Vein graft thrombi, a niche for smooth muscle cell colonization – a hypothesis to explain the asymmetry of intimal hyperplasia

I. BLAAS,* K. HEINZ,* P. WÜRTINGER,† A. TÜRKCAN,‡ C. TEPEKÖYLÜ,* M. GRIMM,* C. DOPPLER,* K. DANZL,* B. MESSNER‡ and D. BERNHARD*

*Cardiac Surgery Research Laboratory, University Clinic for Cardiac Surgery, Medical University of Innsbruck; †Institute of Medical and Chemical Laboratory Diagnostics (ZIMCL), Medical University of Innsbruck, Innsbruck; and ‡Cardiac Surgery Research Laboratory, Department of Cardiac Surgery, Medical University of Vienna, Vienna, Austria

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Essentials
- Vein graft failure is the most frequent late onset complication of coronary artery bypass grafting.
- Cuff technique-based interposition mouse model including new anticoagulation regime was conducted.
- Early vein graft thrombi may serve as a niche for smooth muscle cell colonization.
- The focal character of early thrombi may form the basis for the asymmetry of intimal hyperplasia.

Summary. Background: Autologous saphenous veins are widely used in coronary artery bypass grafting; however, 10 years after surgery, 40% of grafts are completely occluded, and another 30% show reduced blood flow. Objective: In the past, the central processes and signaling pathways responsible for this loss of patency have been identified. However, one central finding in the process of graft failure is so far not understood: the asymmetric character of intimal hyperplasia. It was the goal of the present study to address this aspect. Methods: By the use of a cuff technique-based vein interposition mouse model with a new anticoagulation regime, alterations in vein grafts were analyzed 1 h, 1 day, 2 days, 3 days, 7 days and 21 days after reperfusion by means of immunolabeling, histochemistry, and high-resolution ultrasound. Results: The novel and major finding of this study is that the vein graft thrombus may serve as a niche that is infiltrated and colonized by smooth muscle cells (SMCs). Fibroblast growth factor-1 and platelet-derived growth factor-B may be the SMC-attracting factors in the thrombus. The focal character of early thrombi may define the focal and asymmetric character of vein graft intimal hyperplasia. Conclusions: Inhibiting the formation and reducing the size of early thrombi is an old concept for reducing vein graft failure. However, in light of the present new findings obtained under a clinic-like anticoagulation regime, early vein graft thrombus prevention/size reduction should be revisited in the prevention of graft failure.

Keywords: coronary artery bypass grafting; etiology; thrombosis; vascular graft restenosis; venous intima.

Introduction
Coronary artery bypass grafting (CABG) is an extremely successful surgical treatment option for revascularization in the heart. Nevertheless, the long-term outcome of vein grafts, which make up ~ 50% of grafts used in CABG, is limited because of thrombosis (early), intimal hyperplasia and neointima formation (intermediate), and graft atherosclerosis (late). Central events that have been shown to cause these processes are initial tissue damage, platelet activation and adhesion, inflammation, tissue remodeling, and graft atherosclerosis.

The pathogenesis of vein graft disease (VGD) has been extensively analyzed over the past decades, and is very well summarized in a recent review by Kim et al. [1]. The core of the current concepts for VGD pathogenesis is that surgical handling, resection, intraoperative storage, leakiness checks and the events after reimplantation (re-oxygenation, arterial pressure, and pulse waves) cause
damage to the vein graft (endothelial denudation and disruption of the elastic fiber and muscle system) and alter its physiologic state towards a wound repair/stress response (reduced production of proliferation inhibitors, reduced NO, etc. [1,2]), leading to a prothrombotic and smooth muscle cell (SMC) proliferation-promoting environment. This environment leads to platelet adhesion, thrombus formation, leukocyte adhesion and infiltration, inflammation, and a general proproliferative state, which includes matrix metalloproteinase (MMP) activity and tissue remodeling [1]. Apart from thrombus formation, the SMC response to grafting is thought to be the central element in VGD. The SMC response includes proliferation and migration, and a change in SMC phenotype from quiescent to secretory, which also contributes to intimal hyperplasia [3]. Previous therapeutic approaches targeting these events were: (i) reduction in the graft damage caused by intraoperative handling (e.g. no-touch technique); (ii) reductions in hypoxic and reperfusion stress and damage (e.g. antioxidants [4]); (iii) inhibition of SMC proliferation and migration (e.g. antiproliferative/migratory compounds [5,6]); (iv) inhibition of proinflammatory signaling and inflammation (e.g. immunosuppressants and inhibition of leukocyte adhesion [7]); (v) inhibition of tissue remodeling processes (e.g. MMP–tissue inhibitor of metalloproteinase [8,9] and transforming growth factor signaling [10]); and (vi) inhibition of platelet adhesion and thrombus formation [1,2,11]. Recent studies have suggested that inhibition of platelet adhesion is capable of inhibiting intimal hyperplasia, mainly via the reduction in inflammation resulting from the inhibition of platelet-mediated leukocyte infiltration [11,12]. Note that – apart from standard anticoagulation – there is still no successful clinically applied treatment available that increases the patency of vein grafts. For summaries, see [1,2,13–15].

