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Original Article

Search for optimized conditions for sealing and storage of bypass vessels: influence of preservation solution and filling pressure on the degree of endothelialization

Dominik Roger Weiss[1*], Gerd Juchem[2*], Markus Eblenkamp[3], Bernhard Michael Kemkes[4], Brigitte Gansera[4], Michael Geier[5], Stephan Nees[5]

[1]Department of Transfusion Medicine and Hemostaseology , University of Erlangen- Nuremberg (FAU), 91054 Erlangen, Germany; [2]Department of Cardiac Surgery, University of Munich (LMU), 81377 Munich, Germany; [3]Department of Medical Engineering, Technische Universität München (TUM), 85748 Garching; [4]Department of Cardiac Surgery, Hospital Bogenhausen, 81925 Munich, Germany; [5]Department of Physiology, University of Munich (LMU), 80336 Munich, Germany.

**Both researchers contributed equally to the work.*

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Abstract: The aim of the present study was to develop methods for the rapid assessment of intimal quality of coronary bypass segments of venous origin, and to prevent endothelial damage by improved intraoperative handling of graft segments. Particular attention was paid to the influence of the composition of the preservation solution and the intravasal filling pressure on the degree of endothelialization. Intravasal exposure to Alcian blue at pH<3 resulted in highly specific staining of intimal regions with functionally or structurally damaged endothelium. Standardization of preparation, staining and image acquisition of the intimal surface of graft remnants and subsequent computer-aided planimetry of these images made it possible for the first time to perform rapid serial investigations for quality control of bypass grafts. Using saline as the rinsing and intraoperative storage medium resulted in the loss of more than 50% of the endothelium at intravasal pressures of 0-100 mmHg. Increasing the pressure resulted eventually in complete de-endothelialization. In contrast, grafts incubated in a customized plasma derivative tolerated pressures of up to 200 mmHg with no significant endothelial loss; and even after exposure to 1,000 mmHg (10 times the average mean arterial pressure!) more than 70% of the endothelium were intact and vital. These findings imply strongly that the quality of aortocoronary bypass grafts of venous origin can be improved substantially by the use of a plasma derivative solution for intraoperative preservation and by monitoring and controlling the intravasal pressures reached during sealing and storage.

Keywords: Saphenous vein graft disease, endothelium, pericytes, intima, thrombosis, atherosclerosis, tissue factor, CABG, bypass operation, Alcian blue staining

Introduction

Recent reports have shown that bypass veins stored intraoperatively are characterised by an intimal layer that is strongly procoagulatory following acute stimulation of tissue factor (TF) expression [1]. This is due to the presence of a second, subendothelial intimal tissue leaflet formed by pericytes [2, 3]. Such observations suggest that patients undergoing bypass operations and receiving such vessels are exposed to a substantial risk of thromboembolism. Indeed, a recent clinical study showed that intravasal fibrin thrombi form rapidly in endothelium-denuded saphenous vein segments, explanted for use as aortocoronary bypasses and filled with solutions of defined coagulation factor composition or whole blood [3]. Moreover, the

locally formed thrombin also immediately initiates activation of polymorphonuclear granulocytes (neutrophils) and platelets and their adhesion to the damaged inner wall of the vessel or, more accurately, to the exposed subendothelial pericyte layer. The latter is far more firmly attached to the remaining vessel wall than the endothelium, large areas of which are frequently lost in bypass vessels. The resulting mixed thrombi that include the remaining endothelium represent a substantial hemodynamic flow resistance, such that the resultant shear forces exerted at the moment of bypass reperfusion can result in complete de-endothelialization. This represents a cogent explanation for the reported high incidence of thromboembolic occlusion, especially of aortocoronary grafts of venous origin [4, 5]. Furthermore, since pericytes, without contact to the overlying confluent endothelial cell layer and exposed to growth factors in locally formed serum, react with fulminant growth [2], this concept can also explain the frequently observed longer term stenosis of bypassvessels by wall thickening. Of the aortocoronary bypasses of venous origin that escape acute thromboembolism, 60% occlude completely already within 10 years after implantation, and the remainder show substantial, angiographically demonstrated stenosis [5-7].

We have shown recently that the inappropriate composition of the solutions [saline with or without albumin, histidine-tryptophan-ketoglutarate (HTK) solution or heparinised autologous whole blood] used routinely for locating side branches and/or intraoperative storage of the grafts [3] contributes substantially to the initial endothelial damage in venous bypass segments. In addition this study implied, that the intraoperative handling of the segments, in particular the high pressures used to test for leaks, could also have deleterious effect on the endothelium. Investigations preliminary to the present study showed that employing the surgical standard procedure for sealing tributaries and leak testing (20-ml syringe, manual use) can generate intravasal pressures up to 10 times the average mean arterial pressure in the graft segments (1.000mmHg!). Damage to the endothelium could also occur when the segments are stored intraoperatively in a collapsed state.

In summary, there appears to be substantial justification for the revision and optimisation of procedures for preparation and handling of bypass vessels before their transplantation.

The principal aim of the present study was thus to develop and validate a method for simple assessment of the degree of bypass vessel endothelialization that is suitable for serial clinical studies. A further goal was to determine the influence of filling pressure on endothelial preservation. In this context we used either the routinely employed rinsing solution saline or a customized plasma derivative (Biseko®, Biotest AG, Dreieich, Germany), since the latter closely resembles plasma, the natural environment of the vascular endothelium, and appeared to be far better suited for endothelial preservation in bypass vessels [3].

Our results show that the preservation of the endothelium in venous bypass segments depends not only on the composition of the perfusion and intraoperative storage solution, but also on physical parameters such as perfusion pressure and shear forces.

Materials and methods

All experiments with human tissue were done with written consent of the patients and with approval of the ethical committee of the Munich Municipal Hospital Bogenhausen or the ethical committee of the Ludwig-Maximilians-University of Munich according to the principles expressed in the “Declaration of Helsinki”.

Collection and transport of venous bypass remnants

Saphenous vein remnants were harvested after coronary bypass operations. The samples were immediately transported under aseptic conditions to the laboratory in ice-cold minimal culture medium containing 10 IU/ml heparin and fixed as described elsewhere [3].

Isolation and cultivation of intimal tissue fragments

Intimal tissue was detached from saphenous vein graft remnants after a standardized pressure application protocol (see **Figure 1D**) by a 20-min incubation at 37°C in a protease solution consisting of dispase II and collagenase D (0.09% w/v each; La Roche, Switzerland) in PBS, pH 7.4, supplemented with 0.9% (w/v)

