the implantation of a saphenous vein graft include the deposition of platelets, leukocytes and clotting factors on the damaged endothelium.[10] These processes, which may be potentiated by the adaptive responses of the damaged endothelium to the arterial circulation, influence the maturation and patency of vein bypass grafts. The adhesion and migration of leukocytes into vessels after revascularisation, and subsequent elaboration of inflammatory mediators, contributes to the pathogenesis of intimal thickening, which underlies vein graft stenosis.[11] The recruitment of leukocytes to the vessel wall is a multi-step process.[12] Firstly, the local concentration of leukocytes is increased in response to the secretion of chemoattractants, including MCP-1, which is upregulated by cyclic strain and shear stress.[4,12] Secondly, leukocytes roll along the endothelium encouraged by interactions between leukocyte membrane proteins and the selectins on endothelium[12]: P-selectin is constitutively expressed, whereas E-selectin expression is induced by inflammatory mediators. Thirdly, the firmer leukocyte tethering and adhesion, which precedes emigration into the vessel wall, results from interaction between endothelial cell membrane proteins, particularly ICAM-1 and epitopes on the leukocyte membrane:[12] many of the endothelial cell adhesins, including ICAM-1, are upregulated in response to both shear stress and inflammatory mediators.[5] Therefore, in the newly implanted vein graft altered haemodynamics could potentially modulate the adhesive properties of the intimal surface.

Here we report a simple, robust technique for exposing excised saphenous veins to variable flow conditions using a bypass pump, with validation of vein viability and early results concerning the rapidity with which endothelial expression of proteins involved in leukocyte adhesion are regulated by circumferential deformation rather than shear stress.

Experimental Procedures

Patients

Saphenous vein was harvested from patients undergoing aortocoronary or infrainguinal bypass, amputation or high ligation of saphenous vein for correction of varicose veins, and immediately brought to the laboratory in ice cold, oxygenated Krebs solution.[13] Diseased vein, which did not respond to 10 μM phenylephrine with a contraction of >2 g, was discarded.[12] Smokers and patients with diabetes were excluded. These procedures were approved by the local ethical committee.

Materials

Modified Krebs' solution (NaCl 118.4 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, glucose

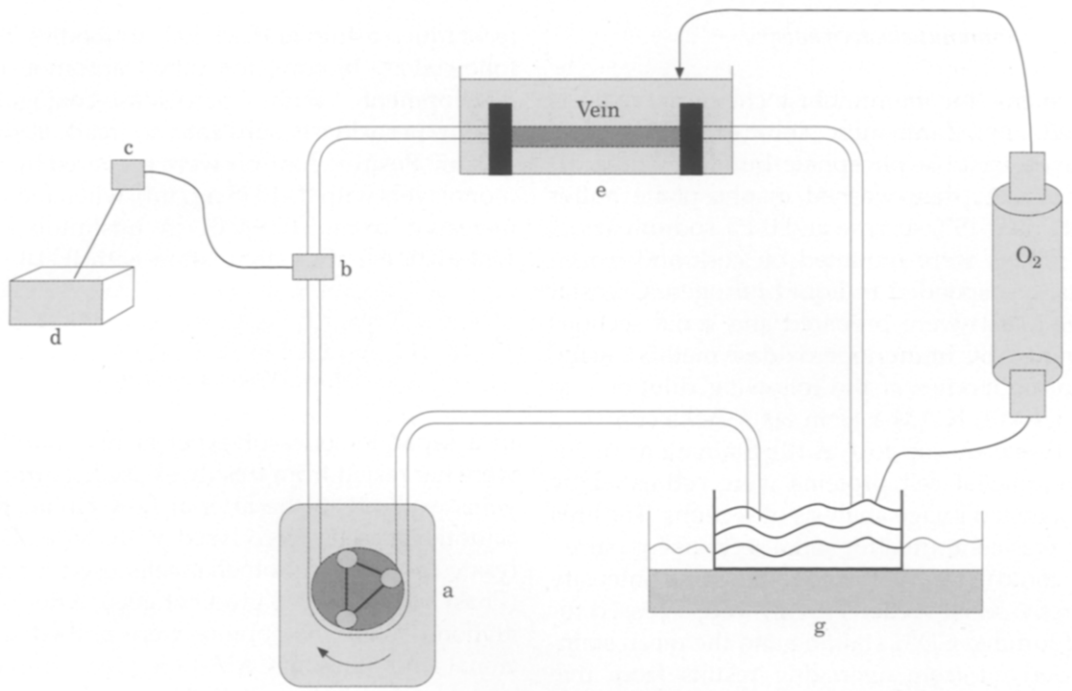

Fig. 1. Diagram of the flow circuit. The circuit consists of the following: (a) Stockert bypass pump, (b) three-way tap, (c) transducer, (d) pressure read-out, (e) vein (stented or unstented) in retaining jig in oxygenated Krebs' solution at 36.5°C, (f) 95% oxygen 5% carbon dioxide cylinder, (g) Krebs' reservoir oxygenated and maintained at 36.5°C.