According to the current concept, intimal hyperplasia is caused by local media SMCs and potentially also by adventitial SMC precursors that infiltrate the intima, to form a neointima and cause, together with proliferating local SMCs, intimal hyperplasia [1,16]. Intimal hyperplasia, early thrombus formation and neointima formation are not thought to be directly interconnected. However, new evidence for such a connection was recently found in a study by Tseng et al., in which the authors demonstrated that a reduction in the platelet adhesion area (by inhibition of integrin αIIbβ3-mediated adhesion) inhibits intimal hyperplasia [11]. The preclinical success of treatments of this type is thought to be attributable to inhibition of graft inflammation by inhibition of leukocyte adhesion to platelets and consequent inhibition of infiltration, which was also suggested by the above study.

In the present study, we sought to extend the current view and provide a novel hypothesis on the genesis of the asymmetric shape of intimal hyperplasia in vein grafts. In testing this hypothesis, old concepts to reduce vein graft failure may be revitalized.

Materials and methods

Ethics statement

All animals received care in compliance with the ‘Principles of laboratory animal care’ of the National Society for Medical Research and the ‘Guide for the care and use of laboratory animals’ prepared by the Institute of Laboratory Animal Resource and published by the National Institutes of Health. This study was approved by the Committee for Animal Testing of the Medical University of Innsbruck and by the Austrian Ministry of Science and Research.

Animal care and experimentation design

In this study, 82 male wild-type C57BL/6N mice (Charles River, Saarland, Germany) with a body weight between 28 g and 30 g were used. All mice were kept at the animal housing facilities of the Medical University of Innsbruck (Zentrale Versuchstieranstalt) and of the Daniel Swarowski Research Institute of the Medical University of Innsbruck at 24 °C under a 12-h light/dark cycle. Mice received a standard diet (R/M-H feed; Sniff, Soest, Germany) and drinking water ad libitum.

All animal handling steps were performed with a standard operation procedure, and were conducted by persons only after an intensive training phase. The animal testing was split into three experiments.

The goal of the first experiment, which included six mice, was to assess the efficacy of the new anticoagulation regime applied in this study. The new anticoagulation setting was intended to be more close to the human clinical setting, in which anticoagulation is conducted during and after surgery [17,18].

The goal of the second experiment, the core study, was the immunohistologic analysis of vein grafts prior to interposition, and 1 h, 1 day, 2 days, 3 days, 7 days and 21 days after interposition of the inferior vena cava (from an isogenic donor mouse) into the left common carotid artery (of a recipient mouse). For each of the seven time points, five animal pairs (donor and recipient) were needed, giving total of 70 mice.

The goal of the third experiment, which included six mice (three donor–recipient pairs), was to assess vein graft wall structure on days 3 and 21 after grafting in the same mice to search for correlates between early (3 day) wall changes and their later manifestation at day 21.

Anesthesia and analgesia

Anesthesia of mice was achieved with a single intraperitoneal injection of xylasol (10 mg per kg body weight)
and ketamine (80 mg per kg body weight). Full anesthesia was achieved when mice did not react to a pinch with forceps at the toes. Postoperative analgesia was achieved by subcutaneous injection of buprenorphine (0.05 mg per kg body weight), and piritramide (one ampulla + 10 mL of 10% glucose solution) per 250 mL of drinking water ad libitum. The drinking water consumption of individual mice was controlled.

Anesthesia for high-resolution ultrasound analyses was achieved by placing mice into a box containing 1.5% isoflurane for 20 s, followed by constant exposure of mice to 1.5% isoflurane with a flow rate of 1 L min\(^{-1}\) via a mask.

