Int J Clin Exp Med (2009) 2, 95-113
www.ijcem.com/IJCEM905001

Original Article

Extensive deendothelialization and thrombogenicity in routinely prepared vein grafts for coronary bypass operations: facts and remedy

Dominik R. Weiss[1*], Gerd Juchem[2*], Bernhard M. Kemkes[3], Brigitte Gansera[3], Stephan Nees[4]

[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 Cardiac Surgery, Hospital Bogenhausen, 81925 Munich, Germany; [4] Department of Physiology, University of Munich (LMU), 80336 Munich, Germany

Received May 16, 2009; accepted May 25, 2009; available online May 28, 2009

Abstract: The objective of this study was to gain deeper insight into the early reasons for saphenous vein graft disease and to find a practical approach to obviate it. Intraoperative storage of freshly explanted venous grafts (45 min, 20°C; n=25 in each case) in saline, saline + 5% albumin, or HTK-solution and also in heparinized autologous blood was poorly tolerated by the endothelium. Large endothelial areas (mostly >75% of total surface) were detached already during brief non-pulsatile flushing just before the transplantation. Contact of deendothelialized areas in graft remnants with defined mixtures of coagulation factors or blood (n=11-17) caused rapid coagulatory processes via expression of tissue factor and assembly of prothrombinase in the subendothelium. Attached platelets and leukocytes accelerated the procoagulatory processes further, and endothelium-dependent anticoagulatory activities were significantly abolished. During pulsatile arterial flow, the resulting blood clots exacerbated the damage of the intima markedly, because they were flushed away tearing off further endothelium. In contrast, storage of venous grafts in a plasma preparation freed from isoagglutinins and coagulation factors preserved the endothelium, which resisted arterial flow and revealed anticoagulatory activity in the presence of antithrombin III and/or protein C. We conclude that gentle preparation and preservation of the vascular endothelium with a suitable storage solution during bypass surgery is a decisive first step to obviate saphenous vein graft disease.

Key words: Saphenous vein graft disease, endothelium, thrombosis, bypass operation, atherosclerosis, pericyte, tissue factor, percutaneous intervention, CABG, subendothelium, vulnerable plaque

Introduction

Despite the superior patency rate using the internal thoracic arteries [1, 2] or vessel segments of other arterial origin [3, 4] as grafts during coronary artery bypass grafting (CABG), the prevailing use of graft segments of venous origin, mostly saphenous vein (SV) grafts (SVG), is in most cases unavoidable. The associated clinical problems are grave. Some 4-500,000 CABG are performed annually in the USA [5], whereby 15-30% of SVG occlude acutely within the first year [6], and about half of these already within the first 2 weeks post-operatively. About 50% of the remaining venous grafts occlude in the following 9 years, with the frequency increasing from year to year, and of those remaining patent after a decade, more than the half shows angio-graphic evidence of severe stenosis [7-9]. The developing pathogenetic processes are subsumed under the term "saphenous vein graft disease (SVGD)" [6, 10]. Should this lead to recurrent ischemia, the resulting thera-peutic challenge is considerable. Revascu-larization options include either repeated CABG or percutaneous interventions (PCI). The former is associated with poorer outcomes, compared with first-time CABG, and increased

* Both researchers contributed equally to the work

perioperative morbidity (3-11%) and mortality (3-7%) [6, 11]. The latter (PCI) achieves at most a postponement of the unavoidable bypass operation. Drug-eluting stents have not improved the situation [12-14]. In view of the substantial total health care costs and the continued threat to many patients, and despite advances in operation techniques, it must be admitted that the prognosis for most CABG, and especially those involving venous grafts, is still fairly limited and thus unsatisfactory.

Given this background, it is clear that the pathogenesis of SVGD is attracting intensive, world-wide research interest [15] in order to improve the quality of coronary grafts of venous origin. Three temporally distinct disease processes can now be identified: acute thrombosis [6], intimal hyperplasia [6, 10, 15], and atherosclerosis [16-18] often followed by infarction [6, 10]. Although these pathological processes are very similar to those in native coronary arteries, there are a number of temporal and histological differences in the atherosclerotically diseased vein segments [6, 19]. One of the major differences is the particularly rapid progressive nature of atherosclerosis in SVG. A further difference is the diffusive, concentric and friable modeling of the plaques. Also conspicuous is the fact that the function of venous grafts ends very frequently by a sudden activation of coagulation in the stenosed regions. In one study for instance, fibrin thrombi were responsible for the occlusion in 69% of the grafts resected in patients undergoing repeated bypass grafting [20].

There is general agreement that the trigger for SVGD is probably damage to, or even loss of, the endothelial coating. Corresponding comparative and quantitative investigations are, however, not yet available in the literature. The multifunctional vascular endothelium [16, 21-23] shields the intima from the blood and is especially endangered in the course of the usual operative manipulations during explantation, sealing and intraoperative storage [24, 25]. This should be alarming, since a key role must be attributed to this tissue with respect to the maintenance of homeostasis within the vessel wall and in the induction of atherosclerotic processes [16]. However, despite the present description of various intimal cell types, their assumed interactions, and elucidation of many of the individual biochemical processes in atherosclerotic lesions [17, 18], the fundamental processes responsible for the initial loss of endothelial barrier functions and the induction of the complex reactions leading to the resultant plaques, are presently still vague, particularly in venous grafts. Crucial questions are, for instance, how an endothelial lesion can immediately lead to induction of fibrin thrombi [6, 20]; why, in the middle to long term, venous grafts almost always develop proliferative wall thickening with progressive lumen narrowing [7, 8]; and why a still developing plaque suddenly ruptures, thus becoming the focus of a fulminant thrombosis that frequently seals the patient's fate [26-29].

The discovery of intimal pericytes (IP) in direct neighbourhood to the luminal endothelium in the subendothelium of arteries and veins may be an important key to a better understanding why thrombotic events are often associated with atherosclerosis. These constitutive IP, first mentioned in the literature some time ago as "pericyte-like cells" [30], apparently express exceptionally high concentrations of tissue factor (TF) and prothrombinase on their surface and therefore resemble pericytes of microvascular origin [31, 32] also in this respect. Should vessels lose their endothelial layer shielding these cells from the blood, the exposed IP could probably become an immediate focus for rapid coagulation processes.

Using a standardized surgical explantation procedure and various common preservation solutions (or also heparinized autologous blood) for the intraoperative storage of venous graft remnants, we were able to show that the widely used routine graft preservation protocols led, in the vast majority of cases, to extensive or even complete loss of the endothelium. As a consequence, the pericyte-like cells in the subendothelium were exposed, which were apparently more firmly anchored to the vascular intima than the endothelium itself. A further objective was to study the hemostasiological consequences of endothelial denudation quantitatively. Finally, we could demonstrate that the high thrombogenicity of deendothelialized venous grafts could be avoided readily by a "no-touch" vessel preparation, carefully controlled perfusion, and by intraoperative storage in a commercially available plasma preparation (PP) freed of isoagglutinins and coagulation factors.

Materials and methods

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

Solutions

Balanced salt solution (BSS-FCS or BSS-BSA, respect.,): 142mM NaCl, 5mM KCl, 1mM NaH_2PO_4, 0.8mM $MgSO_4$, 1.25mM $CaCl_2$, and 55mM glucose, supplemented with 10% v/v FCS or 0.1% (w/v) bovine serum albumin (BSA), respectively; pH 7.4.

Endothelial cell culture medium (ECCM): human endothelial-SFM basal growth medium (Invitrogen, USA) supplemented with 10%v/v FCS, 200 U/ml penicillin, 0.2 mg/ml streptomycin, 1 μg/ml fibronectin, 10 ng/ml EGF, and 20 ng/ml bFGF.

Tris-buffered Krebs-Ringer solution (TKR): 120mM NaCl, 2.7mM KCl, 5mM $CaCl_2$, 50mM trishydroxymethylaminomethane (TRIS), 8.3 mM glucose, 2mM pyruvate, 11.4mM creatine, 20mM taurine, 5mM ribose, 2mM L-aspartic acid, 2mM L-glutamine, 1mM L-arginine, 0.5mM uric acid, adjusted to pH 7.40 with HCl and supplemented with 1mg/ml BSA.

Stopping buffer: 50mM TRIS, 120mM NaCl, 2.7mM KCl and 100mM EDTA, adjusted to pH 10 with HCl and supplemented with 1 mg/ml BSA.

Formaldehyde/glutaraldehyde(FG) fixative: 80 mM NaCl, 50 mM NaH_2PO_4, 4% formaldehyde and 2,5%glutaraldehyde (w/v, each), adjusted to p_H 7.4.

Plasma preparation (PP): commercially available plasma without isoagglutinins and coagulation factors (Biseko® from Biotest AG, 63303 Dreieich, Germany).

Preparation of SV segments

Patients: Between January 2004 and April 2007, a total of 293 patients undergoing first time elective CABG were studied. All participants showed isolated coronary artery disease and received, besides both mammary arteries, at least one additional SVG. Exclusion criteria included patients undergoing emergency CABG, but not those with severe varicosis of the SV, as well - for preparative histological reasons - vein diameters less than 2.5mm.

Surgical techniques: Portions of distal SV were harvested performing a no-touch technique to reduce vein injury. After fixation of flexible vessel cannulae at both ends (Medtronic 30004) and ligation of visible side branches with 4/0 sutures, dissection was performed with great caution. After explantation, the vein segments were tested for leakage by exposure to a moderate intraluminal hydrostatic pressure (approximately 200 mmHg) by means of a syringe connected to the cannula at the distal end of the vein and brief occlusion of the proximal cannula. The vessels were then rinsed in a selected preservation solution (saline, saline plus 5%(w/v) human albumin, HTK-solution, autologous heparinized blood or PP) and stored therein at room temperature until implantation (on average for about 45 min). The present study investigated residual vein remnants (3–8 cm long, diameter > 2.5mm). After the storage period, the bypass remnants were circumfused for 2min with *BSS-BSA* at a flow rate of approximately 250ml/min and either transported in ice-cold DMEM+10%FCS+10 IE/ml heparin into the lab for hemostasiological investigations, or immediately fixed for morphological studies by perfusion with FG-fixative at a transmural pressure gradient of 100 mmHg for 15min.