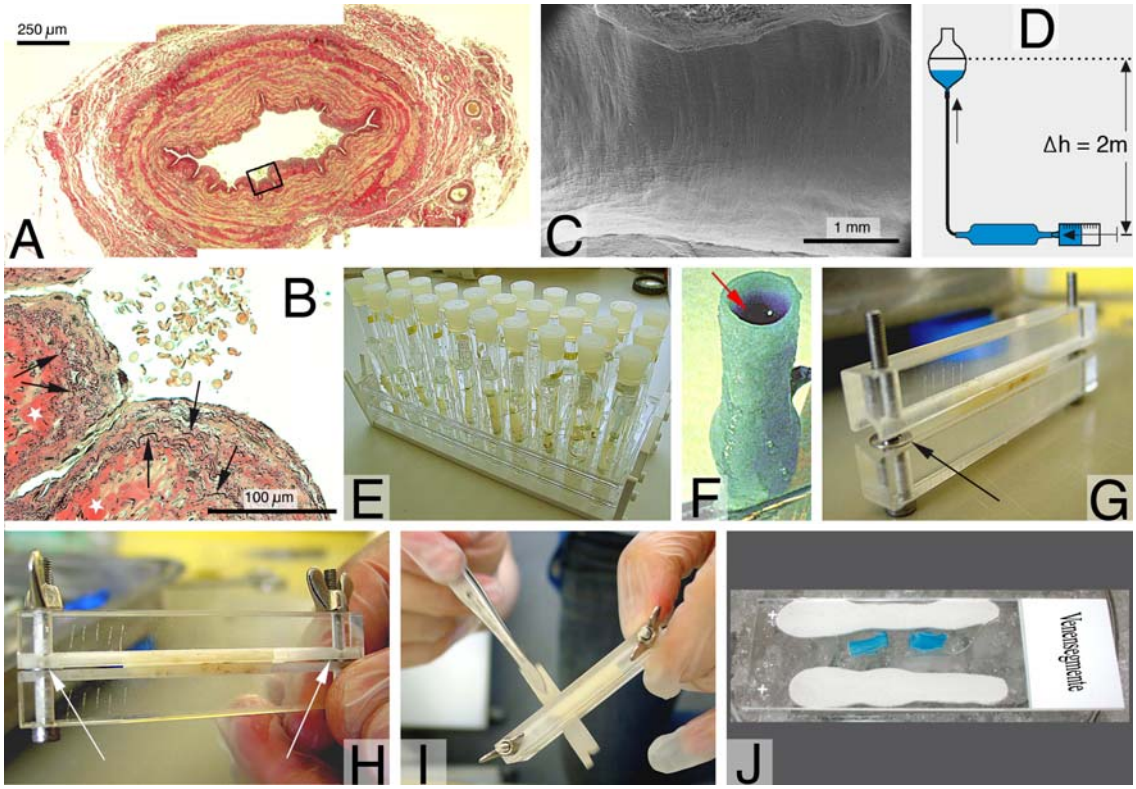

Figure 1. Problems during the preparation of venous graft remnants for histological examination and their easy practical solution. (A) Low-power view (Elastica van Gieson stain) of a vein fixed in an empty state showing the extensively folded inner surface (collagen fibers stain red). The black box is shown in higher power in B. (B) High-power view of the area indicated in A (the internal elastic lamina* is stained red, elastic fibers in the intima black, see arrows).(C) Scanning electron micrograph of a vein fixed at an intravasal pressure of 150 mmHg showing a smooth inner surface. (D) Diagram showing a simple method of bypass segment fixation at a pressure of approx. 150mmHg. (E) Practical long-term storage of bypass remnants in fixative at 4°C until staining and further processing. (F) Specific staining of the intima by intravasal application (red arrow) of the staining solution. (G-I) Procedure for longitudinal sectioning of a fixed bypass remnant. (G) The vessel segment is laid on a Plexiglas block in which a channel, the radius of which corresponds to that of the vessel, has been milled. (H) A second, identical Plexiglas block is inverted over the first. The steel washers (arrows in G and H) act as spacers that allow (I) a razor blade to be passed in the longitudinal direction. (J) Two stained half-veins (blue specimens) are laid flat (intima upwards) on a microscope slide, between two strands of silicon adhesive (see text).

bovine serum albumin. The harvested cells were washed twice with Dulbeccos Minimal Essential Medium including 10%FCS by centrifugation, resuspended in human endothelial-SFM basal growth medium (Invitrogen, USA) supplemented with 10%v/v FCS, 200 U/ml penicillin, 0.2 mg/ml streptomycin,1 mg/ml fibronectin, 10 ng/ml EGF, and 20 ng/ml bFGF, and seeded in Falcon dishes (Corning, NY, USA).

Assessment of viability of freshly isolated intimal tissue

After attachment to their substratum freshly isolated intimal tissue fragments were incubated (37°C, 5% CO_2 in water-saturated air) on the stage of an inverted microscope (Zeiss-Diavert 100, Zeiss, Göttingen, Germany). Series of phase contrast images were acquired using a high-resolution digital camera (AxioCam Color, Zeiss, one frame every 10 min, primary magnification x100) controlled by the manufacturer's software (AxioVision Rel 4.5 SP1, Zeiss), which was also employed to obtain corresponding time lapse videos. After 16h the ratio of the number of spreading cells to the total number of cell nuclei present in the monitored culture area was calculated and expressed as % viability.

Histological Staining Techniques

For staining with Alcian blue, raft remnants were sealed with a clip at one end and mounted vertically. Alcian blue 8GX solution (Fluka Chemie, Buchs, Switzerland) was intravascularly applied, care was taken to avoid intimal damage and adventitial staining. After 15min incubation at room temperature, the dye solution was poured out on paper tissue and excessive dye rinsed with 3% (v/v) aqueous acetic acid.

The immunohistochemical procedures have been described in detail elsewhere [2].

Sectioning and preparation of samples for microscopy

The segments were fixed in a special device (**Figure 1G-I**) and split into longitudinal halves using a razor blade. Two parallel strands (3-mm diameter) of elastic silicon adhesive (Terostat VII, Wackerchemie, Munich, Germany) were applied along the long sides of a dry microscope slide and the vessel halves laid out between the former with their intimal surface upwards and wetted with a few drops of 80% glycerol in water. A further microscope slide was laid over the preparation and pressed gently onto the strands of silicon adhesive avoiding pressure on the vessel halves, **Figure 1J**.

Photography and imaging technique

Stained specimens were illuminated as homogenously as possible with the "visiLED" light system (Zeiss) and viewed with a stereo microscope (Stereo-Discovery V8, Zeiss, Göttingen, Germany). The color images were selected, optimized and captured using a digital-camera (Axiocam Mrc, Zeiss) and the manufacturer's software (AxioVision).

Computer-assisted planimetry

Color images of intimal surfaces were evaluated using either the capabilities of a commercially available image processing software (Photoshop, v. 10, Adobe Systems, San Jose, CA, USA) or by employing a digital morphometry system (S.CORE, S.CO LifeScience, Garching, Germany).

Perfusion and storage of vein graft remnants under defined hydrostatic pressures

Details of the experimental set-up are shown in **Figure 8A**. The electronically controlled pressure transformer (P-1X/D-1X) as well as the pressure transmitter (D-10-P) in the pressure detection unit were supplied by WIKA (Klingenberg, Germany).

Results

Preliminary methodological preparations for clinical studies of endothelial integrity in remnants of coronary bypass grafts of venous origin

Processing of delicate vein segments, with their thin walls and only loosely attached luminal endothelium, for subsequent detailed histological studies in a clinical setting requires a simple technique, since access to sophisticated laboratory equipment is not always available. **Figure 1** illustrates the histological situation and major practical aspects. The outer wall of the human saphenous vein has a low compliance mainly due to the inductile, circumferentially arranged connective tissue of the thick adventitia (**Figure 1A**). On the other hand, due to a low elasticity of the concentric collagen bundles of the media, the compressible nutritive microvessels (Vasa venarum), and particularly by virtue of the high content of elastic fibers in the venous intima (see arrows in **Figure 1B**), the inner surface of the vessel is highly distensible, and thus, in empty bypass segments, heavily folded. An intravasal pressure of about 150 mmHg is necessary to unfold the intima completely (**Figure 1C**), in which state the vessel must be fixed permanently if studies of intimal damage are to include the folded surfaces. Counteracting the strong tension exerted by the intimal elastic fibers requires the use of a cross-linking fixative such as glutaraldehyde. The scheme shown in **Figure 1D** illustrates a convenient and in the clinical context readily implementable protocol for fixation. Preparations fixed in this manner can be stored at 4°C for months for subsequent histological analysis (**Figure 1E**).

To stain the vessel intima selectively, the segment, with the lower end clamped, is mounted vertically and filled with the staining solution. Highly specific staining of the intima can be achieved if contact between the stain and the adventitia is avoided (**Figure 1F**). A reproducible sectioning procedure also avoids preparation-related damage to the intima (**Figure 1G-I**). The

Table 1. Appraisal scheme for various staining procedures

Procedure quality criteria*	Scanning electron microscopy	Immunological methods	Alcian blue staining
High specificity for visualization of endothelium or subendothelium	+	+	+
Color-based delineation of intimal tissue as the basis for computer-aided planimetry	-	+	+
High degree of brilliancy and strong area contrast of stains to enhance photographic sensitivity and to extend the limits of detectability	-	+	+
Simultaneous detection of "structural" and "functional" endothelial lesions	-	+	+
Minimum possible need for fine chemicals; rapid preparation of sections, minimum cost	-	(+)	+
Low microscope technical effort and time requirements	-	+	+
Maximum long-term storage life of stained sections for subsequent verifiability	-	(+)	+
Tolerance to glutaraldehyde as fixative	+	-	+

* criterion: + fulfilled, - not fulfilled

resultant longitudinal half-segments, immersed in 80% glycerin solution are sandwiched flat between two slides, whereby two permanently elastic strips of silicone rubber adhesive serve as spacers. The slides are pressed gently together until the distance between them is just about that of the preparation. Mounted in this manner (**Figure 1J**), the stained preparations can be stored flat (for month, if necessary) until histological analysis. If required, the preparation can also be easily removed from this mounting, for example for scanning electron microscopy.

Staining for selective visualization of intimal damage

Selection of a suitable procedure for visualizing intimal damage, both in the present study and in future serial clinical studies, first required clarification of the criteria that must be fulfilled by such a procedure (**Table 1**, left column). The remainder of Table 1 compares the methods used routinely for studying blood vessels with respect to these criteria.