11.1 mM, $NaHCO_3$ 24.9 mM, $CaCl_2$ 2.5 mM) was made freshly each day. Bradykinin, sodium nitroprusside, phenylephrine, biotinylated horse anti-mouse and sheep anti-rabbit antibodies, avidin and biotin reagents were obtained from Sigma. Monoclonal antibodies for immunohistochemistry were obtained as follows: CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1) and MCP-1 (monocyte chemotactic protein-1) from R&D Systems, U.K., ICAM-1, E-selectin and P-selectin from Serotec, Oxford, U.K.

The flow circuit

Saphenous vein (3–4 cm) was mounted in retaining jig (diameters 2–10 mm available), after removal of a control section of vein for histology, immunohistochemistry and organ bath studies. The jig was then placed in a bypass circuit consisting of a perfusion pump (Stockert), two water baths (one to warm the circulating Krebs' solution and one to warm the stationary solution surrounding the adventitia of the vein), the circuit apparatus and a pressure transducer (S & W Medico Teknik A/S), Fig. 1. Veins were perfused with oxygenated Krebs' solution at 36.5°C (oxygen content 20 ml/l). The veins were exposed either to pulsatile flow (80 cps) at a mean pressure of 100 mmHg (arterial flow) for 90 min (flow rate 200 ml/min) or to non-pulsatile flow at 20 mmHg (venous flow) for 90 min (flow rate 40 ml/min). Externally stented veins were placed inside a 2–4 cm length tube of externally supported polytetrafluorethylene (PTFE), which was non-restrictive, but sized to prevent circumferential distension of the vein during arterial flow. Vein wall motion was monitored by M-mode ultrasonography. At the end of the flow experiment the vein was removed, the ends discarded and the remainder divided for histology, immunohistochemistry and organ bath studies of vasomotor responses. During the circuit procedure, perfusate (10–15 ml) was removed at 15 min intervals, concentrated 10-fold through an Amicon filter with a 3000 molecular weight cut-off, and the concentrate stored at −20°C for analysis of cytokines and other mediators.

Organ chamber studies

Vein rings, for assessment of endothelium-dependent relaxation, were mounted in a 10 ml organ chamber suspended between two 0.2 mm steel wire stirrups, the upper one being attached to a transducer. Responses to phenylephrine, bradykinin and sodium nitroprusside were obtained as described previously.[13]

Immunohistochemistry

Vein specimens for immunohistochemical analysis were fixed in Zamboni's solution (2% paraformaldehyde in 0.1 M phosphate buffer, picric acid) overnight at 4°C, then washed in phosphate buffer saline containing 15% sucrose and 0.1% sodium azide. Fixed specimens were mounted on cork and frozen in isopentane suspended in liquid nitrogen. Cryostat sections (8–10 μM) were prepared and serial sections stained by the ABC immunoperoxidase method, using monoclonal antibodies at the following dilutions: to CD31 (1 in 1000), ICAM-1 (1 in 800), P-selectin (1 in 1000) and E-selectin (up to 1 in 10). Staining areas for specific endothelial cell proteins were estimated by computer-assisted image analysis of sections. The area of staining was computed for serial sections for paired flow and control samples using the same intensity setting. Firstly, serial sections (5–10) were assessed for the area of luminal CD31 staining and the mean staining area derived from averaging results from five sections/sample. Subsequently, further serial sections were assessed for ICAM-1 or selectin staining area, the mean staining area again being derived from averaging the result of five sections/sample. To allow for the small endothelial loss noted from CD31 immunostaining and the dilation that occurs in response to arterial flow, all other results were expressed as percentage of CD31 staining.