**Interposition model for vein grafting**

The surgical procedure in this study is based on the model described in [19], including adaptations of the anticoagulation regime, which are indicated below.

Following anesthesia, the inferior vena cava between the right atrium and the diaphragm was harvested, and subsequently rinsed with and stored in sterile 0.9% NaCl solution containing 3 IU of unfractioned heparin (UFH) mL\(^{-1}\). Recipient mice were anesthetized as above mentioned, and the skin was opened in the region above the right sternocleidomastoid muscle. Subsequently, this muscle was removed, and the carotid sheath was prepared to distinguish the different anatomic structures. The right common carotid artery was then prepared from surrounding tissue, the graft was harvested and directly placed in a sample tube containing 4.5% formaldehyde solution. Five mice were operated on per time point.

**Removal of the vein graft**

Following anesthesia of recipient mice after days 1, 2, 3, 7, and 21 (1-h time point; mice were kept under anesthesia from graft implantation until removal), the region above the carotid sheath was prepared as described previously. After exposure of the grafted inferior vena cava from the surrounding tissue, the graft was harvested and directly placed in a sample tube containing 4.5% formaldehyde solution. Five mice were operated on per time point.

**Hematoxylin and eosin (HE) staining, immunohistochemical staining and immunofluorescence-based evaluation of venous bypass graft sections**

Sample preparation, immunohistochemical staining and immunofluorescence-based staining on vein grafts (peri-anastomotic area) and quantification of signal intensity and cell numbers was performed as described in Data S1. For further details, see [20].

**Quantification of thrombus thickness and thrombus area**

**SMC density**

For details, see Supporting Information.

**High-resolution ultrasound**

Ultrasound analyses (B-mode, M-mode, pulsed-wave [PW]-Doppler and color-Doppler) of vein grafts were conducted under isoflurane anesthesia with a VS-20033 VEVO 1100 Imaging System (VisualSonics; Toronto, Canada). For further details, see Supporting Information, page 3.

**Statistics**

Numerical data were analyzed for a Gaussian distribution with the Shapiro–Wilks test and for equality of variance with Levene’s test for equality of variances. All data analyzed showed a Gaussian distribution. Consequently, more than two groups were analyzed with one-way ANOVA (Bonferroni-adjusted) followed by individual (two-group comparisons) two-sided \(t\)-test comparisons. All data are presented as means ± standard deviations. Asterisks indicate significant differences between groups at \(P < 0.05\).

**Results**

**Histologic changes in vein grafts after implantation**

In order to analyze the morphologic changes in a vein graft after implantation into the arterial system, we analyzed samples prior to implantation and at 1 h, 1 day, 2 days, 3 days, 7 days and 21 days after implantation by means of histochemistry (HE-stained sections; Figs 1 and S1). As can be seen in Fig. S1 (1 h), the first event in the vein graft after implantation was disruption of the venous wall structure. This disruption included the separation of the intima from the media, and partial endothelial denudation. Importantly, 1 h after implantation, endothelial cells were still visible in some areas of the graft’s inner surface. Starting on day 1 (Fig. S1), no endothelial cells could be found on the graft’s inner surface, a state that did not change on day 2 (Fig. 1) and day 3 (Fig. S1). Only on day 7 were parts of the graft’s surface area again covered by endothelial cells. On day 21, endothelial integrity was again fully reached (Fig. 1).
At 1 h after implantation, massive platelet adhesion had already occurred, and remained prominent on the following days (1–3 days). Platelet adhesion at 1 h had a focal character; large areas of the graft’s inner surface did not show adhered platelets. Already on day 1 and also on days 2 and 3, platelets covered the entire inner surface of the grafts. On day 7, the platelet adhesion area was partly covered by endothelial cells, and thrombus tissue was infiltrated by cells. On day 21, almost the entire thrombotic area had been replaced by cellular material.

Neutrophil adhesion was first visible on day 1 after implantation, and neutrophil infiltrates could also be observed at this time point. A similar – but more pronounced state – was seen on day 2, when neutrophils seemed to accumulate between the thrombus bottom and the former intima. Histomorphologically, cellular infiltrates included lymphocytes and macrophages, but the majority of infiltrating cells were neutrophil granulocytes.