The technically very demanding and expensive scanning electron microscopy does not fulfill most of the criteria summarized in **Table 1**. Indeed, for this method the use of glutaraldehyde is mandatory, and assuming that the observer is familiar with the special morphology of the vascular intima, this method principally allows (but only at high magnification and not always reliably) the normal luminal endothelium to be distinguished from the subendothelium (**Figure 2A,B**). Often, however, the conformity of the stretched intimal surface in such magnified scanning electron micrographs would actually tend to hinder the exact demarcation of locally damaged intimal areas as is exemplified by **Figure 2C**. Moreover, such black and white images of the detailed intimal structure do not lend themselves to computer-aided planimetry, and the use of "classical" (manual) planimetry for the morphometric evaluation of sufficiently large intimal areas would only be possible after tedious (and costly) assessment of very large numbers of individual images. In addition, since the method visualizes solely surface morphological structures, and not, for example, the structure in the interior of overlapping endothelial clefts, events such as functional breakdown

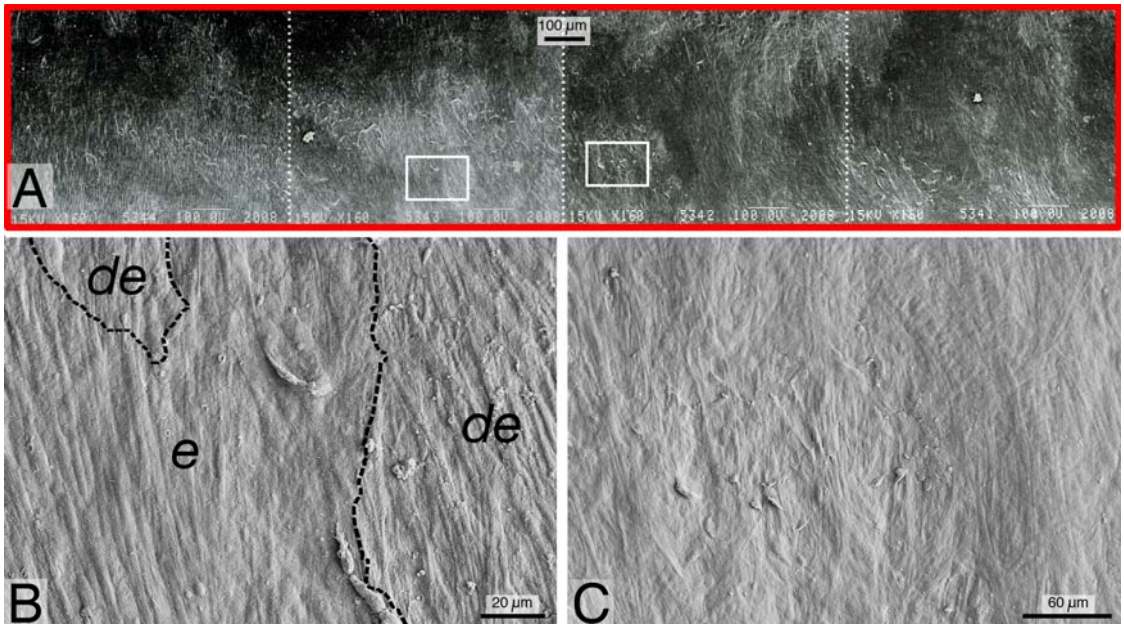

Figure 2. Scanning electron microscopic inspection of the lesioned luminal surface of a human saphenous vein. (A) An overview constructed from 4 low power micrographs shows only blurred contours of the endothelium-denuded regions (which appear as brighter areas). The red frame marks the corresponding areas also in Figs. 4A and 8A. (B) Left frame and (C) right frame from (A) at higher magnification demonstrate the difficulty if not impossibility of distinguishing endothelialized (e) from de-endothelialized (de) areas.

of the endothelial barrier will be overseen in the assessment of intimal damage. Such events could have serious clinical consequences, such as the induction of intravasal thrombosis, and should therefore be adressed in a clinical study.

Immunological stains, with their highly specific, enzyme-coupled or fluorescent antibodies for typical endothelial antigens CD31 (**Figure 3A**), CD141 (**Figure 3B**) and von Willebrand factor-antigen (vWF:Ag, **Figure 3C**) are unsuitable for assessment of areas of intimal damage because of the disadvantageous distribution of the antigens: CD31 and CD141 are restricted to the endothelial joints, vWF:Ag is concentrated in granular organelles and the antibody also clearly stains the endothelial cells' belt-like anchoring structures or remains of their tight junctions, which can be recognized particularly in endothelium-denuded intimal regions (arrows in **Figure 3D**). Similarly unsuitably distributed is the tissue factor (TF) antigen (typical for the intimal pericyte-layer): besides the subendothelially located pericyte net with its more or less large meshes, the TF-containing vesicles and microparticles deposited by these cells in the extracellular matrix are also stained, so that no uniformly stained representation of damaged intimal areas that could be evaluated by computer-aided planimetry (**Figure 3E**) can be achieved. Indeed, the assessment and documentation of the preparations shown (**Figure 3A-E**) was only made possible by the use of technically very demanding confocal microscopy. In contrast to the staining and visualization techniques just discussed, immuno-histochemical staining of smooth muscle cell (SMC) α-actin in the subendothelial pericytes (using an antibody coupled with glucose oxidase, Vector Laboratories, Burlinghame, CA, USA) fulfilled the criteria in **Table 1**, albeit with one important exception. The reaction product (red) could be documented using relatively simple bright field stereomicroscopy. **Figure 3F** shows a representative image. The exception was that this procedure precluded the use of glutaraldehyde, because the cross-linkage mediated by this fixative resulted in the loss of SMC α-actin's typical antigenicity (this occurs with all the antigens of interest in the present study). Thus, although this stain was of interest for other purposes (**Figure 5C**) it was of little use for assessing intimal damage

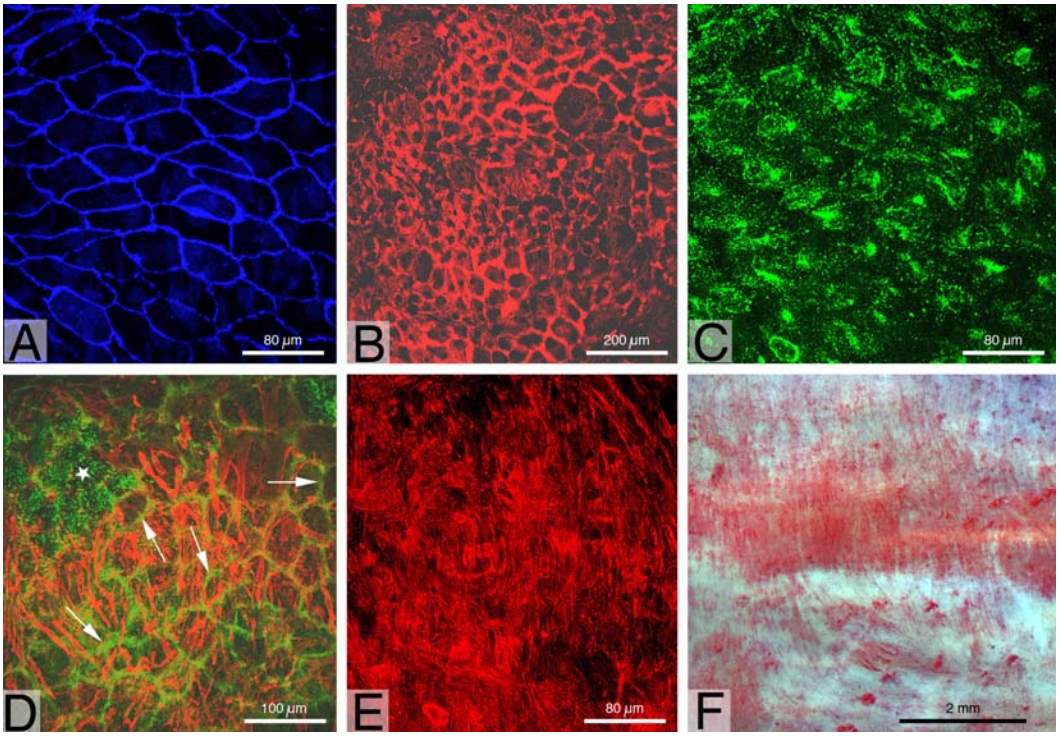

Figure 3. Limits of immunohistological procedures for staining the endothelium or subendothelial areas of vascular intimae with respect to quantification of endothelial damage. Selective staining of the luminal endothelium of a well-preserved section of a formaldehyde-fixed human saphenous vein. (A, B) Staining for CD 141 (A) or CD31 (B): the stains visualize only the cell junctions. (C) Stain for von Willebrand Factor antigen (vWH:Ag): the stain visualizes the endothelial cell Weibl-Palade bodies specifically. (D) Detail of the intimal surface of a heavily damaged vein stained for vWF:Ag (green) und smooth muscle cell α-actin (red). Not only intact islands of endothelium stain clearly (green) for vWH:Ag (*), but also regions of destroyed endothelium, in which remains of the anchoring structures or tight junctions also stain (arrows). The pericytes (stained red) lay 0.5-3 µm deeper in the subendothelium. (E) Completely deendothelialized intima stained for tissue factor. As well as the pericyte net in the subendothelium, numerous vesicles and microparticles produced by the pericytes and deposited in the extracellular matrix are also recognizable, the resulting image is diffuse. (F) Partially de-endothelialized vein intima, stereomicroscopic view in oblique (incident) light after enzyme-immunohistochemical staining for smooth muscle cell (SMC) α-actin (red, intact areas unstained). All other images are confocal fluorescence images.

under the preparatory conditions described above and did not fulfill the criteria compiled in **Table 1**, too.