Tissue ATP concentration

Samples of vein were snap-frozen in a clamp cooled in liquid nitrogen. The tissue was weighed before being pulverised in liquid nitrogen and extracted into 0.9 M perchloric acid. ATP was quantified by a coupled enzyme assay, using at least six separate paired samples for each condition.[14]

Endothelial cell assay to detect upregulation of ICAM-1

Saphenous vein endothelial cells were isolated by digestion with 1% collagenase for 20 min and cultured in RPMI supplemented with 10% pooled human serum and endothelial cell growth factor supplement (Sigma). At second passage cells were seeded into 24 well plates, coated with fibronectin, and grown to confluence. Washed confluent monolayers were incubated under different conditions for up to 6 h, cells fixed and permeabilised with ice-cold methanol and the concentration of ICAM-1 determined by a modified ELISA technique, using monoclonal antibodies to ICAM-1 followed by biotinylated rabbit anti-mouse IgG and development with peroxidase-conjugated-streptavidin/peroxidase substrate to read absorbance at 492 nm. Positive controls were prepared by incubating monolayers with IL-1β (5 ng/ml), when the absorbance increased from 0.02 ± 0.01 in unstimulated cells to 1.40 ± 0.02 6 h after stimulation with IL-1β.

Western blotting

In a separate series of experiments endothelial cells were harvested from freshly excised control veins and veins exposed to the arterial flow circuit for 90 min. Endothelial cells were lysed with 2.5% SDS and the lysate from 500 endothelial cells used for SDS-PAGE (Phast system 8–25% gradient gels). After blotting the Hybond-PVDF membranes were probed with monoclonal antibodies to ICAM-1 (Serotec, 1 in 100 dilution) and processed for enhanced chemiluminescence detection.

Statistical analyses

Differences in the tissue ATP concentrations were compared by Student's paired *t*-test. Differences in vasomotor responses, reported as mean ± S.E.M., were compared by Student's paired *t*-test and one-way analysis of variance. Immunostaining areas (mean ± S.E.M.) were also compared using Student's paired *t*-test.

Results

Tissue ATP concentration

The tissue concentration of ATP was 320 ± 11 nmol/g weight in freshly excised vein ($n=8$) and increased following exposure to arterial flow for 90 min to 566 ± 60 nmol/g ($n=8$, paired *t*-test, $p=0.003$) in unstented veins and to 421 ± 49 nmol/g in externally stented veins ($n=8$, paired t-test, $p=0.002$).

Vasomotor responses

Veins exposed to arterial flow for 90 min were more sensitive than controls to phenylephrine, the EC_{50} decreased from 9 ± 1 μM to 1 ± 0.2 μM after 90 min of

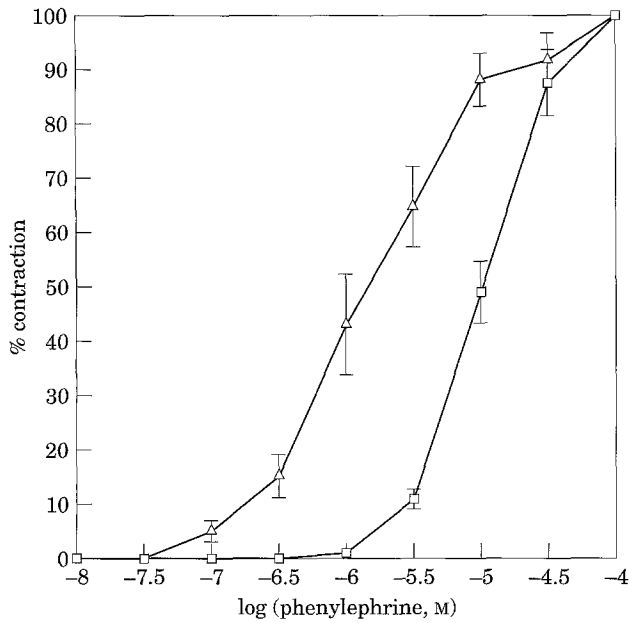

Fig. 2. Phenylephrine-induced contraction. The concentration responses of freshly excised vein (□) and vein exposed to arterial flow for 90 min (△) are shown. Points represent the mean of six paired samples and vertical bars are standard error of the mean.