**SMCs infiltrate and potentially replace the thrombus**

Figure 2A,B shows intensive brown staining (von Willebrand factor) of the endothelial layer on days 7 and 21.
colonized by SMCs (Fig. 2B,D). The thickness of thrombus tissue luminal to the basal membrane increased significantly over time (Fig. 2E). Until day 7, the increase in thrombus thickness was caused by real thrombotic processes; the further increase in thrombus thickness on day 21 was caused by classic intimal hyperplasia (SMC infiltration and proliferation in the entire intima; also see Fig. 1D,F,H). The colonization of the early thrombus (between the original basal membrane and the endothelial layer) by SMCs was quantified (Fig. 2F). Twenty-one days after implantation, the massive infiltration and proliferation of SMCs had led to a significant increase in SMC density in the area that may have stemmed from the initial thrombus (also see Fig. 2D,F).

### Analysis of intrathrombus non-SMC infiltrates and growth factors

In order to test the hypothesis that SMCs may infiltrate focal thrombi in vein grafts, we tested for the occurrence of the SMC chemoattractive growth factors fibroblast growth factor-1 (FGF-1) and platelet-derived growth factor-B (PDGF-B) in the thrombus. Intrathrombus expression analyses of FGF-1 (Fig. 3A,E) revealed a sharp and significant increase in FGF-1 expression from 1 h after reperfusion (no expression) to day 1 after reperfusion. Starting at 2 days, FGF-1 expression decreased gradually. PDGF-B was already present in the thrombi at 1 h, but to a small extent. No significant increase in intrathrombus PDGF-B could be observed on day 1 after reperfusion. Only on day 2 was a sharp increase in intrathrombus PDGF-B observed, which gradually decreased over the subsequent days. Seven days after reperfusion, the time point when SMC infiltration had started, PDGF-B levels were back in the same range as 1 h after reperfusion. Neither the expression of intrathrombus FGF-1 nor that of PDGF-B in the first days was associated with the occurrence of cell nuclei. However, PDGF-B expression on day 2 was associated with cells (neutrophils; see below and Fig. 3C) adhering to the thrombus surface. Interestingly, 21 days after reperfusion, the original vein graft media SMCs were also colonized with PDGF-B (Fig. 3A).

In order to extend the analysis of intrathrombus cell infiltrates beyond SMCs and to test for a potential bone marrow origin of SMCs, immunofluorescence analyses were performed against CD3 (T cells), CD20 (B cells), CD68 (macrophages), NIMP-R14 (neutrophils), and CD34 (bone marrow-derived stem cells). Our analyses showed that intrathrombus cell infiltrates other than SMCs occurred, with the exception of neutrophils (Fig. 3C,D), to a very small extent (Fig. 3B,D), particularly until day 7, when a significant number of SMCs could already be observed in the intrathrombus area. Only on day 21 did the number of intrathrombus T cells show a non-significant increase (Fig. 3B, right row, second image from top, and Fig. 3D). Importantly, the intrathrombus expression of CD34 was also extremely low/not detectable, suggesting no relevant involvement of bone marrow-derived (stem) cells in SMC colonization of the thrombus. It is of note that the expression profile of CD34 over time was identical to the profile of CD68 (shape and amount of cells).

The number of thrombus-associated neutrophils (NIMP-R14+) showed a sharp peak on day 2 (Fig. 3C, D; also see Fig. 1C,D). Neutrophils occurred mainly on the thrombus surface and at the lining between the original intima and the bottom of the thrombus (central image in the bottom row of Fig. 3C); intrathrombus neutrophils could hardly be detected. As mentioned above, the expression of PDGF-B was associated with neutrophils, both mainly occurring on day 2 after grafting.

### High-resolution ultrasound-based analyses of vein grafts

Because all of the above results are based on histologic samples for which mice had to be killed, and therefore represent independent samples that do not allow for time-dependent observation of processes occurring in a defined area (thrombus) of the same vein graft, we...
decided to study vein grafts over time in individual mice by using high-resolution ultrasound. The results of these analyses provide evidence that graft segments with increased thickness of a tissue that may represent thrombus on day 3 after grafting are correlated with increased thickness of tissue of the same graft segments that may represent vessel wall on day 21.