Preparation of porcine ear veins: pig ears were obtained immediately after slaughter in the local abattoir and flushed free of blood (via a syringe and the major auricularis vein) with ice-cold DMEM+10%FCS+10 IE/ml heparin. After transport on ice into the lab, a segment without side branches was identified and tied off from the rest of the vessel. After warming up to 37°C, both ends of the segment were cannulated, filled with especially composed coagulation assay mixtures and processed as is described below.

Isolation and cultivation of VEC

Freshly obtained residual segments of coronary bypass grafts were stored in heparinized, ice-cold BSS-FCS and transported to the lab.

The endothelium could then be almost selectively detached 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) BSA. After gentle flushing with PBS the cells were washed twice with ECCM by centrifugation, resuspended in ECCM, and cultured in Falcon dishes or on FBS-pre-coated polyester Transwell inserts (Corning, NY, USA).

Time-lapse microcinematography

Confluent endothelial layers were incubated (37°C, 5% CO_2 in water-saturated air) on the stage of an inverted microscope (Zeiss-Diavert 100). Series of images were acquired using a high-resolution digital camera (Zeiss AxioCam Color, one frame every 2 min) controlled by the manufacturer's software (Zeiss AxioVision Rel 4.5 SP1), which was also employed for image analysis.

Quantitation of endothelialization

Vein segments were fixed [33] (under approximately 100 mmHg intraluminal pressure) and silver impregnated [34] essentially as has been reported previously. Digital images of the luminal surface were acquired at a primary magnification of 100x using bright field microscopy. Care was taken to ensure that all borders of successive individual images overlapped. The individual images were then digitally assembled to a fused image of the entire intimal surface. The deendothelialized areas could be distinguished readily from intact intima due to their dark brown staining. These lesioned intimal areas were quantified using computer-based planimetry techniques and expressed as a percentage of the total area.

Hemostasiological assays

Chemicals: Coagulation factors II, VII, X, and IX were contained in the prothrombincomplex (PPSB)-solution Octaplex 500 (Octapharma, Langenfeld, Germany). Factors V_a, VII_a, and X_a was obtained from American Diagnostica (Pfungstadt, Germany), factor VIII from Biotest AG (Dreieich, Germany). The X_a-substrate S-2222 (Chromogenix) was purchased from Haemochrome Diagno-stica (Essen, Germany).

Antithrombin III (AT-III) was purchased from Sigma-Aldrich (Munich, Germany), protein C (PC) was generously supplied by Immuno Heidelberg, Germany.

Photometric tests for Faxtor X activation: The following procedures are basically modifications of methods described by Le [35]. Briefly, the functional activity of VIIa-TF complexes on the endothelium-denuded luminal surface of the "preserved" vein segments was quantified by their ability to activate factor X after filling the vein lumen with appropriate assays. In a series of experiments, veins were filled with prewarmed Octaplex solution adjusted to 2 IU/ml factor X (dilution with TKR) and supplemented with 400 ng/ml factor VII_a. The concentrations of factors II, VII and IX in the Octaplex solution were roughly similar to that of factor X. In other experiments, we added V_a und VIII and adjusted all coagulation factors to a final concentration of 400 ng/ml. In inhibitory experiments, AT-III or PC were added at 200 ng/ml.

After incubating a vein segment for 45min at 37°C, its content was collected in a cup, the segment rinsed into the same cup with approximately twice the incubation volume of TKR, and the total volume *V* determined by weighing. Aliquots (200 µl) of this mixture were then added to 70 µl ice-cold stopping buffer and centrifuged at 10,000 *g*. The amount of X_a in 100 µl aliquots was determined in triplicate by mixing with 100 µl substrate solution (1 mg S-2222/ml H_2O) and measuring the initial rate of colour development at 405 nm. These values were converted to $pmol \cdot ml^{-1} \cdot min^{-1}$ X_a from a standard curve obtained by adding 100 µl S-2222 solution to 100 µl of serial dilutions of an aliquot of the Octaplex solution that had been incubated with sufficient thromboplastin (Thromborel S, Dade-Behring, Eschborn, Germany) to reach a final X_a concentration of 256 nM. Values were expressed subsequently as pmoles X_a produced per minute per millilitre volume *V*. The dilution error inherent in this calculation was corrected for by the photometric determination of the actual intravascular volume of the vein segment (*V**). The latter was determined by filling the segment at a pressure of 100 mmHg via a 3-way tap with a solution of Evans blue of known light extinction in TKR-solution containing 100 µM papaverin, diluting the blue compartment after an incubation period of 5min at room temperature with the known volume of TKR in a connected flask and correcting the

calculated volume for the known volume of the connectors. The ratio V^*/V was the dilution factor for the assay solution. Assuming a cylindrical luminal form, the intimal "surface area" could also be estimated from V^*, the measured length, and the factor X activation rate.

Scanning electron microscopy and quantification of platelet and/or PMN adhesion

Specimens were fixed in FG-fixative over night, post-fixed for 2h in 1% (w/v) aqueous OsO_4, dehydrated using a graded ethanol series, and dried in the critical point dryer (Balzer CPP 020, Bal-Tec, Walluf, Germany) with liquid CO_2. After mounting on aluminium stubs the samples were immediately sputter-coated with gold (Polaron E 5000, Plano, Marburg, Germany). Specimens were examined at 15 kV on a scanning electron microscope (JSM-35 CF, JEOL, Tokyo, Japan). The luminal vessel surface was examined perpendicularly. Platelets and PMN in defined areas were counted directly on the microscope monitor until the SEM was < ±15 %.

Statistics

The values for the hydrodynamic conductivity of endothelial cell layers during incubation in various preservation solutions were graphically expressed as means ± SEM. Differences of all other experimental group data were analysed and displayed via boxplots, in order to indicate the degree of dispersion and skewness in the data via the spacings between the different parts of the boxes, as well as outliners. Comparisons of two group means were performed with the Student's *t* test, differences were considered significant at a probability level of <5% (P<0.05).

Results

Morphology and barrier function of cultivated endothelial layers of venous origin incubated in various graft preservation solutions

Figure 1 shows the effect of incubating confluently cultured human SV endothelium in media used clinically for intraoperative storage ("preservation") of SVG during CABG. Tissue architecture was documented by digital time-lapse microcinematography.

Figure 1A shows exemplarily that the endothelial sheets began to disintegrate immediately in saline. After only 10 min, the individual endothelial cells (EC) had lost contact to their neighbours and began to die (indicated by rounding) and, after 2 h, more than 40% of the cells were dead. Addition of 5% albumin to saline (not shown) improved the situation somewhat, although after 2-5 h cells died also in increasing numbers. Likewise, the tissue also failed to maintain its integrity in HTK-solution (Bretsch-neider's cardioplegic solution) [36] (**Figure 1B**). After already 20 min the endothelial clefts were widely open and after 2 h the still flat cells appeared "flaked" and paralyzed in the time-lapse recordings. Only in a commercially available PP that contained all major plasma components apart from isoagglutinins and coagulation factors (**Figure 1C**), could a long-term stability and survival be observed. Increasing the incubation temperature to 37°C, which in the other solutions merely accelerated tissue break-down, actually elicited cell division and proliferation.

The above morphological alterations were also reflected in the barrier function of the tissue layers. Using a simple experimental approach and a sensitive measuring device (inset **Figure 2**), the extent of transendothelial filtration under a constant hydrostatic pressure gradient (the "hydrodynamic conductivity") could be measured and recorded continuously. The curves shown in **Figure 2**, obtained at room temperature, illustrate that only the endothelium kept in PP maintained its barrier function (low and constant hydraulic conductivity $L_P = 3 \pm 0.7 \cdot 10^{-8}$ cm/sec • cm $_{H2O}$).

Condition of the intima of SV segments after sealing their tributaries and intraoperative storage in various solutions

Figure 3 demonstrates the morphological appearance of intimal areas of differently preserved SV grafts after silver impregnation. Panel A shows the characteristic pattern of the interendothelial junctions in a high-quality graft (preservation in PP). Endothelium-denuded areas with a conserved subendothelium stained clearly dark-brown (**Figure 3B,C**). Loss of the extracellular matrix (ECM) that normally enveloped cocoon-like the IP, allowed the demarcation of the cellular network of the latter in such regions (**Figure 3D,E**). These clearly star-like, branched cells