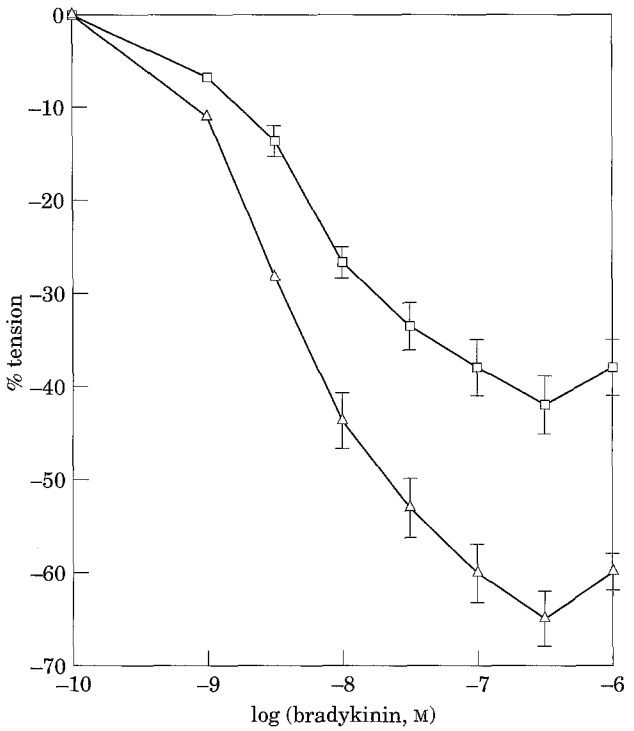

Fig. 3. Endothelium-dependent relaxation induced by bradykinin. The responses of freshly excised vein (□) and vein exposed to arterial flow for 90 min (△) to increasing concentrations of bradykinin (1–0.0001 μM) are shown. Points represent the mean of six paired samples and vertical bars are standard error of the mean.

arterial flow, $n=6$, $p=0.008$ (Fig. 2). After sub-maximal precontraction of vein rings with phenylephrine, sodium nitroprusside effected a concentration-dependent relaxation (endothelium-independent relaxation): the EC_{50} for sodium nitroprusside of veins exposed to either venous or arterial flow for 90 min was closely similar to the EC_{50} for control veins. Bradykinin was used to investigate the endothelium-dependent responsiveness of precontracted vein rings. Maximum endothelial-dependent relaxation, observed at 0.1 μM bradykinin, was $42.0 \pm 4\%$ in freshly excised veins ($n=6$) and $65.4 \pm 3.4\%$ in veins exposed to arterial flow for 90 min ($n=6$), $p = 0.009$ (Fig. 3).

Immunohistochemistry

CD31 was expressed abundantly on the endothelium (Fig. 4). Exposure of veins to arterial (but not venous) flow for 90 min resulted in some endothelial loss (5–10%), although the staining area for CD31 was not reduced significantly (Fig. 5). Immunostaining for P-selectin and ICAM-1 was estimated by assessing the ratio of specific protein staining area to the staining area for CD31 on serial sections and comparing all measurements for veins exposed to flow with paired controls. There was no change in the ratio of staining area of P-selectin/CD31 under any of the different flow conditions (arterial stented/unstented or venous). On freshly excised saphenous vein and vein exposed to a venous flow circuit there was intermittent staining on the endothelial surface for ICAM-1 (Fig. 6a). The staining pattern for ICAM-1 was similar after externally stented veins were exposed to arterial flow for 90 min. In contrast there was increased staining for ICAM-1 after unstented veins were exposed to arterial flow for 45 min (Fig. 6b). The staining ratios, ICAM-1/CD31, for the different conditions are shown in Fig. 6c. Arterial flow effects a two-fold increase in ICAM-1 staining ratio for unsupported veins, $p<0.01$, but no change in staining ratio for externally stented veins. No endothelial staining for E-selectin was observed, even at high concentrations of antibody, in freshly excised vein or vein exposed to arterial flow for up to 90 min. Qualitative support for the observation that there was a two-fold upregulation in ICAM-1 after exposure of unsupported, but not externally stented, vein to arterial flow was obtained from Western blotting: increased staining for ICAM-1 was observed only in endothelial cells prepared from unsupported vein exposed to arterial flow conditions for 90 min.