Discussion

The occurrence of vein graft neointima formation, intimal hyperplasia, vein graft atherosclerosis and, ultimately, graft failure remains a significant problem in CABG in humans. To investigate pathophysiologic processes and to develop therapies that increase the patency of grafts, several animal models have been established. A frequently used model – also used in the present study – is the mouse veingraft interposition model using the cuff technique, which was developed by Zou et al. in 1998 [19]. Like all animal models, this model has several limitations related to species-specific differences, including physiology and anatomy, including the size of structures and the associated handling differences between mouse and human vessels. Vessel size also has a significant impact on various other parameters, e.g. reduced compound diffusion distances, which may increase pharmacologic success in the mouse as compared with the human setting. Despite important limitations, central aspects regarding the fate of a vein after interposition into the arterial system in the mouse are similar or even identical to the human situation (see Introduction). There is, however, a general need to adjust animal model systems to the human clinical setting. Regarding anticoagulation in the clinical setting, it is recommended that the stable CABG patient is not anticoagulated prior to surgery, but is heparinized during and after surgery [17,18]. In the vein graft mouse model used in this study, there is no clear consensus regarding anticoagulation, and heparin use in the past was mainly limited to the vein graft donor mouse, to intraoperative storage solutions, or as a constituent of wound-flushing solutions in the recipient mouse [21]. If heparin was applied to the recipient mouse, 100 IU UFH kg$^{-1}$ body weight was usually applied (for example, a 30-g mouse receives 3 IU UFH in total) intravenously [11], which is known to be rapidly metabolized. As the human patient remains anticoagulated also after surgery, we have adapted the model system described by Zou et al. by applying 20 IU UFH per mouse (28–30 g body weight) as a subcutaneous depot, which, at the time of reperfusion of the interposed vein graft, resulted in 5.24 IU UFH mL$^{-1}$ plasma (also see Fig. S2). Although subcutaneous UFH has reduced bioavailability (compensated for by increasing the dose), its half-life is known to be significantly increased. Clearly, there is still room for further improvements, but we think that this adaptation, particularly because thrombus formation is central to vein graft disease, makes the model system more similar to the human clinical setting. Importantly, as it cannot be excluded that the anticoagulation regime affects thrombus stability and architecture, we suggest that heparin concentrations

![Fig. 4. Schematic sketch of the events leading to asymmetric intimal hyperplasia.](image)
should be analyzed in future studies, irrespective of the anticoagulation regime applied.

The processes occurring in the vein graft after reperfusion have been intensively studied and well described in the past (e.g. [2,11,19]). As some observations made in the present study partly differed from previous findings, or were not described previously, the findings of the present study need to be discussed (also see schematic sketch in Fig. 4).

One hour after perfusion, massive mechanical damage can be observed, including partial de-endothelialization. In areas where endothelial cells have detached, a focal and bladder-shaped accumulation of platelets and thrombus tissue occurs. At this time point, thrombi are free of cellular infiltrates, and growth factor expression is absent or very low. One day after reperfusion, the focal platelet and thrombus accumulation remains bladder-shaped. In addition, a thin lining of platelets/thrombus covers the entire graft surface. Importantly, FGF-1, a well-known growth and chemoattractant factor [22], is present inside the thrombus in the absence of cellular infiltrates (absence of cell nuclei). Intrathrombus FGF-1 may serve as a signal for neutrophils, which appear in massive amounts on day 2, potentially via neutrophil fibroblast growth factor receptor 2, as was suggested by Haddad et al. [23]. On day 2, neutrophils are found attached to the thrombus surface, and are present between the previous intima lining and the thrombus bottom [2]. Neutrophils are not localized inside the thrombus. The occurrence of neutrophils is paralleled by the expression of platelet-derived growth factor, which can be found inside the thrombus but is mainly colocalized with neutrophils, which may suggest that neutrophils are attracted by PDGF-B, as has already been shown [24]. The synthesis of PDGF-B may be directly induced by FGF-1 stimulation [25], and neutrophil-triggered release of PDGF-B from platelets or other cell types may be at play [26]. Importantly, PDGF-B and FGF-1 are well-known chemoattractants and proliferation inducers for various cell types, including SMCs [27–29]. On day 3, bladder-shaped thrombi have flattened, and now appear as a more sickle-shaped thickened thrombus layer, still with a clear focal character. The thrombus is still hardly colonized by cells, neutrophils have disappeared, and FGF-1 and PDGF-B expression is slightly decreased. FGF-1 is now massively present on the thrombus surface, probably via affinity for heparin-like glycosaminoglycans [30]. One week after grafting, endothelial cells start to cover the surface of sickle-shaped thrombi, and SMCs can be found between the previous (old) intima and the newly forming endothelial layer. SMCs appear as infiltrates originating from the vessel