The most suitable procedure for selective demarcation of damaged intima proved to be staining with Alcian blue. This stain, when applied solely from the intravasal compartment, fulfilled all the criteria listed in **Table 1**. **Figure 4A** shows the inner surface of a vein preparation in incident light. The numerous deendothelialized patches, stained blue due to the penetration and binding of the dye selectively in the intima (**Figure 4B**), are clearly distinguishable from unstained regions with intact endothelium (which shields the intima from the dye, **Figure 4C**). This claim can be verified using scanning electron microscopy for further identification and characterization of the respective areas (**Figure 4D,E**).

This example emphasizes also again the unsuitability of scanning electron micrographs for quantitative morphometric analysis of intimal damage. This becomes clear by a comparison of the Alcian blue-stained intimal area within the red frame (**Figure 4A**) with the scanning electron microscopic overview shown in **Figure 2A**, which represents the same intimal area. In stark contrast to the single low-power image of the Alcian blue-stained preparation with the sharply demarcated lesions within a large intimal area (**Figure 4A**), the blurred grey contours of scanning electron microscopic images do not allow

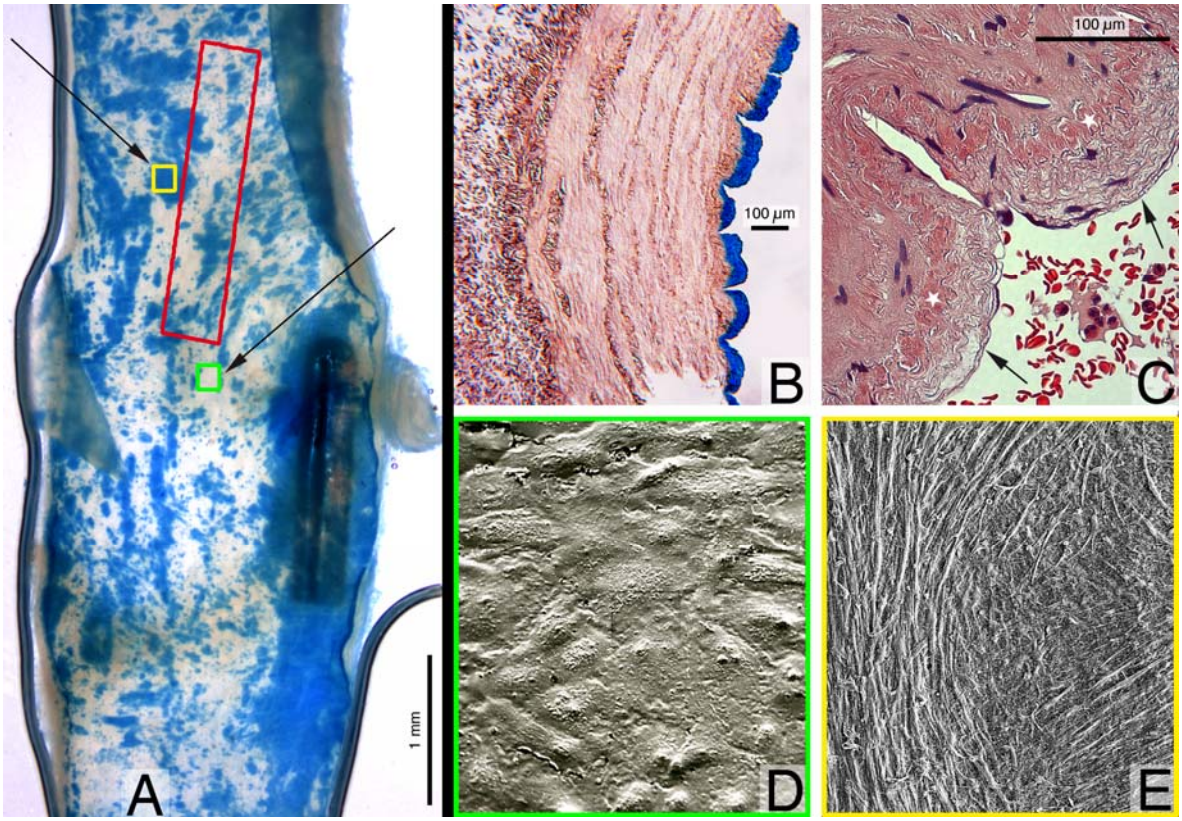

Figure 4. Highly specific visualization of deendothelialized areas in venous bypass vessels using simple Alcian blue staining as the basis for computer-aided quantification. (A) Stereo micrograph of the intimal surface in incident light. The area within the red frame is identical with that represented by the scanning electron micrograph in Figure2A. (B) Cross-section through a completely deendothelialized venous wall after Alcian blue and Giemsa staining, (C) analogous image of a highly magnified venous intima with continuously preserved luminal endothelium (arrows). (E,F) High power scanning electron micrographs of the regions within the small yellow and green frame in Figure 4A, respectively (arrows, note corresponding frame colors).

precise computer-aided planimetry (see below). Hundreds of high power scanning electron micrographs would be necessary to evaluate the quality of such a relatively large intimal area as shown in **Figure 4A**, and this could be done only by use of classical, extremely tedious planimetry techniques.

Validation of the use of Alcian blue as a stain for highly specific demarcation of deendothelialized intimal areas

Comparison of the Alcian blue stain with other staining methods further substantiated the specificity of Alcian blue. **Figure 5A**, for example, shows an endothelium-denuded intimal area visualized by silver impregnation, **Figure 5B** the same area stained with Alcian blue after selective removal of the silver precipitate. Both areas are absolutely congruent. As documented in **Figure 3F**, with an antibody coupled to glucose oxidase, the deendothelialized intimal surface in another vein preparation stained red. The subsequent staining of such specimens with Alcian blue generated in all (formerly red) areas a dark violet as the mixed color, as is seen here in **Figure 5C**. Had the areas stained by the two methods not been congruent, individual areas of red or blue would be visible in **Figure 5C**, which is clearly not the case.

Moreover, in vascular regions with particularly turbulent flow, Alcian blue also stained the narrow zones directly below intercellular clefts as a result of partial leakage and penetration of the stain into the local subendothelium. Such local breakdown of the normally very tight venous endothelial barrier occurrs, for instance, in the