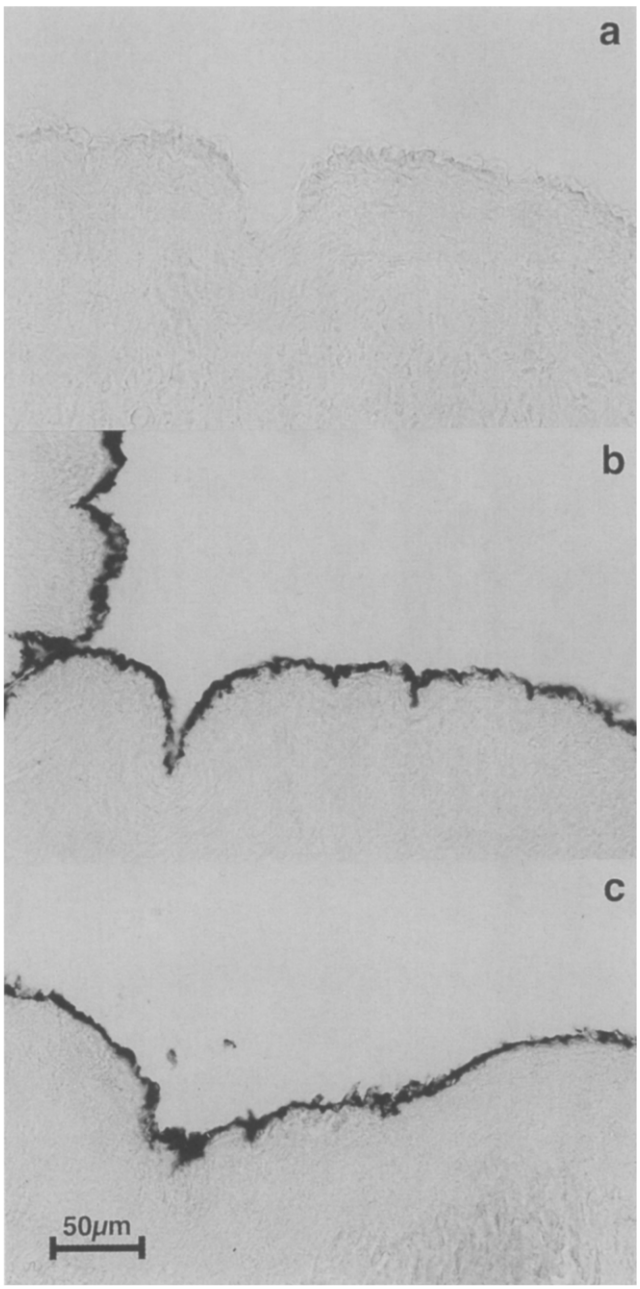

Fig. 4. Immunostaining for CD31. (a) Negative control with primary antibody omitted, (b) freshly excised vein and (c) vein exposed to arterial flow for 90 min.

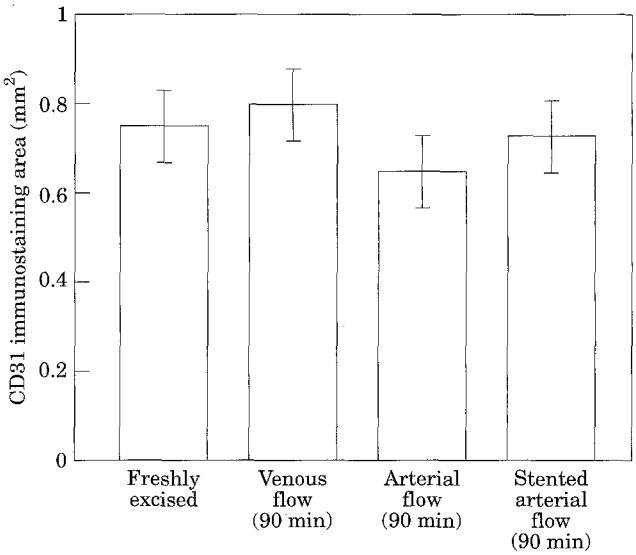

Fig. 5. Luminal staining areas (mm^2) for CD31. The staining areas for freshly excised vein, vein exposed to venous flow, and vein exposed to arterial flow for 90 min with and without external PTFE stents to limit circumferential (or radial) deformation are shown. Bars represent the mean of five sections examined from each of eight paired samples; vertical lines are standard error of the means.

Analysis of vein perfusate

To investigate the possibility that arterial flow increased the expression of a growth factor or cytokine which stimulated the rapid upregulation of ICAM-1, monolayers of cultured human saphenous vein endothelial cells were incubated with concentrated vein perfusate for up to 6 h. Interleukin-1β effected a 6–10 fold increase in ICAM-1 staining over this time period, whereas there was no increase in ICAM-1 staining after incubation with concentrated vein perfusate. Further, no significant MCP-1 was detected in the concentrated perfusate, using an ELISA (R&D Systems) with a detection limit of 30 pg/ml. Therefore, there is no evidence that the upregulation in ICAM-1 in veins exposed to arterial flow is mediated by a soluble factor.