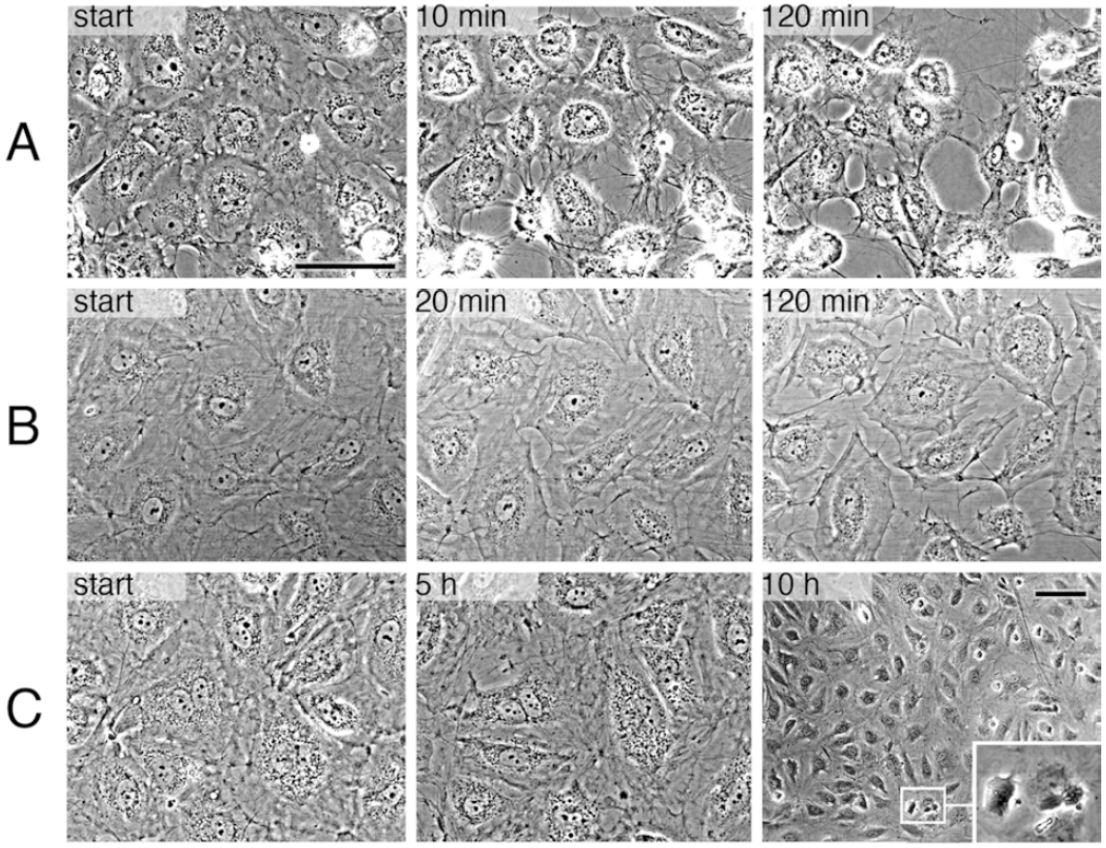


Figure 1: Behaviour of cultured EC from adult human SV during incubation in various graft preservation media. Incubation at room temperature in saline (row A: 0, 10 and 120 min), HTK (row B: 0, 20 and 120 min) or PP (row C: 0, 5 h and 10 h (37°C, the inset shows dividing cells)). Common bar (80μm), with an exception in the last picture in row C (bar, 250μm).

could be distinguished readily from the paving stone-like pattern of the endothelium. Given this histological experience and using computer-based planimetry techniques, we were able to quantify the degree of endothelialization, expressed as a percentage of the entire luminal surface. Four quality classes I–IV for vein grafts were defined with 75-100%, 50-75%, 25-50% or 0-25% endothelial coverage respectively.

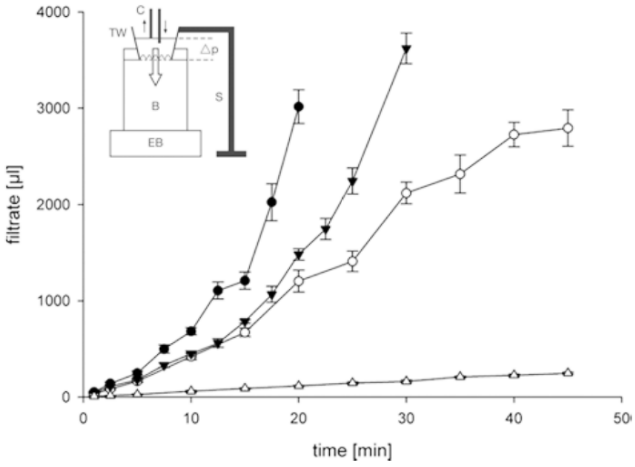


Figure 2: Permeability of cultured confluent endothelial layers exposed to graft preservation solutions. Inset: schematic drawing of apparatus, TW Transwell culture filled with preservation medium (PM), S stand to which TW is attached, C cannulae maintaining a constant pressure gradient Δp of 1.5cm H_2O via peristaltic pump and reservoir, B beaker filled with resp. solution, filtrate (boxed arrow) is monitored with an electronic balance EB. Time course of transendothelial filtrate of: saline (solid circles), HTK (solid triangles), saline + 5% albumin (open circles), PP (open triangles). Means ±SEM, n=8.

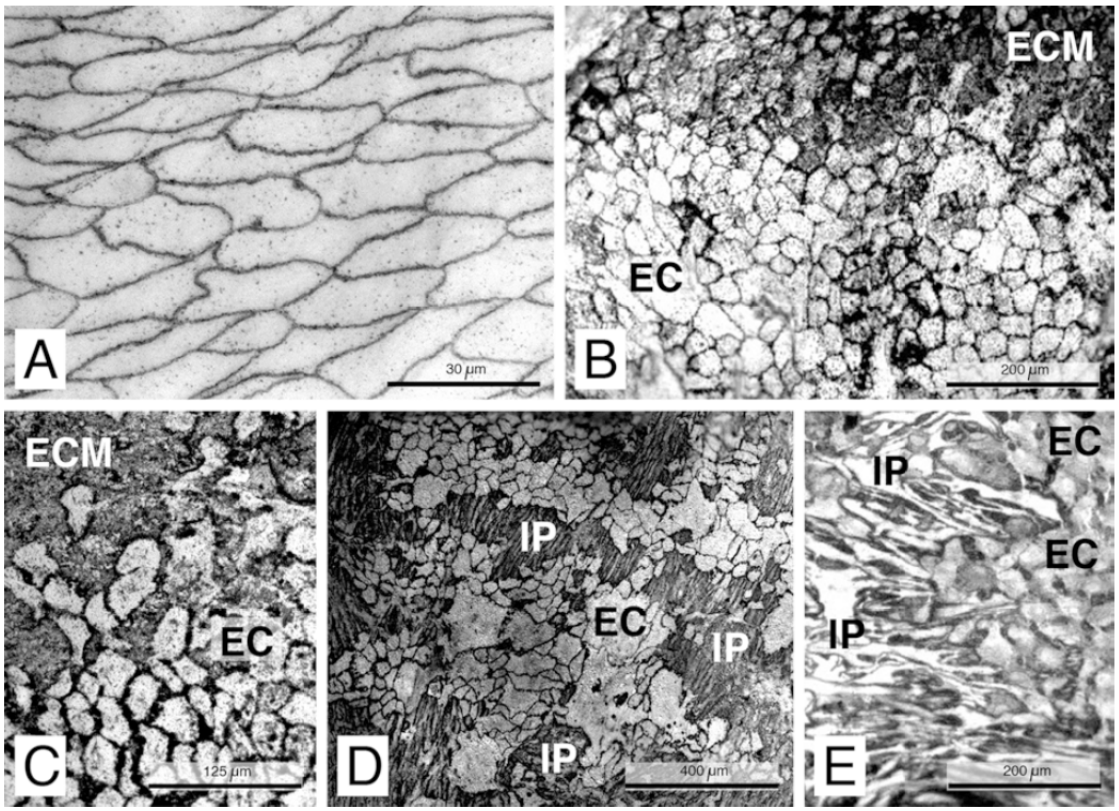


Figure 3: Intimal surface of surgically prepared SVG after silver impregnation. (A) almost entirely preserved endothelium in a class I vein; (B) partially disrupted endothelial sheet with preserved subendothelial ECM (class II vein); (C) enlarged view of a vein surface similar to B; (D) a vein intima similar to B, but with disrupted ECM; (E) enlarged view of a vein surface similar to D. *EC* endothelial cells, *P* platelets, *IP* intimal pericytes.

Table 1 summarizes the state of the intima of SV grafts after storage in various preservation media. Storage in surgically widely used saline, saline + 5% albumin, or HTK-solution for 45 min at room temperature, followed by brief rinsing with the same medium resulted in massive damage to and loss of the endothelium: approximately $^2/_3$ of the samples were classified as IV; about $^1/_3$ were in class III. Astonishingly few grafts could be classified in higher classes. In stark contrast, grafts stored in and rinsed with the PP displayed an almost entirely preserved endothelial barrier and belonged in their majority to class I.

Activation of coagulation in porcine ear veins after storage in various solutions

Since for human vein segments obtained under the conditions in the operation room it

Table 1: Quality and distribution of SVG between classes I–IV (see text) after their intraoperative preservation in various storage solutions. Listed are in each case the numbers and the percentages (values in brackets) of samples in the respective class, 25 SVG were stored in each solution).

Quality Category	Saline	Saline + 5% Albumin	HTK-solution	Plasma Derivative
Class I	1 (4)	0	0	19 (76)
Class II	2 (8)	2 (8)	2 (8)	6 (24)
Class III	7 (28)	8 (32)	7 (28)	0
Class IV	15 (60)	15 (60)	16 (64)	0