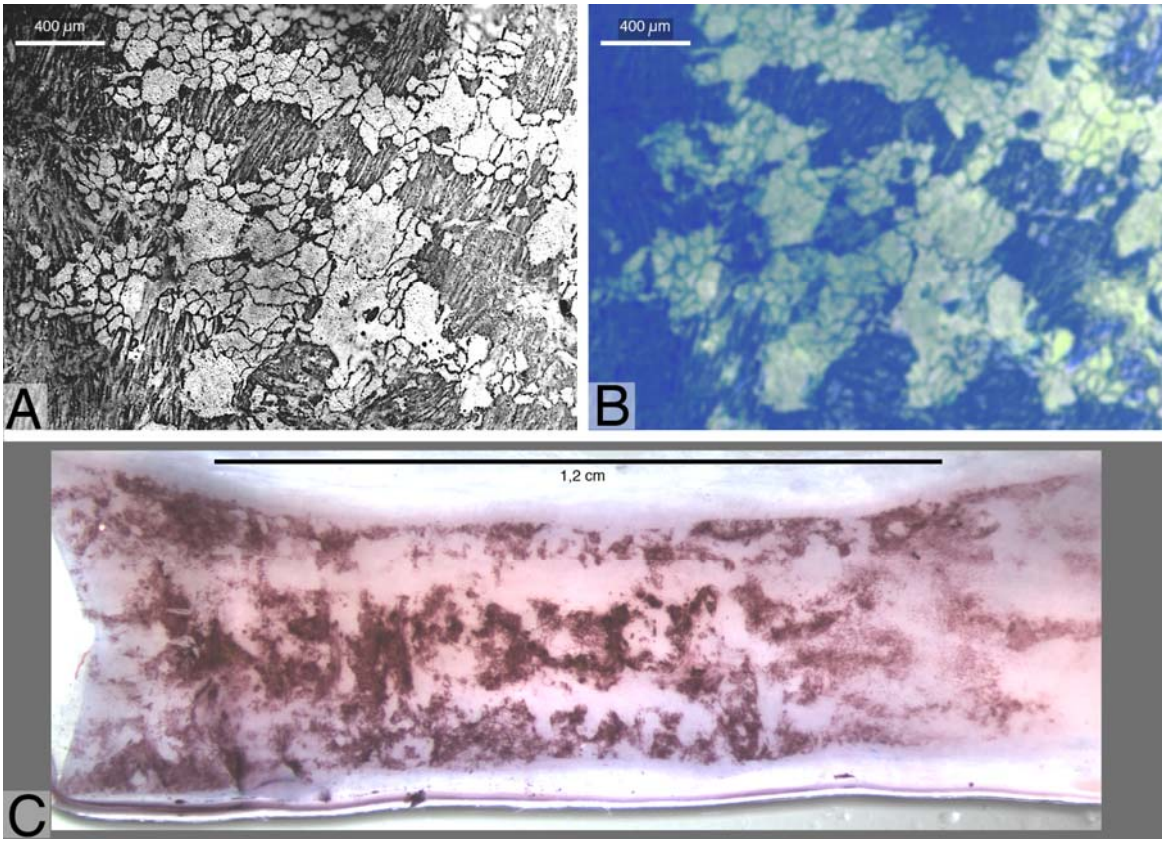

Figure 5. Validation of the Alcian blue staining method for highly specific visualization of intimal damage by comparison with argentophilic or immunohistochemical stainings. (A) Black demarcation of lesioned intimal areas in a human saphenous vein after preservation in saline and silver staining, (B) same areas bind the dye Alcian blue after selective removal of silver. (C) Intimal surface of another damaged vein first stained immunohistochemically for smooth muscle cell α-actin (red) and subsequently with Alcian blue. Dark violet results as the mixed color of the congruently stained lesions.

vicinity of vessel branches (**Figure 6A,B**) due to locally extreme shear forces (caused e.g. by flushing at too high perfusion pressures, see **Table 2**), or in dissected arteries in which the surgical manipulation (wounding by sectioning, release of inflammatory mediators, incubation in an unphysiological milieu) results in strong contraction not only of the inner media and the intima, but also of the individual arterial endothelial cells (**Figure 6 C,D**). Thus, since intact endothelial cells themselves definitively do not stain with Alcian blue, this dye is suitable for visualizing not only structural damage to the intima (i.e. endothelium-denuded areas), but also functional intimal damage (intimal regions with abnormally high endothelial permeability).

Quantification of the deendothelialized fraction of the total intimal surface area using computer -aided planimetry

Digital stereophotographic images obtained under optimal exposure conditions (as in **Figure 7A**) served, after conversion, as the basis for tedious traditional ("cut out and weigh") planimetric quantification of the damaged intimal area. **Figure 7B** illustrates the computational basis for determining the fractional area of damage using histograms generated by Adobe's Photoshop (after black/white conversion and definition of an optimal threshhold). **Figure 7C** shows the same image after conversion to blue/yellow using the computer assisted morphometric S.CORE technology. In this example (optimal image of the intimal surface) the calculated degrees of endothelialization were very similar, the three very different methods resulted in comparable results.

However, the illumination, the histological structure or the preparative processing of prepara-

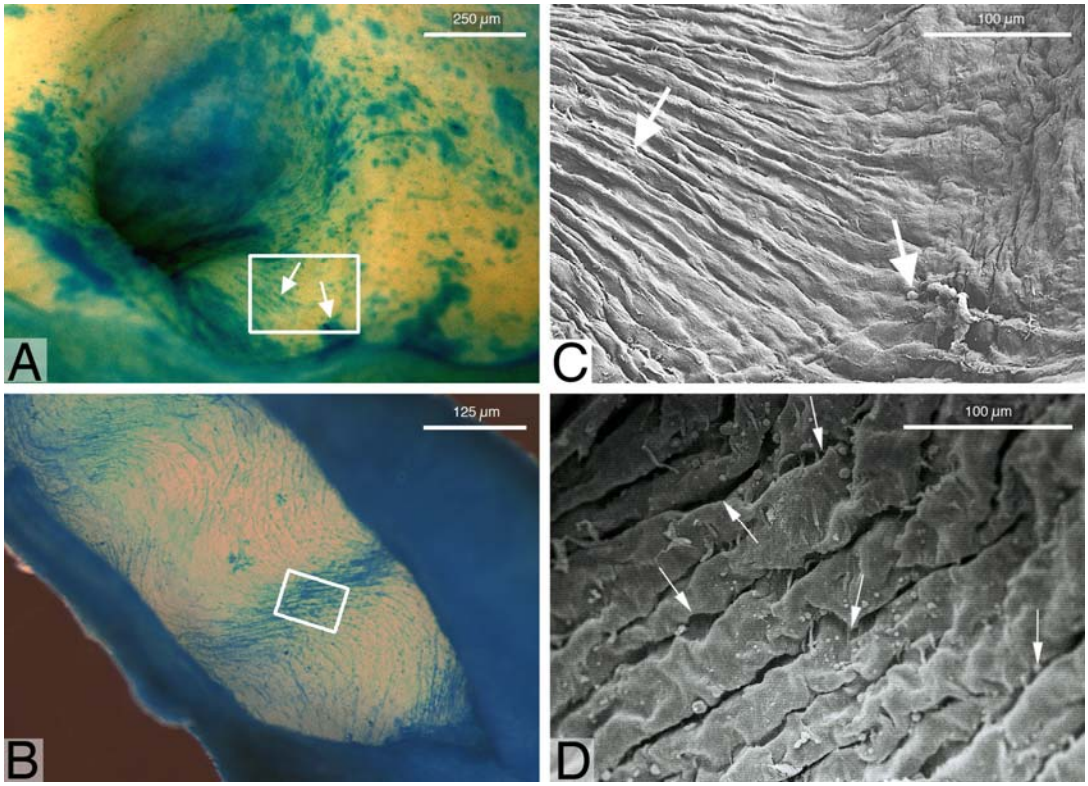

Figure 6. Demonstration of selective Alcian blue staining of widely open intercellular junctions in vessel areas with particularly stressed endothelium (A,B) Intimal surface of a saphenous vein in the vicinity of the ostium of a tributary, where the endothelium was stressed locally by high shear forces and/or turbulent flow. (A) Stereomicroscopic overview, (B) scanning electron microscopic enlargement of the frame in Figure A demonstrating the locally disturbed endothelium with opened junctions and a small mechanical lesion (white arrows point to the respective areas, which are marked selectively by Alcian blue in (B). (C,D) Histological inspection of an internal thoracic artery bypass remnant. In case of complete absence of vasodilators in the incubation medium such vessels are typically hypercontracted. (C) Stereomicroscopic overview of the artery after complete immersion in Alcian blue and subsequent removal of excess stain. The adventitia (which contains polyanions like the subendothelial extracellular matrix of the intima) is stained homogenously blue, but the intima of the partially incised vessel is exclusively stained only in certain intercellular regions. (D) High-power scanning electron micrograph of the frame in C to substantiate the conclusion that the selective striped staining in C was due to the opening of endothelial clefts. of the hypercontracted and thus distorted intima (white arrows).

tions under routine conditions can be considerably less satisfactory than in the foregoing example. In the case of a primary image as in **Figure 7D**, a purely threshold-based system (such as the Photoshop procedure, cf. **Figure 7E**) yields deendothelialization values that are far too high. In contrast, the more comprehensive S.CORE analysis system delivers reliable values, consistent with those obtained by carefully performed, time consuming classical planimetry (**Figure 7F**).