Discussion

The use of saphenous vein as an arterial bypass conduit has been one of the most important developments in the history of cardiovascular surgery. The early success of vein bypass surgery may not be durable, the primary one-year patency of saphenous vein grafts in the infrainguinal circulation being ~70% and in the coronary circulation even lower.[15,16] The adaptation of the saphenous vein to the new haemodynamic environment is pivotal to the maturation of the vein graft, and the responses in the tissue may be different from those which can be studied in isolated cell monolayers. Therefore we have developed an *ex vivo* technique to study the early adaptive responses of saphenous vein endothelium: in one variant of the technique veins are supported by external stenting with non-restrictive PTFE grafts, which permits dissection of the differential effects of circumferential (or radial) stress and longitudinal fluid shear stress.

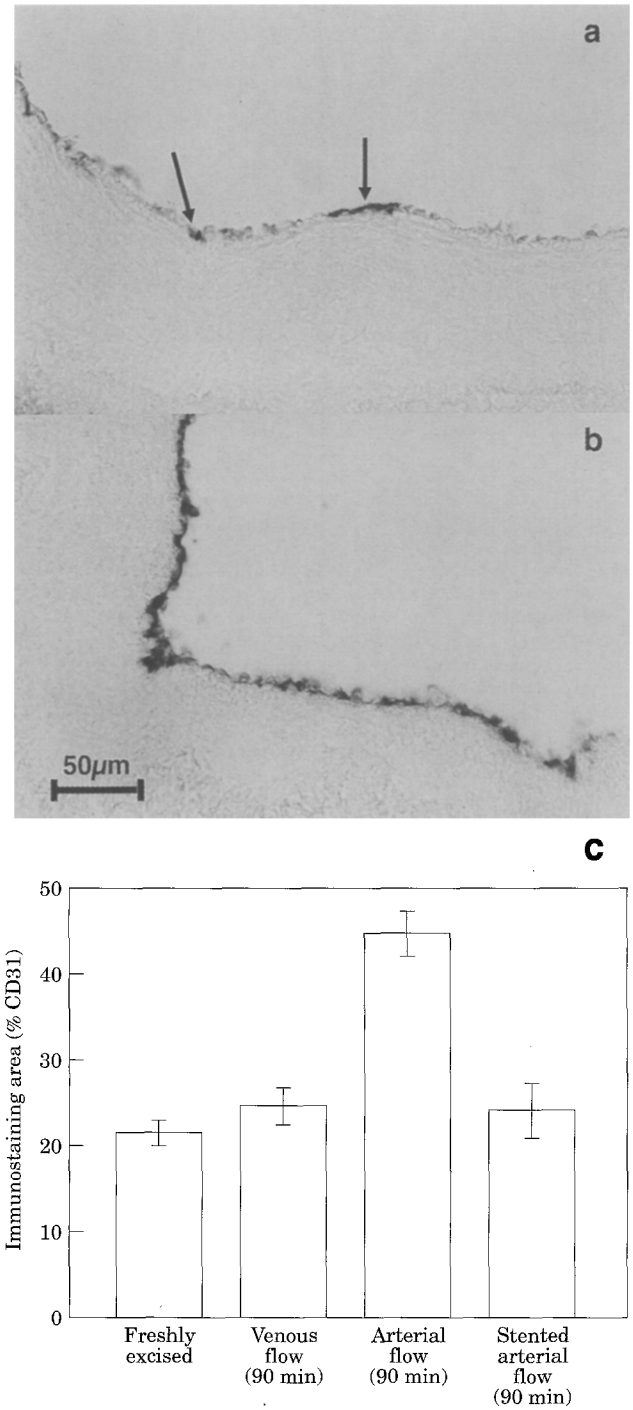

Fig. 6. Immunostaining for ICAM-1. (a) Freshly excised vein and (b) vein exposed to arterial flow for 45 min. (c) Histogram of luminal immunostaining area for ICAM-1 on freshly excised vein, vein exposed to venous flow, and vein exposed to arterial flow for 90 min with and without external PTFE stents to limit circumferential and radial deformation. Bars represent the mean of five sections examined from each of eight paired samples; vertical lines are standard error of the means. Staining area is expressed as % of total CD31 staining (see methods).