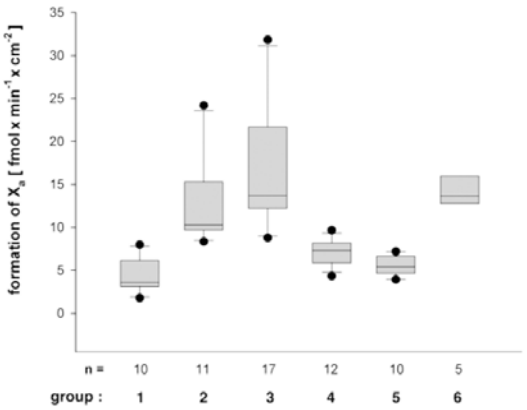


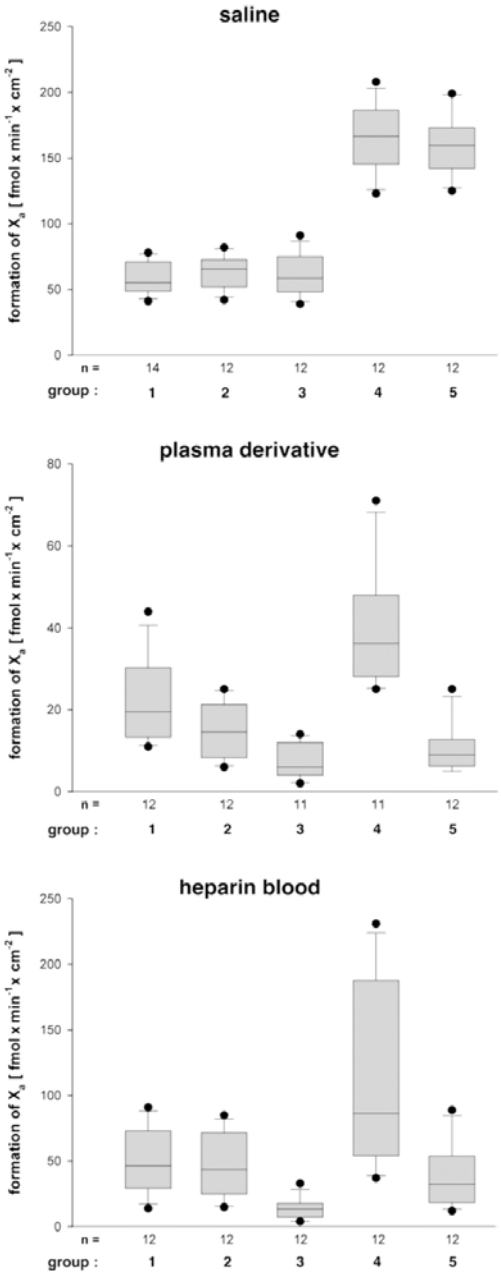
Figure 4: TF-mediated activation of coagulation factor X in porcine ear veins after intraluminal exposure to various storage solutions for 90 min. Experiments in groups 1-4 were performed at room temperature. Group 1: control, no prior exposure to preservation solution; Groups 2-4: FX activation after exposure to saline+ 5% albumin (group 2), saline (group 3), and PP (group 4); Groups 5-6: veins were treated as above (group 5: after exposure to PP; group 6: saline), but then perfused for 60 min at arterial pulsatile pressure with (rich) endothelial cell culture medium at 37°C (recovery period). Thereafter FX activation was assessed as above. Box-and-whisker plots; n values below the abscissa; P<0.001 groups 2-4 ; P=0,01 group 5 vs. control (Student's t-test).

might not be possible to distinguish intimal damage occurring during storage from mechanical damage during handling and sealing, we initially sought a vein preparation that could be studied without prior explantation („ideal no-touch vein "). A suitable object was the major auricularis vein in pig ears, which could be filled via fixed cannulae with incubation solutions optimized for assessment of coagulation factor X (FX) activation. The rationale behind this was that FXa would be formed only after direct contact between FX and TF, the latter being expressed selectively on the subendothelial IP and consequently accessible only at the site of an endothelial lesion. **Figure 4** summarizes the results of such studies. The basal rate of formation of FXa in freshly obtained veins without prior exposure to a "preservation medium" was 4.1±0.8 fmol/min per cm^2 (group 1). After a 90-min pre-incubation at room temperature in saline containing 5% albumin the median FXa formation rate more than doubled (2), and with saline alone tripled or quadrupled (3). Closer inspection of the box-plots shows that in these two groups a trend to higher values was always evident. On the other hand, the FXa formation rates in veins pre-incubated with the PP (4) proved to be only slightly higher (albeit significantly, $p<0.05$) than control and the distribution of the data was symmetric. Surprisingly, analogous experiments after pre-incubation with the HTK-solution could not be carried out, because the wall permeability of the pig veins increased so much that the subsequently added FX test solution simply leaked away into the surrounding connective tissue.

In a further approach, the conditions directly after implantation of veins as aorto-coronary grafts were imitated in the lab. Porcine ear veins, after exposure to the given preservation media as described above, were perfused for 60 min at 37°C under arterial conditions of pulsatile pressure and flow, with a rich cell culture medium prior to assessment of FXa formation. After this recovery period, FXa formation rate in veins formerly exposed to PP (group 5) was no longer significantly different from control, whilst in those exposed to saline (group 6) the FXa activation rate proved to be unchanged compared with group 3. These experiments carried out on ideal "no-touch veins" clearly demonstrated the superior preservation potency of the plasma preparation (PP) on intimal integrity in these pig vessels. Assuming that the degree of activation of FX in the ear veins reflected the degree of endothelial disruption in the individual experiments, these coagulation data yielded a qualitatively similar classification of endothelial integrity in the respective animal veins as in human vein grafts after exposure to the various media as was evaluated histologically (**Table 1**). This finding encouraged us to carry out analogous coagulation studies on human SV segments, in this case also varying the combinations of factors included in the test assay and examining the role of AT-III and PC, important cofactors for the initiation of anticoagulatory actions of healthy endothelium.

The upper panel of **Figure 5** summarizes the studies on such vein segments after intravascular pre-incubation („preservation") with saline. The data of group 1 correspond to those under group 3 of the porcine vessels in **Figure 4**, although the rate of activation of FX in the human vessels proved to be about 3 times higher. Allowing the vein segments to

recover prior to saline exposure in a rich culture medium at 37°C (group 2) or the addition of AT-III (group 3) did not alter the rate of formation of FXa. On the other hand, inclusion of factors IX, VIII, V_a and II accelerated the FXa formation considerably (group 4). This was apparently due to the arrangement of prothrombokinase-complexes at the surface of IP, a prothrombotic event that could not be significantly counteracted by the addition of Protein C (group 5).



The middle panel of **Figure 5** summarizes analogous studies on vein segments after prior intravascular exposure to the PP. All values proved to be considerably lower than after exposure to saline and, moreover, addition of AT-III or PC inhibited FXa formation now substantially.

The lower panel in **Figure 5** shows that FXa formation in vein segments pre-incubated with heparinized, matched whole blood was comparable to that in the veins exposed to saline, although now inhibition by AT-III and PC was marked.

Adhesion of neutrophils (PMN) and platelets to the luminal surface of SV segments after exposure to different storage solutions and subsequent exposure to heparinized, matched whole blood

Whilst all the storage solutions studied resulted in remarkable adhesion of PMN to the luminal surface (**Figure 6**, upper panel), adhesion of these leukocytes after exposure to the PP was highly significantly less (group 4). After exposure to all solutions the numbers of adhering platelets (**Figure 6**, lower panel, different scale of the ordinate!) were impressively high, with the exception of the experiments with PP as a storage solution (group 4).

Surprisingly, intravascular pre-incubation with heparinized, matched whole blood (group 5 in both panels) resulted in adhesion of both cell types in numbers very similar to those observed after prior exposure to the crystalloid preservation media and subsequent exposure to the blood.

Scanning electron microscopic evidence for thrombotic processes in vein grafts previously

Figure 5: TF-mediated activation of coagulation factor X in human SVG after intraluminal exposure to saline (upper panel), PP (middle panel), or heparinized autologous whole blood (lower panel) for 45 min at room temperature. The intraluminal contents were subsequently replaced by the following hemostasiological assay mixtures (concentration of each component close to its normal physiological concentration in plasma): Group 1: FVII/$FVII_a$ + X; Group 2: as in group 1, but after perfusion for 60 min at arterial pulsatile pressure with endothelial cell culture medium (see methods) at 37°C; Group 3: as group 1, but with AT-III added; Group 4: FVII/FVIIa +FX + FXI + FVIII + FVa + FII; Group 5: as before, but with PC added. Box-and-whisker plots; n values below the abscissa; $P<0.001$ vs. group 4 as control (Student's-t-test).

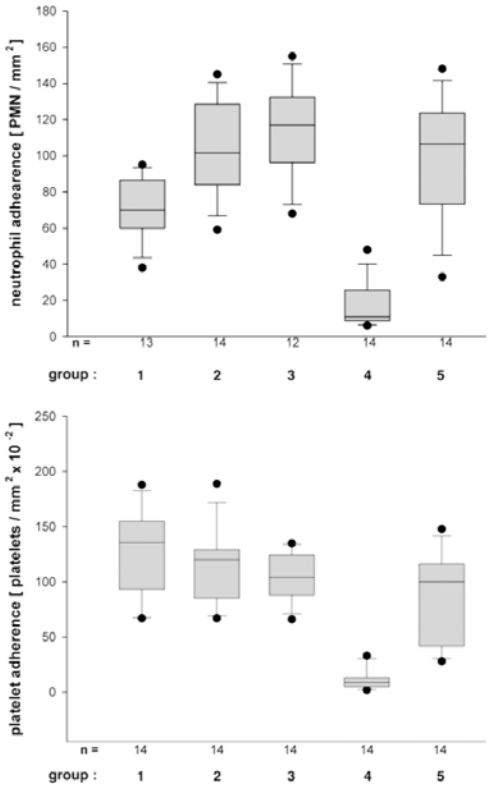


Figure 6: Adhesion of neutrophils (upper panel) and platelets (lower panel) to the intimal surface of SVG after incubation in different storage solutions and subsequent exposure to whole blood. SV segments were stored for 45 min at room temperature in saline (group 1), saline + 5% albumin (group 2), HTK (group 3), PP (group 4), or heparinized, autologous blood (group 5). Subsequently, the intraluminal contents of the veins of groups 1-4 were replaced by heparinized, matched whole blood for a further 10 min prior to brief flushing with BSS-BSA, fixation, critical point drying and sputtering with gold. Segments stored in blood (group 5) were fixed and prepared immediately after the 45-min exposure period. The adherent PMN and platelets were counted by SEM. Box-and-whisker plots; n values below the abscissa. P<0.001 for all groups vs. group 4 as control (Student's t-test).

exposed to various storage media and subsequently to native, matched whole blood

The data just described, imply that endothelial damage and exposure of prothrombotic subendothelial structures following usual intraoperative storage protocols of vein segments should result in an even increased thrombogenicity of their inner surface, when the "preserved grafts" are perfused with freshly drawn, native blood (capability of the lesioned intima to recruit PMN and platelets, which can amplify coagulatory processes; reduced ability to assemble anti-coagulatory activities or components). This would be the case after their implantation and reperfusion in the patient, especially after the usual neutralization of the intraoperatively applied systemic heparin by protamine sulphate at the end of cardiopulmonary bypass. These conditions were imitated in vitro and documented by high-power scanning electron microscopy (SEM). 10 VSG were examined in each group.