Generation and recording of intravasal pressure in short bypass remnants

On the basis of the above histological findings, we constructed the perfusion system shown in **Figure 8A** to allow reproducible examination of intimal integrity under the influence of raised intravasal pressure and with various rinsing or preservation solutions. The pressure within a given vascular segment was registered by sensitive transmitters and recorded on line. Experiments with five different pressure limits were performed. **Figure 8B** shows a representative pressure time course in a vein segment exposed to the plasma derivative at a pressure of 500 mmHg with subsequent repeated acute pressure reductions over an interval of 5min at room temperature, thus imitating the procedure used commonly in bypass surgery to find and ligate the small side branches in a venous by-

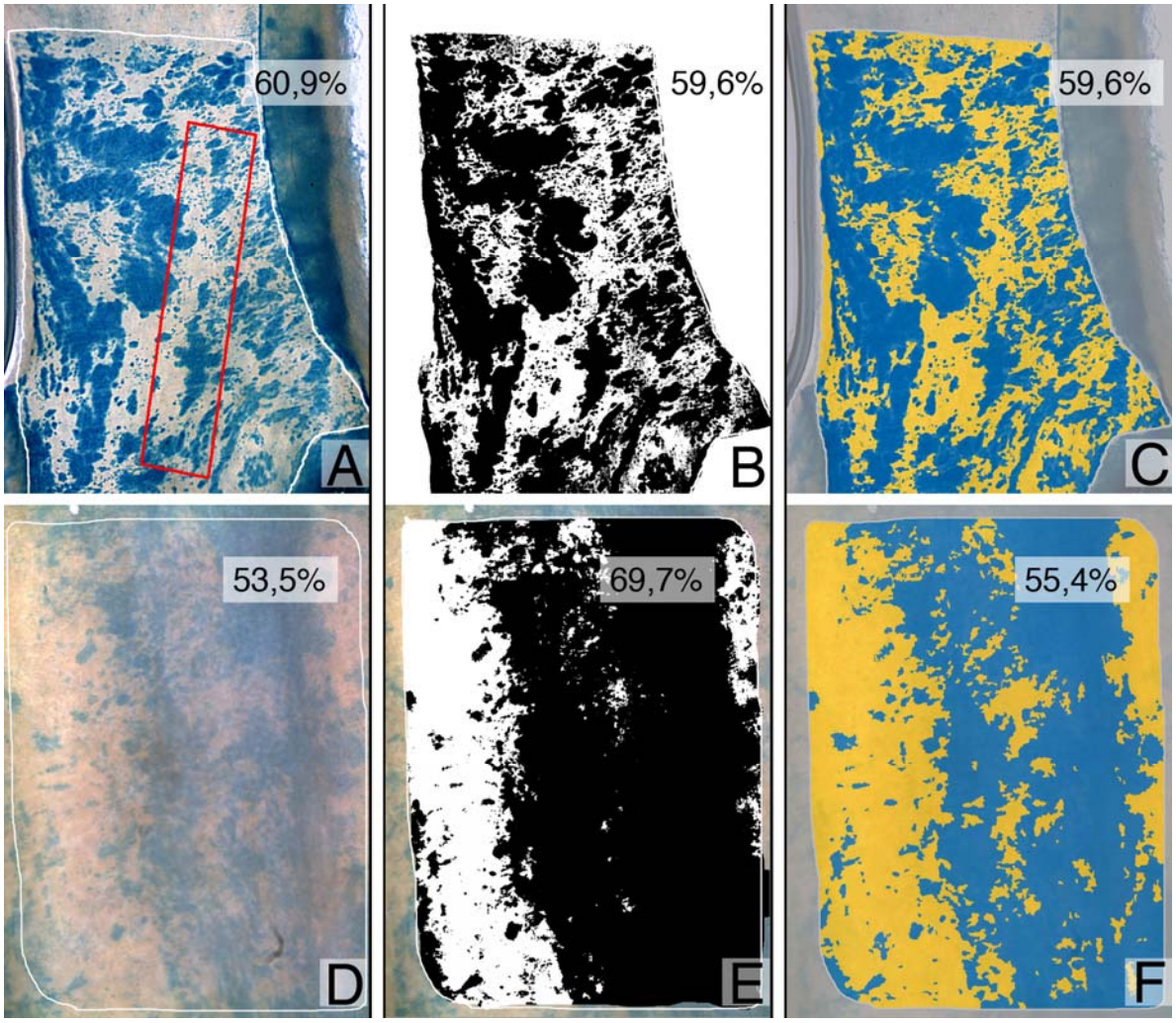

Figure 7. Conversion of digital stereophotographic images of the blood vessel inner surface as the basis for computer -aided quantification of intimal damage. (A) Stereomicrograph of the intimal surface of a human saphenous vein segment after standardized incubation in saline at an intravasal pressure of 100mmHg and subsequent staining with alcian blue in homogenous incident light. (B) Same image after threshold-based black/white conversion prior to evaluation using the histogram technique of Adobe's Photoshop software. (C) Conversion to a blue/yellow-image using the morphometric S.CORE technology. The numbers in each panel indicate the relative percentage of deendothelialization measured by the different methods (D) Stereomicrograph of an inadequately prepared and photographed vein segment after analogous treatment with saline and alcian blue, (E,F) analogous conversions and calculations as above.

pass segment (high and mostly unmonitored pressure generated by manual filling with a syringe and still open tributaries). Following this "manipulation" phase, segments were stored for 45 min in the same filling solution, during which, as is exemplarily shown in **Figure 8C**, the pressure fell continuously and asymptotically. Thereafter, segments were rinsed briefly, and fixed with glutaraldehyde at the same counterpressure. Intimal integrity was subsequently determined morphometrically as described above.

Influence of intravasal pressure and filling medium on intimal integrity and endothelial viability during intraoperative sealing and storage, simultaneously validation of various planimetry methods

Table 2 summarizes the experimental data. In-

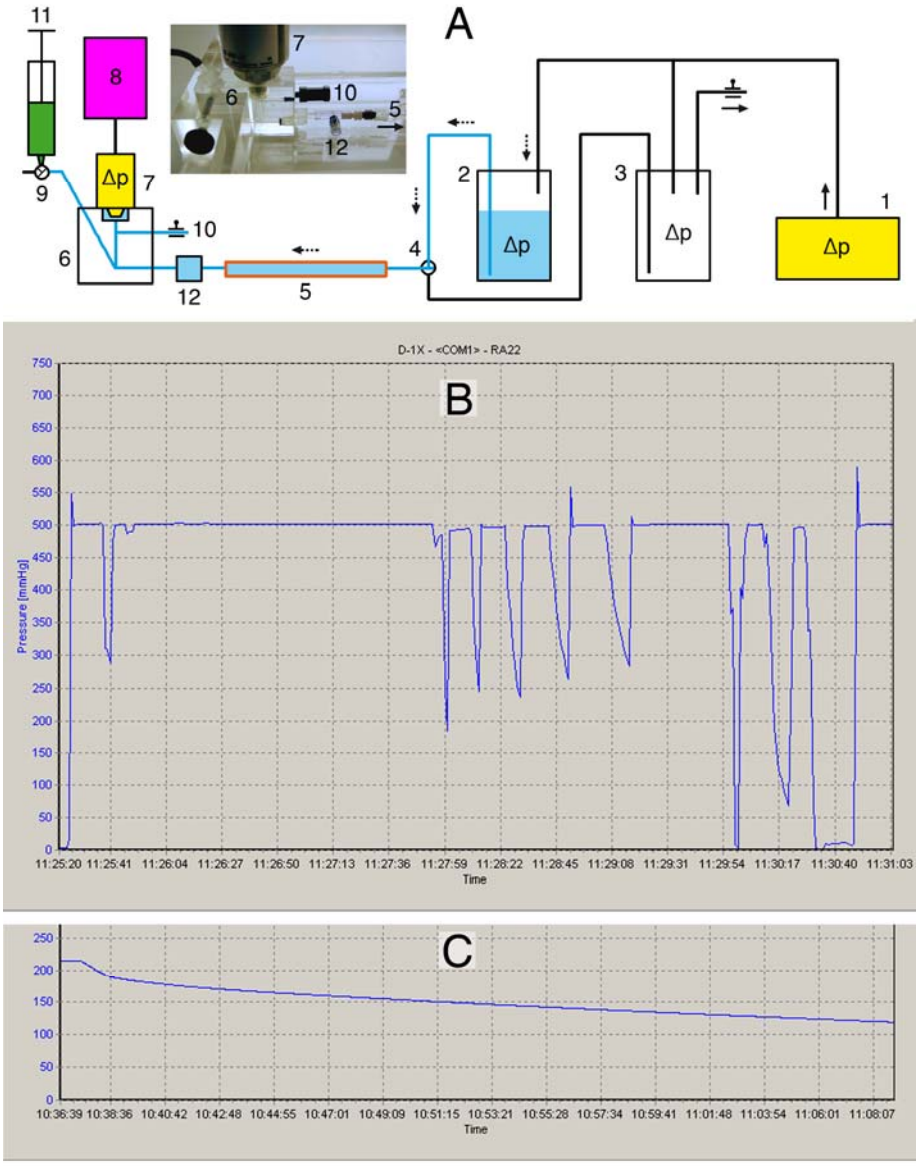

Figure 8. Manipulation and storage of saphenous vein segments under defined intravasal hydrostatic pressure prior to histological assessment of their intimae. (A) Diagram of the experimental set-up. A constant pressure generated by an electronically controlled transformer (1) is transmitted via tubing to two reservoirs (2, 3). Reservoir (2) contains the preservation solution, which passes via a 3-way tap (4) to the vessel under study (5) and then to a detection unit (6) where the pressure is detected by a transmitter (7). The transducer signal is registered on-line continuously by computer (8). The 3-way tap (9) and the stopper (10) are opened to fill the vein and the detection unit. By means of tap (9) pressure drops can be elicited as desired, shutting the tap allows the pressure to recover. At the end of the experiment, the taps (9) and (4) are set so as to allow passage of glutaraldehyde (or. in other experiments a protease mixture to detach the endothelium) from a syringe (11) at the set pressure in the vessel (5) and, if required to reservoir 3. (12) one way valve. (C) Online recording of intravasal pressure showing repeated filling-maneuvers to imitate the pressure variations that occur during the usual sealing procedure of the tributaries. (D) Typical slow pressure fall during storage of a vessel which had been filled at 200 mmHg.