Application of this technique suggests that the regulation of endothelial gene expression by haemodynamic forces is more rapid in the vein than in cultured venous endothelial cells. For instance, we observed a more than two-fold upregulation of ICAM-1 on venous endothelium after only 90 min of exposure to arterial flow compared with the 4–6 h required to upregulate ICAM-1 on monolayers of cultured venous endothelial cells in response to shear stress.[5] Further, this rapid upregulation of ICAM-1 on saphenous vein endothelium in response to arterial flow appears to be a result of exposure to circumferential (or radial) stress rather than shear stress, because the upregulation was not observed in externally stented veins.

The principal differences between our *ex vivo* technique which we report here and those recently published[7–9] include the availability of variable size mounting jigs to cope with veins of different dimensions and the use of Krebs' solution for the perfusate, rather than tissue culture medium supported by serum. The technique we describe uses a large circulating fluid volume, much of which is necessary to prime the bypass pump. The use of Krebs' solution is economical and facilitates cleanliness of the system. The use of a large circulating volume also minimises the concentration of accumulating waste products, which in the *in vivo* situation would be filtered at the glomerulus or detoxified in the liver. Whilst the use of Krebs' solution is acceptable for short-term experiments, such as those described herein, it is unlikely to be suitable for long-term experiments. The other techniques recently described for perfusing vein segments have depended on histology and/or vasomotor responses to assess vein viability.[7–9] We have used both histology and vasomotor responses, and have additionally monitored tissue ATP concentrations. Interestingly, the tissue ATP concentrations increase significantly after exposure of vein to arterial flow; we suggest that this is a result of work hypertrophy. The increased tissue concentrations of ATP also might underlie the increased sensitivity of vein rings to phenylephrine, since the amount of ATP available to support muscle contraction has increased. The EC_{50} for phenylephrine decreased by an order of magnitude after veins had been exposed to arterial flow (Fig. 2). A similar increase in sensitivity to norepinephrine in veins exposed to pulsatile arterial flow *in vitro* for 48 h was observed by Schwartz *et al.*[7] There is further agreement between our results and those of Schwartz *et al.*[7] that the relaxation of the smooth muscle in response to sodium nitroprusside was unchanged in response to pulsatile arterial flow *in vitro*. We also observed that the endothelium-dependent relaxation

in response to agonist challenge (bradykinin) was increased significantly after exposure of veins to arterial flow conditions for 90 min: this may be a result of the upregulation of endothelial nitric oxide synthase, which is recognised to be upregulated by flow and shear stress.[1,2] Immunostaining for CD31 also demonstrated that there was only a small degree of endothelial loss in our model. Therefore, we have validated a technique for evaluating the short-term responses of saphenous vein endothelium to arterial flow conditions.

The first endothelial epitope we investigated was ICAM-1, a cell membrane protein of the immunoglobulin superfamily with a critical role in leukocyte emigration, known to be upregulated by shear stress in endothelial monolayers.[5,12] In veins exposed to arterial flow for 90 min there was a significant (more than two-fold) increase in the staining area ratio of ICAM-/CD31, without change in the staining area ratio of P-selectin/CD31. This increase in immunostaining was only observed in unsupported veins; no significant increase in ICAM-1 staining was observed in externally stented veins. The principal difference between unsupported veins and externally stented veins is the extent of circumferential (or radial) deformation with each fluid pulse. The nine mechanical factors altered by exposing vein grafts to pulsatile arterial flow have been discussed by Dobrin *et al.*[17]: circumferential, radial and longitudinal deformations can be separated from pulsatile deformation and blood flow velocity (shear). In the experimental situation of canine infrainguinal vein grafts, intimal hyperplasia of vein grafts was associated with low blood flow velocity and shear stress, whilst medial thickening was associated with increasing circumferential deformation.[17] Moreover, abolition of the circumferential deformation by external stenting of vein grafts has been reported to reduce medial thickening in experimental vein grafts.[18,19] Therefore it is particularly exciting that the experimental model we have developed permits discrimination of circumferential stresses from longitudinal or shear stresses.

Work is in progress to investigate how the regulation of other proteins of saphenous vein endothelium, involved in thrombosis and leukocyte adhesion, is influenced by exposure to pulsatile arterial flow and circumferential stresses. Knowledge of the magnitude of these changes and their time course should provide the much needed scientific basis for new therapeutic interventions to improve the patency of vein bypass grafts.

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