Picture series A in **Figure 7** shows exemplarily that saline-preserved grafts were indeed highly pro-thrombotic. Their surface was practically denuded of endothelium, and a 10-min perfusion with freshly drawn, matched whole blood at 37°C was sufficient to generate numerous microthrombi consisting of detached EC, PMN and platelets, many of them enveloped by fibrin fibers. Subsequent pulsatile perfusion of such grafts with balanced salt solution supplemented with 10%v/v FCS (BSS-FCS) for 10min under arterial pressure and flow conditions (pictures not shown) resulted in detachment of the microthrombi and an accompanying demolition of the endothelium to which these thrombi firmly adhered. Such findings were consistently found throughout the vessels inspected in this group.

Picture series B in **Figure 7** demonstrates that grafts preserved in HTK-solution showed the same "flaked" endothelial cell morphology with widely opened clefts as was already noted as a typical criterion of cultured venous endothelium during incubation in the same solution (**Figure 1B**). During inspection with SEM, numerous adherent platelets and PMN could be observed particularly in the microdomains of clefts between the partly contracted EC, where they obviously interacted with subendothelial structures. Again, subsequent pulsatile perfusion under arterial conditions increased the degree of deendothelialization significantly.

Grafts preserved in PP, on the other hand, (picture series C in **Figure 7**) retained their endothelial integrity as noted above. Compared with the two foregoing preservation protocols only loosely adherent PMN and

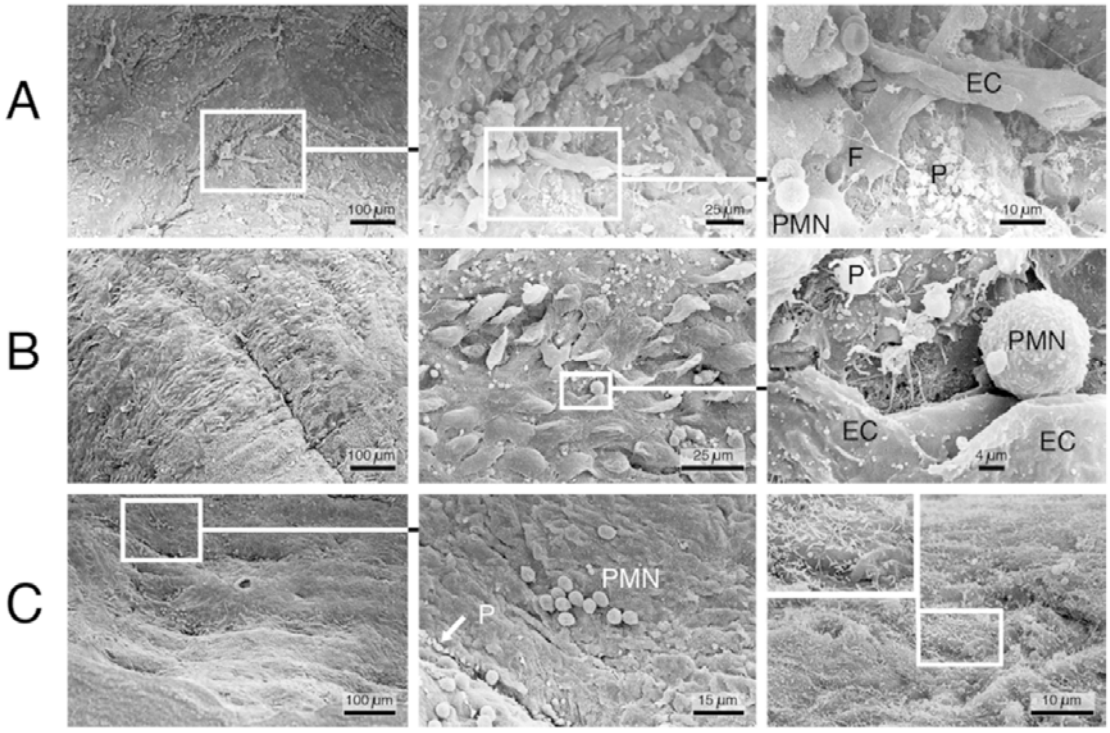


Figure 7: SEM images of the luminal surface of SVG after intraluminal exposure to different storage solutions for 45 min and subsequent slow perfusion with freshly drawn, matched whole blood for 10 min (37°C). (A) storage in saline. Left: overview showing a deteriorating intimal surface; middle and right panels: successive higher power views of the boxes. The right panel shows numerous mixed microthrombi consisting of neutrophils (*PMN*), platelet aggregates (*P*), endothelial cells (*EC*), and fibrin (*F*). (B) Storage in HTK. Left: overview showing the typically and generally widely opened endothelial clefts after contraction of the individual EC developing in this medium; right: higher power view of the box shown in the middle panel revealing the numerous flattened *PMN* and *P* adhering in the furrow-like regions between the EC. Adherent platelets develop numerous pseudopodia and appear to recruit further neutrophils. (C) Storage in PP. Left: overview demonstrating an intact endothelial surface; middle: higher power view of box showing a loose chain of platelets (P) fixed to a fibrin thread (arrow), *PMN* neutrophils. Right: inset (upper left) shows a very high power view of the box which reveals the abundant and well-preserved microvilli of the endothelial plasmalemma.

practically no anchored platelets could be observed. If at all present, rare microthrombi consisting only of solitary fibrin threads and a few attached, apparently only reversibly activated platelets were seen close to endothelial clefts. The EC displayed no morphological signs of activation, and possessed abundant microvilli on their surface, possibly the backups for the multifunctional gel-like glycocalyx of the vital cells. The endothelial tissue of such grafts survived subsequent perfusion with BSS-FCS under arterial conditions readily.

Intraoperative storage of grafts in heparinized autologous whole blood is a preferred protocol in some centers for cardiac surgery. The above hemostasiological studies (**Figure 5**), however, suggested strongly that such segments, too, will be pro-thrombotic after the storage period in vitro (on an average 45 min). This was again histologically examined in grafts of 10 patients, 50 IU heparin were added per ml blood. A time-dependend coagulation process could in fact be demonstrated at the surface of adherent platelets - particularly in areas in which the intercellular junctions of the largely intact endothelium were partly open - and also on exposed IP (**Figure 8**). Thus, despite the presence of effective heparin concentrations, loosely organized, mixed thrombi were

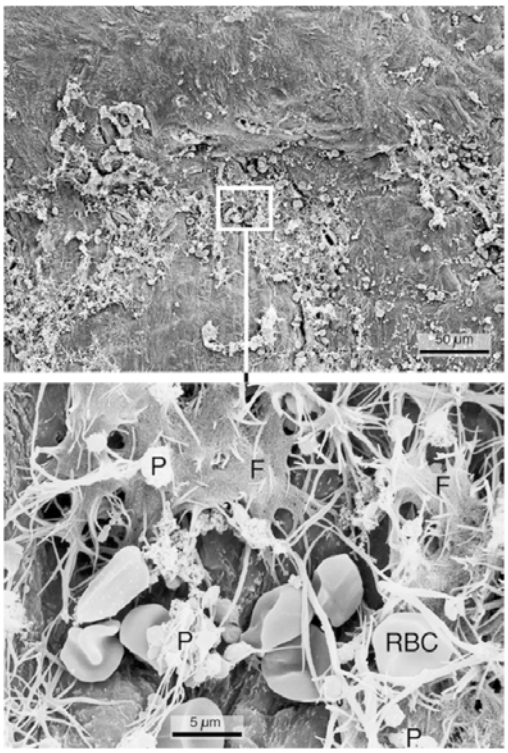


Figure 8: SEM images of the intimal surface of SVG after storage in effectively heparinized, autologous blood for 45 min. Top panel: overview. In de-endothelialized areas of the intima loosely organized, mixed thrombi can be recognized. Lower panel: higher power view of the box as indicated. Platelets (*P*), red blood cells (*RBC*) and exposed, here partially hidden pericyte-like cells organize fibrillar growth of branched fibrin threads, first locally, then spreading out like thorns or burs. On this pillar-like framework, fibrin nets (*F*) were formed, in which numerous RBC, platelets and often also PMN (not present in this picture) were lodged.

surprisingly generated in these discrete micro-domains of the intimal surface.

The situation deteriorated dramatically during subsequent incubation at 37°C with freshly drawn, matched whole blood. Now the adherent platelets and shortly later also the IP below the increasingly contracted endothelium became catalytic foci of fulminant coagulatory and pro-aggregatory processes. Within 10 min (**Figure 9A**) the contracting and detaching EC were enveloped into mixed thrombi of fibrin, platelets and neutrophils, and the subsequent pulsatile perfusion with BSS-FCS under arterial pressure conditions led finally to complete deendothelialization (**Figure 9B**) and the exposure of a highly proaggregatory surface to the perfusate.

Discussion

The present study addresses the acute morphological and hemostasiological consequences of extensive endothelial denudation and simultaneous exposition of the subendothelium in explanted SVG, particularly after the intraoperative storage of the latter in crystalloid solutions and at the moment of reperfusion with native whole blood under arterial conditions.