creasing intravasal pressure was clearly associated with a decreasing degree of endothelialization in both the saline and plasma-derivative-filled segments. In saline-filled segments, the

Table 2. Influence of intravasal pressure during sealing and storage on the degree of intimal endothelialization in vessel segments filled with either saline or plasma derivative. The degree of endothelialization was determined quantitatively from digital stereophotographic images of the intimal surface by means of classical planimetry (1), from histograms after image conversion to black and white (Adobe Photoshop software) (2), or after conversion of the images to blue/yellow using the S.CORE technology (3).

intravasal pressure [mmHg]	storage in saline degree of endothelialization [% of total intimal surface] mean values ± SD n=5 per method			storage in plasma derivative degree of endothelialization [% of total intimal surface] mean values ± SD n=5 per method		
	method of quantitation			method of quantitation		
	1	2	3	1	2	3
50	58 ± 3	51 ± 8	61 ± 1	96 ± 4	93 ± 9	93 ± 7
100	46 ± 11	52 ± 14	53 ± 3	98 ± 2	96 ± 3	98 ± 2
200	44 ± 7	42 ± 11	42 ± 9	95 ± 5	91 ± 6	92 ± 5
500	8 ± 5	11 ± 8	15 ± 1	81 ± 13	70 ± 14	81 ± 9
1000	3 ± 3	3 ± 3	3 ± 2	73 ± 13	62 ± 22	74 ± 13

degree of endothelialization was only about 50% at intravasal pressures of 50-100 mmHg and a steep decline in the degree of endothelialization to practically zero was observed at higher pressures (see also **Figure 9A**). In contrast, the degree of endothelialization in the plasma-derivative-filled segments was reduced significantly only at pressures in excess of 200 mmHg, but was still very high (**Table 2**). Scanning electron micrographs of the intima from the plasma derivative-filled veins at high pressure showed that the normally widely overlapping intercellular regions had been drawn apart, but apparently relatively gently, so that all clefts of the now very flattened endothelial sheet remained closed (**Figure 9B**) and casual damage to the individual endothelial cells (**Figure 9C**) was rare. Both computer-aided planimetric procedures yielded practically identical results (**Table 2**), which, moreover, were fully consistent with data obtained from representative intima images and evaluated in the classical manner ("cut out and weigh").

Systematic evaluation of the viability of the endothelium remaining in the vessel segments showed that using saline as the filling medium caused almost complete cell death. In contrast, the majority of endothelial cells in segments filled with plasma derivative survived pressures even as high as 1,000 mmHg (i.e. 10 times average mean arterial pressure!) and showed the typical morphology during attachment and spreading in cell culture (**Figure 9D-G**). Moreover, they revealed the same growth behavior as described recently [2].

Discussion

Preparation of bypass remnants for morphometric evaluation of their intimal surface

To our knowledge, staining and preparation procedures practicable for use in clinical studies for quantitative 2-dimensional assessment of the intima in bypass vessels have not yet been

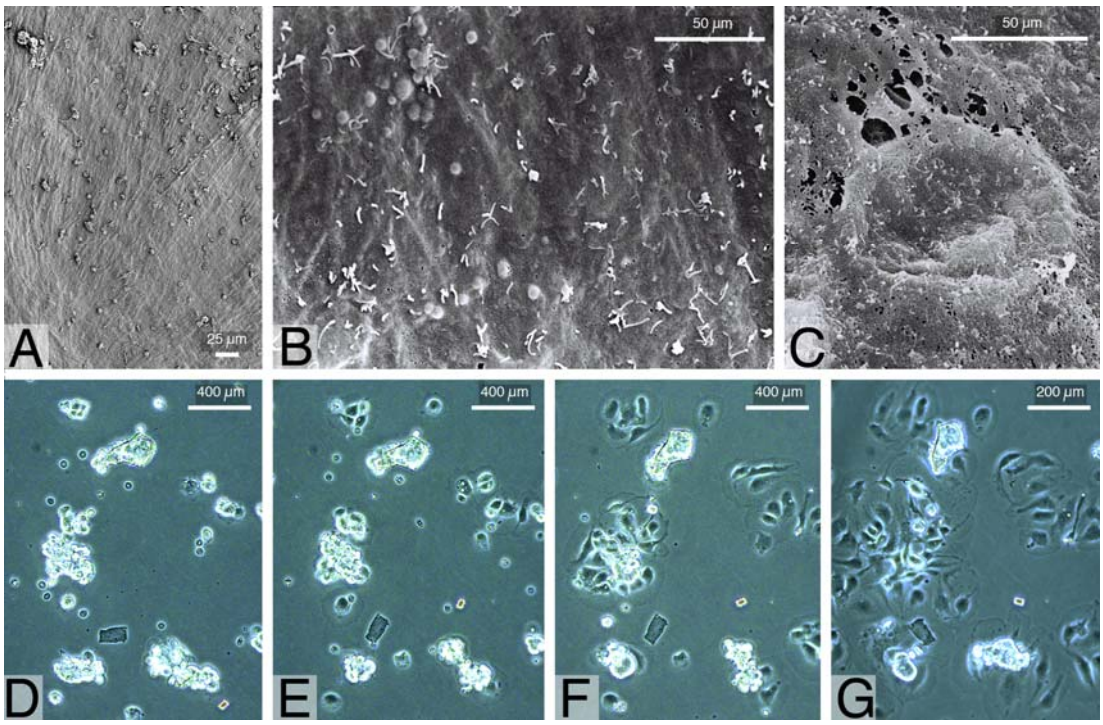

Figure 9. Microscopic evaluation of endothelial cell architecture and viability in venous segments after filling and incubation at a pressure of 500mmHg according to the standardized protocol of the present study. (A) Scanning electron micrograph of the intimal surface of a vessel after an experiment with saline as filling medium. (B) Analogous experiment after filling with plasma derivative. (C) Demonstration of the only scanty endothelial damage in the former experiment. (D-G) Phase contrast micrographs of proteolytically detached intimal tissue fragments 4, 8, 12 or 16 h after attachment to the culture dish. The viability in this culture was 88%.

described in the literature. The reliability of the results to be obtained will depend critically on the standardization of all steps in the harvesting, preparation, staining and photographic image acquisition of the tissue samples. The procedures described in the present study create a basis for such future studies and allow the rapid investigation of bypass remnants in pathology laboratories. This in turn raises the possibility to objective quality control of surgical explantation and bypass preparation procedures and for planning clinical studies with the goal of determining the best means of intraoperative sealing, preservation and storage of bypass segments.

Alcian blue: a highly specific and stable stain for demarcation of deendothelialized intimal areas

Alcian blue 8GX is a well known histological stain [8]. The attachment of 4 tetramethylisothiouronium groups to the phthalocyanine ring of the strongly cationic dye molecule make the compound to big to fit between the coils of the DNA helix [9]. Moreover, the weak electrostatic attraction of the dye between phosphate groups in DNA and RNA is abolished at pH<6, and (due to the given molecular distances within the nucleic acid backbone) there can be also no close-range interaction (due to van der Waals forces) between the aromatic rings of the dye and the purine and pyrimidine rings of the nucleic acids. The access of the relatively large Alcian blue molecule to the phosphate groups of nucleic acids is possibly also hindered by the presence of associated nucleoproteins. For all these reasons the dye stains nuclei only at pH>6 [10]. On the other hand, Alcian blue 8GX binds strongly to carbonyl and sulphate-ester groups of glycosaminoglycanes [11] at pH <3 (which is the approximate pH of the commercially available dye solution), but selectively to the latter at pH 1.0. In addition to its specificity another advantage of this dye over other cationic dyes is that it is not extracted from stained samples by water, ethanol, weak acids, or solutions usually employed for histological counter-

Table 3. Influence of intravasal pressure during sealing and storage on the degree of endothelial viability in vein segments filled with either saline or plasma derivative

intravasal pressure [mmHg]	saline endothelial viability [% initially seeded cells] mean values ± SD n=3 per segment	plasma preparation endothelial viability [% initially seeded cells] mean values ± SD n=3 per segment
50	segment 1: 22 ± 8 segment 2: 20 ± 14	segment 1: 92 ± 3 segment 2: 95 ± 4
100	segment 1: 20 ± 5 segment 2: 22 ± 6	segment 1: 95 ± 5 segment 2: 92 ± 4
200	segment 1: 16 ± 3 segment 2: 10 ± 4	segment 1: 93 ± 5 segment 2: 92 ± 5
500	segment 1: 6 ± 6 segment 2: 6 ± 6	segment 1: 95 ± 3 segment 2: 91 ± 3
1000	segment 1: 2 ± 2 segment 2: 2 ± 2	segment 1: 90 ± 5 segment 2: 88 ± 4

staining. Alcian blue-stained bypass remnants can thus be stored for long periods (months) in diluted glutaraldehyde solutions.