Fig. 5. High-resolution ultrasound-based follow-up of vein grafts. Representative ultrasound-based images of the native common carotid artery prior to surgery (control), the vein graft (inferior vena cava) 3 days (3d) after interposition and the same graft 3 weeks (21d) after interposition are shown. The upper-row images are identical to the images at the bottom. Yellow arrows indicate the location of the cuffs; the distance between the tips of two white arrows indicates vessel wall thickness; dotted lines indicate the vein graft lumen, and hatched areas indicated potential correlations between thrombus area at day 3 and thickened vessel wall on day 21. The scale on the right side of the images indicates millimeters, and diagrams at the bottom of the images show electrocardiograms and breathing rates of mice during the measurement. The numbers in the circles (1, and 2) refer to the same areas of the graft at day 3 and 21.
lumen as well as from the original media. The absence of CD34-positive cells in the entire process suggests that SMCs are of local tissue origin, i.e. the vein graft media, rather than descendants from the bone marrow. This was also suggested in previous studies [1,16]. FGF-1 and PDGF-B levels have further decreased. Three weeks after grafting, the endothelial layer is again fully intact, and thrombus tissue is hardly visible. SMCs appear in massive amounts in the subendothelial area, and form intima asymmetry with sickle-shaped thickening. The observation of high PDGF-B expression at day 21 may indicate PDGF-B-based autocrine growth stimulation of SMCs, as was previously reported in atheroma and balloon-injured vessels [31,32]. In the thickened areas, von Willebrand factor, which is initially released in response to stress and endothelial damage, binds to factor VIII, and is an important stabilizer of thrombi [33], is still present in a scattered manner between SMCs. The increase in the number of CD3-positive cells on day 21 may indicate the start of a further phase of transformation in the sickle-shaped thickened vessel wall. Importantly, the late appearance of CD3-positive, CD20-positive, CD68-positive and CD34-positive cells suggests that these cells are not directly involved in thrombus formation or in the later attraction/proliferation stimulation of SMCs.

In our previous in vitro models used to study intimal hyperplasia in vein grafts (human saphenous vein) and arteries (radial artery) [34,35], no asymmetric intimal hyperplasia could be produced, suggesting that factors that are not located in the vessel wall are responsible for the asymmetry of intima hyperplasia. Accordingly, it is important to consider that, when intimal hyperplasia pathophysiology and therapy are studied by the use of vascular wall tissue or isolated cells in vitro, the processes observed occur in the absence of factors that are relevant to the entire process in vivo.

The ultrasound analyses (Fig. 5) reported in this study do not fully prove the concept of SMC colonization of thrombi as the basis for asymmetric intimal hyperplasia. This is mainly because of methodological limitations; specifically, it was not possible to clearly differentiate between vessel wall and perivascular scar tissue at 21 days. Importantly, these analyses did not result in data that contradict our concept, but could provide further evidence in support of our hypothesis. Clearly, further studies (e.g. pharmacologic interference) are needed to strengthen our concept.

In summary – on the basis of an experimental animal setting for vein grafting, which, owing to more intense anticoagulation, is more close to the clinical setting than previous studies – we suggest that the asymmetric character of intimal hyperplasia in vein grafts is based on the formation of sickle-shaped thrombi on the graft’s inner surface, which – mediated by growth factors (FGF-1 and PDGF-B) – leads to the infiltration and ultimate colonization of asymmetric thrombi by local SMCs. We suggest that this process is the basis for the asymmetric charter of intimal hyperplasia in (vein) grafts in vivo.

Addendum

I. Blaas was responsible for performance of experiments, experimental design, and discussion of data. K. Heinz and P. Würtinger were responsible for performance of experiments, and experimental design. A. Türkcan was responsible for performance of experiments, and analysis of data. C. Tepeköylü was responsible for performance of experiments, and discussion of data. M. Grimm was responsible for discussion of data, and study design. C. Doppler was responsible for performance of experiments, and data analyses. K. Danzl was responsible for performance of experiments, and discussion of data. B. Messner was responsible for experimental design, discussion of data, and image analysis. D. Bernhard was responsible for study and experimental design, discussion of data, and drafting of the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and methods.
Fig. S1. Histological overview on time dependent changes in vein grafts after implantation.
Fig. S2. Determination of plasma heparin in mice.

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