Incubation of cultured human venous EC in saline - the solution used most frequently worldwide for intraoperative graft storage - results in a rapid and dramatic loss of vitality. This is at first astonishing, because one characteristic of EC is their ability of rapid metabolic adaptation to substrate deprivation [37]. ATP levels in EC are closely controlled, so that energy-dependent processes can be curbed rapidly and intracellular ATP levels change little, e.g. after incubation for some hours in balanced salt solutions (pH7.4, 37°C) without added glucose [38] or under hypoxic conditions [39]. It is thus unlikely that the observed rounding and rapid death of cultured EC in saline was primarily due to the complete absence of energy substrates in this "preservation medium". More probable is a role for the inhibition of the Na^+/K^+-ATPase induced by the absence of K^+. The vital importance of this ion pump for the maintenance of cell membrane potential, cell osmoregulation and cell form is well known [40]. In this situation of an almost completely abolished osmoregulation, it is also particularly hazardous for the cells that the medium – due to the absence of any high-molecular weight constituents – does not develop oncotic pressure. Other possible contributors to endothelial cell demise in this insufficient "preservation solution" saline may have included the complete absence of Ca^{++}ions. Moreover, the lack of certain essential plasma proteins may have led to a markedly shrinked endothelial glycocalyx. Physiologically - together with a variety of bound plasma proteins - the glycocalyx forms the important endothelial surface layer [41] with a thickness of approximately 0.5 µm, acting in situ as an exclusion zone for all blood cells. Amongst other vital tasks this layer

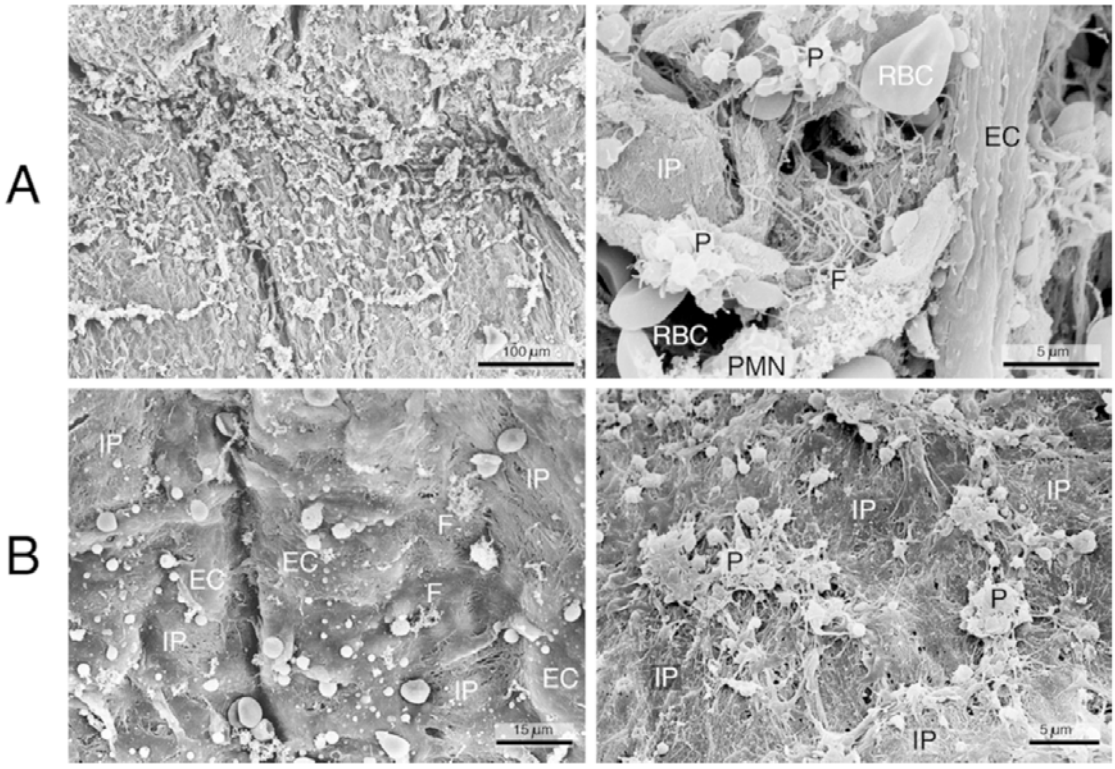


Figure 9: SEM of the intimal surface of SVG after storage in heparinized, autologous blood for 45 min at room temperature and subsequent incubation for 10 min with freshly drawn, matched blood (37°C) without anticoagulants. A, left panel: Low-power view showing extensive thrombotic deposits on the intima after the storage period. Right: a higher power view of such material shows numerous aggregating platelets (*P*), which are anchored to threads or deposits of fibrin (*F*) organizing from the surface of exposed intimal pericytes (*IP*) or the extracellular matrix between contracted endothelial cells (*EC*). B, left panel: after only 3 min pulsatile perfusion with BSS-BSA, many contracting EC are seen and the IP are increasingly exposed to the perfusion medium. Right panel: View 5 min after adding platelet rich plasma to the perfusate. Large aggregates of platelets are apparent at the endothelium-denuded luminal surface, which are very firmly anchored to the IP: both withstanded the shear forces easily.

reduces the shear forces in a perfused vessel considerably [42] and acts like a molecular filter [43-46]. On all accounts and consistent with the observations on cultured endothelial layers were our observations on SVG stored in saline and subsequently briefly flushed with balanced salt solution supplemented with serum albumin: extensive, and in the majority of cases almost complete, endothelial denudation. It must be stated again that the surgical protocol employed for vessel isolation and our regular supervision of the procedure make it highly unlikely, that the damage was a consequence of rigorous handling during explantation and sealing.

The inherent robustness of vascular endothelial tissue, with respect to metabolic stress and its ability to survive energy depletion for longer periods of time, is apparently also compromised by intraoperative storage for 45min at room temperature in hyperkaliaemic ("cardioplegic") solutions designed primarily for cardioprotection (HTK-solution). Take up of K^+ on the $Na^+/K^+/2Cl$-Co-transporter as was described for glial cells [47], would lead indirectly to continuous energy consumption when the Na^+ is subsequently pumped out. Under such conditions, it would thus be unlikely that the EC are able to reduce their metabolism to a state of "hibernation", at least not without being cooled strongly. Although these questions have not yet been studied systematically in EC, our observations on cultured SV endothelium incubated at room temperature in HTK-solution were clear: the

rapid breakdown of the barrier function was documented microcinematographically with respect to the "flaked" morphology (**Figure 1B**) and by measurement of the time course of changes in hydraulic conductivity (**Figure 2**). SVG incubated under similar conditions showed a devastatingly high degree of de-endothelialization, similar to that seen in saline-stored graft segments (**Figure 7**).

As might be expected from the above, the endothelial damage seen in purely crystalloid solutions in vitro was not prevented by the addition of a high albumin concentration to saline. On the other hand, the vessel intima and endothelial structure were largely conserved after storage in a commercially available plasma preparation (Biseko®) freed of isoagglutinins or coagulation factors. Not only does this PP contain physiological concentrations of diverse carbohydrate substrates and K^+, and a sufficient Ca^{++}-concentration to prevent cellular activation processes while maintaining the endothelial cytoarchitecture, the PP is also buffered physiologically and contains (with the exceptions mentioned above) all other plasma proteins, including, apparently, growth factors. It can thus develop and maintain a nearly physiological oncotic pressure. About 75% of all SVG stored in this solution showed almost complete endothelial conservation, so that this solution alone deserved the designation as a "true graft preservation solution" in our hands.

In a separate series of experiments, the effect of the various storage solutions on the integrity of the endothelium of the porcine ear vein was examined by measurement of the rate of FX activation, which could only be catalyzed by the exposed TF in deendothelialized regions of the intima. The worst results were again obtained for the crystalloid solutions. The addition of 5% albumin brought about no improvement. Again, the best results were achieved with the PP. Thus, a functional classification of endothelium's integrity, after exposure to these solutions and on the basis of a coagulation assay, yielded essentially the same results as obtained in the histological studies on human SVG. These results, together with the fact that the porcine ear veins were studied practically in situ, i.e. without exposure to direct surgical preparative stress, emphasize again the importance of the composition of the solution in which the grafts are stored intraoperatively for the maintenance of endothelial integrity.

The foregoing aspects and the impressive histological demonstration of high concentrations of TF in the intima of human venous grafts [48], which considerably increased during storage, encouraged us to perform quantitative hemostasiological studies also on SVG under defined in vitro conditions. The corresponding results emphasized all the more the necessity for improved endothelial conservation during bypass surgery.

First to the role of the platelets and PMNs that have evolutionary predecessor cells with both haemostatic and non-specific immunological functions [49]. Since the vessel endothelium is the actual "container" of the blood in the body, it is convenient that, in the context of endothelial injury, both cell types can be rapidly activated by inflammatory mediators [50] and co-operate closely with each other [51, 52] and the endothelium [53] to prevent bleeding and infection. Platelets have a particularly high degree of affinity to certain subendothelial structures [54] and can recruit PMN via formation of fibrin nets once they become activated [55]. Platelet reactivity is also considerably enhanced by red blood cells [56]. All these facts are particularly relevant for explanted, intraoperatively stored and then implanted bypass grafts that are necessarily exposed to inflammatory mediators released from the cut surfaces. Moreover, it can be assumed that the IP will also be activated under such circumstances. At least this would agree well with the already mentioned cardiosurgical observations, where a high expression of TF in the venous intima even under basal conditions has been demonstrated that was further enhanced strongly during intraoperative storage [48].

Heparin effectuates its anti-coagulatory potency by binding to AT-III and through a greatly accelerated rate of inactivation of the proteolytic enzymes of blood coagulation by this serpin [57]. However, all of the humoral and cellular interactions between platelets, PMN and the subendothelium still occur without any restraint in the presence of heparin. Even additionally added selective platelet inhibitors, such as acetyl-salicylic acid or persantin, do not block these interactions [58]. Thus, it is not surprising that after fixation of vessel segments, that had been stored in

heparinized, autologous blood, enormous numbers of platelets and PMNs were seen adhering to exposed subendothelial regions and even to an otherwise seemingly normal endothelium; a fact which has already been reported by others [59]. In bypass grafts stored in the insufficient storage solutions discussed above, the first contact with blood led immediately to particularly excessive adhesion of platelets and PMN to their lesioned intima and probably also to activation of the intimal pericytes.