With respect to blood vessels Alcian blue has been used for specific staining of freshly isolated intimal sheets ("Häutchen-preparations" [12]), the endothelial glycocalyx [13], and for demonstrating the presence of acidic mucopolysaccharides in the intima of microvessels [14] or experimentally injured [15] or atherosclerotic [16] aortae. As far as we are aware, Alcian blue staining has to date been used in this context only on cross-sections of vessels, so that its value for demarcation of intimal damage has not yet been recognized. Control experiments including scanning electron microscopy, silver staining and immunohistological staining reproducibly verified those areas of the vascular intima stained by Alcian blue at pH 2-3 as areas with damaged endothelium. Moreover, Alcian blue applied intravasally at low pH also clearly visualized even smallest increases in endothelial permeability, for instance those resulting from the opening of endothelial clefts ("functional" damage, not resulting from injury to the endothelium, but rather from the loss of the latter's reactive barrier function, in contrast to "structural" damage resulting from mechanically induced barrier loss). In view of the presence of strongly thrombogenic subendothelial pericytes in blood vessels [2, 3], clinical studies aimed at improving bypass quality will need to quantify and document such functional intimal leaks also, since wide-open endothelial intercellular clefts can result in induction of intravasal thrombosis just as well as mechanical damage to the endothelium.

Quality assessment of bypass vessels using computer-assisted image analysis and quantitative planimetry

Automatic and objective image analysis for the assessment of biological preparations is technically demanding in general, and all the more so for the blood vessel samples analysed during clinical studies.

Numerous factors, which can be eliminated only in part by standardization of preparation, staining and photographic conditions can lead to substantial variation in image quality. The factors include variations in tissue thickness (which result in variations in the intrinsic tissue

color), differences in surface structure (resulting in reflections and shadows), differences in staining intensity due to local tissue inhomogeneity, and the occurrence of non-constant anatomical structures (vessel branches, thrombotic depositions etc.).

Standardization notwithstanding, considerable variation in the visualization of the inner vessel surface remains, in particular when dealing with larger series of samples obtained under the conditions in an operation theatre. For this reason, conventional image analysis techniques, which assess the damaged (i.e. stained) areas as those areas in which the value of a given colour (or, in the case of the use of Photoshop histograms, the intensity of grey) exceeds a predefined threshold, are not completely reliable. That this did not apply in the present study, however, speaks for the strict observation of the standardization protocols, in particular for the homogeneity of illumination achieved with the illumination system, and the resulting high quality of the digital images (see Methods).

The automatic morphometry system S.CORE combines a threshold-based system with object-oriented analysis technology and can analyze even poorly illuminated intimal surfaces with a high degree of reliability. This is made possible by the program's recognition of color intensity relative to the surroundings as well as the recognition of characteristic morphological structures that can occur when the endothelium is damaged (e.g. clumped, sharp-edged structures around fibrin depositions). The technical capabilities of the S.CORE analysis unit are based on the combination of various software components, each optimized for the user's specific demands (e.g. object recognition, data processing, statistics etc.). Via interfaces, the individual software components are combined to an integrated software package, in which the strengths of the individual components are bundled. The result is an analysis unit that allows an extremely high degree of automaticity for the most varied tasks. A particular advantage is that the user can access, via the Internet, S.CO LifeScience's powerful central image analysis system with the combined high-performance computers of the Technical University of Munich. The procedure for the quantification of intimal lesions described here can thus principally be employed anywhere in the world.

Endothelial integrity and viability in coronary bypass vessels of venous origin. Influence of filling solution and intravasal pressure during sealing and intraoperative storage

The clear dependency of endothelial integrity in bypass vessels on the composition of the solution used for intraoperative preservation seen in the present study confirms that seen earlier [3]. Saline, in contrast to the plasma derivative, proved to be by no means a "preservation" or "physiological salt" solution. At least after exposure to saline, at even low intravasal pressure, large areas of endothelial denudation were observed, which give rise to an alarming thrombogenicity of the investigated graft remnants [3].

Indeed, in view of this earlier study, it is not surprising that adverse physical conditions during preparation, sealing and intraoperative storage up to implantation have deleterious effects on intimal integrity too, and that this is intimately related to the poor clinical prognosis of aorto-coronary grafts of venous origin [4-7]. One factor that immediately attracts attention in this respect is the extreme variation in intravasal pressure that can occur when freshly explanted veins are filled under pressure with saline, histidine-tryptophan-ketoglutarate (HTK) solution or other "preservation" solutions to detect and ligate or clip side branches. In general this is done manually using a syringe and pressures in excess of 1,000 mmHg can be generated easily. However, since the pressure at which the human saphenous vein bursts is considerable higher [17] (a fact which can now be easily explained by the well developed connective tissue cuff of the adventitia and the multiple rings of collagen fibre bundles, compare Fig1A) is not immediately apparent that the pressure generated is in any way critical. A further key point for intraoperative lesioning of the bypass vessels is probably the usual practice of storing the prepared grafts prior to implantation in an empty state. Since empty veins collapse completely due to their thin walls and the elastic retraction of their intimae, the opposing intimal surfaces come into contact with each other, whereby the resultant frictional and suctional forces lead to large areas of endothelial rupture. Since it therefore seemed advantageous to store grafts in a filled state prior to implantation, we always included a 45-min storage phase at a defined filling pressure in our pressure experiments. The

initially applied pressure in this phase always fell characteristically, i.e. slowly and asymptotically. Although we did not follow this up, the explanation may lay in microscopic transmural veins that drain the Vena venarum and empty into the vascular lumen. Such tributaries could therefore account for slow retrograde outflow from the vein lumen, as has been hypothesized repeatedly in the past [18-22]. In any case, the rate of loss of intravasal volume from the stored segments was significantly larger than could have been expected on the basis of the known hydraulic conductivity of intact venous endothelium [23]. In addition, the segments never collapsed completely within the storage period, thus excluding frictional forces as a cause of endothelial damage.

In saline-"preserved" grafts filled to pressures in excess of 100 mmHg we already observed a significant decline in the degree of endothelialization. Apparently not only the recently demonstrated inadequacies of this "preservation solution" [3], but also the unphysiologically high intravasal pressures contribute to this decline. The stretch exerted on the whole vessel wall may well result in excessive tension in the well developed junctional complexes of the rather tight intercellular clefts of the luminal endothelium, leading to loss of plasmalemmal integrity and reduced endothelial viability. Surprisingly, this effect was markedly reduced in vessels filled with the plasma derivative, the composition of which closely approached that of the native "incubation" solution of the endothelium: autologous blood plasma. During preservation in the plasma derivative the degree of endothelialization declined only at pressures over 250 mmHg. Indeed, even at pressures up to 1,000 mmHg, more than 70% of the endothelium in the plasma-preserved grafts remained intact and, after proteolytic detachment of the remaining endothelial cells with collagenase, the latter were shown to be vital, in contrast to analogously isolated cells from saline-filled veins. Presumably the complex mixture of proteins in the plasma preparation plays an important structure-preserving role under these circumstances. Indeed, albumin, present in the plasma in high concentration, is known to reduce shear forces at the inner blood vessel wall, and numerous other plasma proteins form integrated components of the endothelial glycocalyx [24-30] without which the vascular endothelium is unlikely to be able to maintain its barrier function and its anchorage to the remaining vessel wall.

In summary, the quality of bypass vessels depends heavily not only on the composition of the preservation medium, but also on the pressure at which this medium is kept in the vessel. Development of a device for pressure-controlled perfusion of blood vessels would be of very great advantage in this respect.

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Please address correspondence to: Stephan Nees, MD, Department of Physiology, University of Munich (LMU), Schillerstr. 44, 80336 Munich, Germany, TEl: +49 6022 9508, Fax: +49 6022 710641, E-mail: stephan.nees@med.uni-muenchen.de

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