The uncontrolled attachment of activated PMNs alone may be already sufficient to damage the vessel endothelium locally by the release of aggressive oxygen metabolites [60] and/or hydrolytic enzymes [61]. The massive adhesion and activation of platelets at the endothelial surface, however, has even further-reaching pathological consequences, involving also the subendothelial pericytes exposed by endothelial denudation of macrovessels, as became evident during our electon microscopic investigation of lesioned intimal regions (e.g. **Figure 8, 9**). As noted above, at least microvascular pericytes express constitutively TF and prothrombinase on their surface [31, 32]. In the very moment where lesioned bypass grafts with adhering platelets and PMN are reperfused with warm, fully coagulable blood (in the clinical setting this will occur at the moment the surgeon completes cardiopulmonary bypass and the systemic heparinization is counteracted by the administration of protamine sulphate), several plasma coagulation factors are promptly activated as a consequence of TF exposure and start the subsequent “initiation phase” [62, 63] of the coagulatory process [64-66]. The locally formed thrombin leads, since this protease is the most potent platelet activator known, to activation within milliseconds [67] of all adherent platelets in the vicinity. The latter blood cells serve not only to cover or to plug damaged vessel areas temporarily, but also to recruit and concentrate subsequent pro-coagulant events at their surface. Platelet focussed pro-coagulatory reactions include the release of bioactive peptides and proteins that recruit additional platelets and the components of the non-specific immune system (including PMN) [68], expression of specific, high-affinity membrane receptors for coagulation proteases [69, 70], release of zymogens such as factor V [71], and the extreme amplification of the initial formation of thrombin by providing a huge catalytic surface (integrated over the total surface area of all the individual platelets anchored to a wound surface it may comprise square meters)—for the soon massively propagated formation of fibrin clots [64-66]. The consistently documented formation of loosely organized, fibrin-rich thrombi at the surface of SVG stored in autologous, effectively heparinized blood (**Figure 8**) can be explained by the fact that receptor-bound, activated coagulation factors cannot be inhibited by heparin-dependent inhibitors like AT-III [72]. The following "propagation phase of coagulation" [62] is especially characterized by rapid and quantitative thrombin generation facilitated by the enhanced concentration of FX_a made available through the assembly and function of the factor $VIII_a$-factor IX_a complex, and the arrangement of the prothrombinase-complex (involving X_a, V_a and prothrombin) on the phospholipid-rich surface of activated platelets [64-66, 69, 70]. The physiological significance of binding factors $VIII_a$ and IX_a to activated platelets to effect FX activation is best exemplified by the kinetic parameters defining this process [73]. The functional consequence of this complex is an almost unbelievable 13 million-fold increase in catalytic activity compared to the activity of the factors in fluid phase.

Given the above, it can be readily understood why inadequately preserved SVG during reperfusion with native, coagulable whole blood coagulation processes are initiated so vigorously that all damaged structures of the graft intima are rapidly covered and enveloped by a firm fibrin net. According to our results this also included SVG preserved in autologous, heparinized blood. Under arterial flow conditions, such mixed clots exacerbated the already considerable damage markedly, because they were flushed away tearing off large areas of endothelium. In our in vitro experiments this became manifest already after a reperfusion period under pulsatile arterial flow conditions of only 10 min. Although the extent to which these processes have an impact on implanted grafts in situ needs to be examined systematically, preferably in appropriate animal models [74], the clinical incidence of acute thromboembolic graft occlusion - up to 30% in the first postoperative year-, however, speaks already for itself [6].

Against this alarming background, the question arises as to why or how the majority of patients, in whom such inadequately preserved grafts were implanted and in which these processes necessarily can be assumed to occur, still survives. However, as threatening as the pro-coagulatory mechanisms in the graft lumen just described may be, all the more powerful and effective are the anti-thrombotic activities of the (hopefully healthy) microvascular bed downstream. These can be divided into anti-aggregatory (platelet-inhibiting), anti-coagulatory (coagulation-inhibiting), and pro-fibrinolytic (fibrinolysis-promoting) activities [23, 75], physiologically catalysed exclusively at the endothelial surface. Since the latter increases enormously in the downstream capillary network from purely geometrical reasons, such that 1 ml blood is in contact with 5,000 cm^2 capillary endothelial surface (calculated from [76, 77]), even large numbers of microthrombi, washed in with the blood flow and trapped in the coronary microcirculation, can be completely dissolved within a few hours or at least days (e.g. particularly during the postoperative stay of the patients in the intensive care unit). Nevertheless, to have to rely on a patient's intrinsic antithrombotic defense mechanisms for the success of a complicated and sophisticated surgical intervention cannot be regarded as a satisfactory situation. And even after the acute thrombotic danger has gone, the exposed subendothelial cells in the deendothelialized areas of the graft, activated by the continuous local formation of serum, remain a threat to the patient: on the one hand as a local focus of unrestrained thrombosis, on the other through their time-dependent, uncontrolled proliferation and resultant wall thickening and lumen reduction. Although these aspects also require systematic study, preferentially in appropriate animal models, the middle- to long-term prognosis for transplanted venous grafts also speaks for itself [7-9].

What clinical conclusions should be drawn from the foregoing? More cautious preparation and, above all, preservation of the graft segments is certainly imperative. The better prognoses for grafts of arterial origin imply that preserving the endothelium is worthwhile. Although arterial vessels apparently also contain subendothelial pericytes [30], their endothelium is more firmly anchored to the particularly richly developed and thick subendothelium than in SVG. Furthermore, dissected arterial segments do not collapse during intraoperative storage in the preservation medium, thus avoiding potential mechanical damage to the endothelium. The arterial bypass vessels with the best prognosis are the internal thoracic arteries, which are neither explanted nor stored in preservation solutions, but are simply diverted.

Due to all our observations, explanted segments are ideally preserved intraoperatively in the commercially available PP, which is still mainly used to fight the liquid loss of patients suffering from severe burns, but which now appears also as a tailor-cut bypass preservation solution. The cost of such a preparation is nothing when compared to that of a bypass operation and all its ramifications, not to speak about the costs and risks of coronary reoperations. Given that these relationships have long been recognized in many surgical centres, and have been compiled in detail in recent reviews [24, 25], it is somewhat surprising that vessel transplantations are still often performed with insufficient preservation protocols. To help to overcome this unsatisfactory situation further, we are presently preparing for a controlled clinical study, which is designed to prove the value of intact venous grafts also in the patient.

Address correspondence to: Stephan Nees, MD, PhD, Department of Physiology, University of Munich (LMU), 80336 Munich, Germany. E-mail: Stephan.Nees@med.uni-muenchen.de

References

[1] Damgaard S, Steinbruchel DA and Kjaergard HK. An update on internal mammary artery grafting for coronary artery disease. Curr Opin Cardiol 2005; 20: 521-524.
[2] Gansera B, Schiller M, Kiask T, Angelis L, Neumaier-Prauser P and Kemkes BM. Internal thoracic artery vs. vein grafts--postoperative angiographic findings in symptomatic patients after 1000 days. Thorac Cardiovasc Surg 2003; 51: 239-243.
[3] Manabe S and Sunamori M. Radial artery graft for coronary artery bypass surgery: biological characteristics and clinical outcome. J Card Surg 2006; 21: 102-114; 115.
[4] Raja SG. Composite arterial grafting. Expert Rev Cardiovasc Ther 2006; 4: 523-533.
[5] Epstein AJ, Rathore SS, Krumholz HM and Volpp KG. Volume-based referral for cardiovascular procedures in the United States: a

cross-sectional regression analysis. BMC Health Serv Res 2005; 5: 42.
[6] Peykar S, Angiolillo DJ, Bass TA and Costa MA. Saphenous vein graft disease. Minerva Cardioangiol 2004; 52: 379-390.
[7] Bourassa MG. Fate of venous grafts: the past, the present and the future. J Am Coll Cardiol 1991; 17: 1081-1083.
[8] Fitzgibbon GM, Kafka HP, Leach AJ, Keon WJ, Hooper GD and Burton JR. Coronary bypass graft fate and patient outcome: angiographic follow-up of 5,065 grafts related to survival and reoperation in 1,388 patients during 25 years. J Am Coll Cardiol 1996; 28: 616-626.
[9] Shuhaiber JH, Evans AN, Massad MG and Geha AS. Mechanisms and future directions for prevention of vein graft failure in coronary bypass surgery. Eur J Cardiothorac Surg 2002; 22: 387-396.
[10] Motwani JG and Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. Circulation 1998; 97: 916-931.
[11] Weintraub WS, Jones EL, Morris DC, King SB, 3rd, Guyton RA and Craver JM. Outcome of reoperative coronary bypass surgery versus coronary angioplasty after previous bypass surgery. Circulation 1997; 95: 868-877.
[12] Htay T and Liu MW. Drug-eluting stent: a review and update. Vasc Health Risk Manag 2005; 1: 263-276.
[13] Krucoff MW, Boam A and Schultz DG. Drug-Eluting Stents "Deliver Heartburn." How Do We Spell Relief Going Forward? Circulation 2007;
[14] Laskey WK, Yancy CW and Maisel WH. Thrombosis in coronary drug-eluting stents: report from the meeting of the Circulatory System Medical Devices Advisory Panel of the Food and Drug Administration Center for Devices and Radiologic Health, December 7-8, 2006. Circulation 2007; 115: 2352-2357.
[15] Mitra AK, Gangahar DM and Agrawal DK. Cellular, molecular and immunological mechanisms in the pathophysiology of vein graft intimal hyperplasia. Immunol Cell Biol 2006; 84: 115-124.
[16] Martins e Silva J and Saldanha C. Arterial endothelium and atherothrombogenesis. I--Intact endothelium in vascular and blood homeostasis. Rev Port Cardiol 2006; 25: 1061-1083.
[17] Desideri G and Ferri C. Endothelial activation. Sliding door to atherosclerosis. Curr Pharm Des 2005; 11: 2163-2175.
[18] Newby AC and Zaltsman AB. Molecular mechanisms in intimal hyperplasia. J Pathol 2000; 190: 300-309.
[19] Cox JL, Chiasson DA and Gotlieb AI. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences between veins and arteries. Prog Cardiovasc Dis 1991; 34: 45-68.
[20] Solymoss BC, Nadeau P, Millette D and Campeau L. Late thrombosis of saphenous vein coronary bypass grafts related to risk factors. Circulation 1988; 78: I140-143.
[21] Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res 2007; 100: 158-173.
[22] Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. Circ Res 2007; 100: 174-190.
[23] Verhamme P and Hoylaerts MF. The pivotal role of the endothelium in haemostasis and thrombosis. Acta Clin Belg 2006; 61: 213-219.
[24] Boyle EM, Jr., Lille ST, Allaire E, Clowes AW and Verrier ED. Endothelial cell injury in cardiovascular surgery: atherosclerosis. Ann Thorac Surg 1997; 63: 885-894.
[25] Steen S. Preservation of the endothelium in cardiovascular surgery-some practical suggestions--a review. Scand Cardiovasc J 2001; 35: 297-301.
[26] Boos CJ and Lip GY. Blood clotting, inflammation, and thrombosis in cardiovascular events: perspectives. Front Biosci 2006; 11: 328-336.
[27] Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005; 352: 1685-1695.
[28] Libby P and Theroux P. Pathophysiology of coronary artery disease. Circulation 2005; 111: 3481-3488.
[29] Viles-Gonzalez JF, Fuster V and Badimon JJ. Links between inflammation and thrombogenicity in atherosclerosis. Curr Mol Med 2006; 6: 489-499.
[30] Andreeva ER, Pugach IM, Gordon D and Orekhov AN. Continuous subendothelial network formed by pericyte-like cells in human vascular bed. Tissue Cell 1998; 30: 127-135.
[31] Bouchard BA, Shatos MA and Tracy PB. Human brain pericytes differentially regulate expression of procoagulant enzyme complexes comprising the extrinsic pathway of blood coagulation. Arterioscler Thromb Vasc Biol 1997; 17: 1-9.
[32] Osterud B and Bjorklid E. Sources of tissue factor. Semin Thromb Hemost 2006; 32: 11-23.
[33] Gundry SR, Jones M, Ishihara T and Ferrans VJ. Optimal preparation techniques for human saphenous vein grafts. Surgery 1980; 88: 785-794.
[34] Poole JC, Sanders AG and Florey HW. The regeneration of aortic endothelium. J Pathol Bacteriol 1958; 75: 133-143.
[35] Le DT, Rapaport SI and Rao LV. Relations between factor VIIa binding and expression of factor VIIa/tissue factor catalytic activity on cell surfaces. J Biol Chem 1992; 267: 15447-15454.
[36] Bretschneider HJ. Myocardial protection. Thorac Cardiovasc Surg 1980; 28: 295-302.
[37] Culic O, Decking UK and Schrader J. Metabolic adaptation of endothelial cells to substrate

deprivation. Am J Physiol 1999; 276: C1061-1068.
[38] Culic O, Gruwel ML and Schrader J. Energy turnover of vascular endothelial cells. Am J Physiol 1997; 273: C205-213.
[39] Tretyakov AV and Farber HW. Endothelial cell tolerance to hypoxia. Potential role of purine nucleotide phosphates. J Clin Invest 1995; 95: 738-744.
[40] Clausen T. The sodium pump keeps us going. Ann N Y Acad Sci 2003; 986: 595-602.
[41] Pries AR, Secomb TW and Gaehtgens P. The endothelial surface layer. Pflugers Arch 2000; 440: 653-666.
[42] Secomb TW, Hsu R and Pries AR. Effect of the endothelial surface layer on transmission of fluid shear stress to endothelial cells. Biorheology 2001; 38: 143-150.
[43] Jacob M, Bruegger D, Rehm M, Welsch U, Conzen P and Becker BF. Contrasting effects of colloid and crystalloid resuscitation fluids on cardiac vascular permeability. Anesthesiology 2006; 104: 1223-1231.
[44] Pries AR and Kuebler WM. Normal endothelium. Handb Exp Pharmacol 2006; 1-40.
[45] Gouverneur M, Berg B, Nieuwdorp M, Stroes E and Vink H. Vasculoprotective properties of the endothelial glycocalyx: effects of fluid shear stress. J Intern Med 2006; 259: 393-400.
[46] Nieuwdorp M, Meuwese MC, Vink H, Hoekstra JB, Kastelein JJ and Stroes ES. The endothelial glycocalyx: a potential barrier between health and vascular disease. Curr Opin Lipidol 2005; 16: 507-511.
[47] Amedee T, Robert A and Coles JA. Potassium homeostasis and glial energy metabolism. Glia 1997; 21: 46-55.
[48] Poston RS, Gu J, Brown JM, Gammie JS, White C, Nie L, Pierson RN, 3rd and Griffith BP. Endothelial injury and acquired aspirin resistance as promoters of regional thrombin formation and early vein graft failure after coronary artery bypass grafting. J Thorac Cardiovasc Surg 2006; 131: 122-130.
[49] Opal SM. Phylogenetic and functional relationships between coagulation and the innate immune response. Crit Care Med 2000; 28: S77-80.
[50] Chlopicki S, Lomnicka M and Gryglewski RJ. Obligatory role of lipid mediators in platelet-neutrophil adhesion. Thromb Res 2003; 110: 287-292.
[51] de Gaetano G, Cerletti C and Evangelista V. Recent advances in platelet-polymorpho-nuclear leukocyte interaction. Haemostasis 1999; 29: 41-49.
[52] Zarbock A, Polanowska-Grabowska RK and Ley K. Platelet-neutrophil-interactions: linking hemostasis and inflammation. Blood Rev 2007; 21: 99-111.
[53] Siminiak T, Flores NA and Sheridan DJ. Neutrophil interactions with endothelium and platelets: possible role in the development of cardiovascular injury. Eur Heart J 1995; 16: 160-170.
[54] Chen J and Lopez JA. Interactions of platelets with subendothelium and endothelium. Microcirculation 2005; 12: 235-246.
[55] Kuijper PH, Gallardo Torres HI, van der Linden JA, Lammers JW, Sixma JJ, Zwaginga JJ and Koenderman L. Neutrophil adhesion to fibrinogen and fibrin under flow conditions is diminished by activation and L-selectin shedding. Blood 1997; 89: 2131-2138.
[56] Marcus AJ and Safier LB. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. Faseb J 1993; 7: 516-522.
[57] Imberty A, Lortat-Jacob H and Perez S. Structural view of glycosaminoglycan-protein interactions. Carbohydr Res 2007; 342: 430-439.
[58] Zhao L, Bath P and Heptinstall S. Effects of combining three different antiplatelet agents on platelets and leukocytes in whole blood in vitro. Br J Pharmacol 2001; 134: 353-358.
[59] Schlitt A, Pruefer D, Buerke U, Russ M, Dahm M, Oelert H, Werdan K and Buerke M. Neutrophil adherence to activated saphenous vein and mammary endothelium after graft preparation. Ann Thorac Surg 2006; 81: 1262-1268.
[60] Kaminski KA, Bonda TA, Korecki J and Musial WJ. Oxidative stress and neutrophil activation--the two keystones of ischemia/reperfusion injury. Int J Cardiol 2002; 86: 41-59.
[61] Thomas EL, Lehrer RI and Rest RF. Human neutrophil antimicrobial activity. Rev Infect Dis 1988; 10 Suppl 2: S450-456.
[62] Butenas S, van 't Veer C and Mann KG. Evaluation of the initiation phase of blood coagulation using ultrasensitive assays for serine proteases. J Biol Chem 1997; 272: 21527-21533.
[63] Lawson JH, Kalafatis M, Stram S and Mann KG. A model for the tissue factor pathway to thrombin. I. An empirical study. J Biol Chem 1994; 269: 23357-23366.
[64] Green D. Coagulation cascade. Hemodial Int 2006; 10 Suppl 2: S2-4.
[65] Arnout J, Hoylaerts MF and Lijnen HR. Haemostasis. Handb Exp Pharmacol 2006; 1-41.
[66] Hoffman M and Monroe DM. Coagulation 2006: a modern view of hemostasis. Hematol Oncol Clin North Am 2007; 21: 1-11.
[67] Born GV and Kratzer MA. Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. J Physiol 1984; 354: 419-429.
[68] Gawaz M, Langer H and May AE. Platelets in inflammation and atherogenesis. J Clin Invest 2005; 115: 3378-3384.
[69] Walsh PN. Platelet coagulation-protein

interactions. Semin Thromb Hemost 2004; 30: 461-471.
[70] Jurk K and Kehrel BE. Platelets: physiology and biochemistry. Semin Thromb Hemost 2005; 31: 381-392.
[71] Tracy PB, Eide LL, Bowie EJ and Mann KG. Radioimmunoassay of factor V in human plasma and platelets. Blood 1982; 60: 59-63.
[72] Olson S, Bjork I and Shore J. Kinetic characterization of heparin-catalyzed inhibition of blood coagulation proteinases by antithrombin. Methods of Enzymology 1993; 222: 525-559.
[73] Rawala-Sheikh R, Ahmad SS, Ashby B and Walsh PN. Kinetics of coagulation factor X activation by platelet-bound factor IXa. Biochemistry 1990; 29: 2606-2611.
[74] Schachner T, Laufer G and Bonatti J. In vivo (animal) models of vein graft disease. Eur J Cardiothorac Surg 2006; 30: 451-463.
[75] van Hinsbergh VW. The endothelium: vascular control of haemostasis. Eur J Obstet Gynecol Reprod Biol 2001; 95: 198-201.
[76] Anversa P, Levicky V, Beghi C, McDonald SL and Kikkawa Y. Morphometry of exercise-induced right ventricular hypertrophy in the rat. Circ Res 1983; 52: 57-64.
[77] Bassingthwaighte JB, Yipintsoi T and Harvey RB. Microvasculature of the dog left ventricular myocardium. Microvasc Res 1974; 7: 229-249.