Introduction

Increasing numbers of patients with end-stage renal disease are surviving worldwide because of improved dialysis access techniques. Since being introduced in 1966 [1], arteriovenous fistulas (AVFs) have been commonly used as the vascular access of choice [2]. However, the 1-year patency rates are ∼63% [3], and recurrent fistula thrombosis is an important cause of morbidity and mortality among those patients. Neointimal hyperplasia is considered to be the major cause of AVF failure, resulting in vein wall thickening, stenosis and, ultimately, occlusion [4]. Whereas excessive wall thickening is an undesired response to arterialized blood flow, moderate wall thickening is important for ‘fistula maturation’ that makes recurrent dialysis cannulation principally possible. Morphologically, neointimal hyperplasia is based on a proliferation of smooth muscle cells (SMC) combined with matrix deposition. Nonetheless, the underlying molecular mechanisms are still poorly understood.

Despite improved surgical and puncture techniques, no adjuvant strategies such as medical therapies have so far demonstrated efficacy to preserve fistula patency. A reliable rodent AVF model can be helpful in understanding and preventing the pathological mechanism of fistula failure. Therefore, a few different central and peripheral AVF models have been recently described in the mouse [5] and rat [6–9]. In the past, the analysis of morphological and functional changes in fistula veins was bound to invasive techniques. Moreover, the killing of animals was hereby necessary to perform longitudinal assessments.

Ultrasound, and in particular Doppler ultrasound has been shown to be a valuable tool to investigate the rat vasculature [10]. However, the scientific value of this method has not yet been demonstrated in an experimental AVF model. Therefore, the aim of this study is to develop a novel AVF rat model that shows typical features of fistula maturation seen in patients and that also allows longitudinal in vivo monitoring of fistula veins by high-resolution ultrasound.
Antibody was clone 1A4 (Dako, Glostrup, Denmark). Slides were washed in PBS and incubated with a rat tissue-adsorbed, biotinylated secondary antimouse antibody (Vector laboratories, Burlingame, CA, USA).

**Experimental groups**

A total of 18 animals (3 shams and 15 AVF) were part of the study. Fifteen rats underwent AVF surgery. Four AVF rats were post-operatively examined with ultrasound (US) on Day 1, Day 7 (Week 1), Day 14 (Week 2), Day 21 (Week 3), Day 42 (Week 6) and Day 84 (Week 12). At these times and on Day 140 (Week 20), AVFs and contralateral femoral vessels were harvested for histological and immunohistological examination. Three rats underwent invasive haemodynamic assessment on Day 1 and Day 21 (Week 3).

**Microsurgical procedure**

The procedure was performed on rats anaesthetized by isoflurane inhalation (5% induction and 1.5% maintenance, isoflurane at 1.5 l O2/min) thereby allowing spontaneous breathing. Animals were placed in a supine position on a heating pad (TR-200, Fine Science Tools, Heidelberg, Germany). The entire procedure was performed using a dissection microscope (M650, Leica Microsystems, Wetzlar, Germany) with 16-fold or 25-fold optical magnification. Surgical access was conducted via a 3 cm oblique skin incision of the right groin. After subcutaneous dissection, the femoral artery and vein were prepared. Following distal vein ligation using silk 8-0, a 1.5 mm longitudinal venous incision was made. After gentle arterial clamping, a corresponding longitudinal femoral artery arteriotomy was anastomosed to the femoral vein incision in an end to side manner using 10-0 monofilament (Ethilon, Ethicon, Norderstedt, Germany) running suture (Figure 1). A dilatation of the arterialized vein could be observed quickly after clamp removal. Finally, the skin was closed with 3-0 continuous suture (Vicryl, Ethicon, Norderstedt, Germany).

**High-resolution ultrasound**

The afferent femoral artery and fistula vein were visualized with a 15-MHz transducer (SONOS 2500, Philips, Amsterdam, The Netherlands). After identifying the vessel by its characteristic flow pattern, the position of the probe was optimized to show clear vessel wall/lumen interfaces and longitudinal B-mode images were recorded. Diameters were measured by automated analysis software (Brachial Analyzer, Medical Imaging Applications, Iowa City, IA, USA).

**Assessment of haemodynamic parameters**

Haemodynamic parameters were assessed invasively by a method similar to that described by Fries et al. [11]. Briefly, cardiac output was determined with the transpulmonary thermodilution technique using a thermodilution microprobe (IT-18, Physitemp Instruments, Clifton, NJ, USA) advanced into the thoracic aorta via the left femoral artery. A 1 mm flowmeter (1 RB, Transonic Systems, Ithaca, NY, USA) was placed around the fistula vein for continuous measurement of AVF flow.

**Histological and morphometrical procedures**

For tissue harvesting, anaesthesia was administered as described above. The incision in both groins was followed by cardiac puncture to flush vessels first with a saline solution and then with 4% formaldehyde in phosphate-buffered saline (PBS, pH 7.4). The AVF in the right groin and contralateral femoral artery and vein were gently removed and fixed in 4% formaldehyde in PBS for histological and immunohistological analyses. After overnight fixation, the specimens were processed and embedded in paraffin using standard techniques. Longitudinal sections (4 µm) were stained with haematoxylin-eosin (HE) and Elastic-van Gieson (EVG) for morphometrical analysis at the site of the anastomosis. After anastomosis site identification, neighbouring intima–media thickness in the downstream fistula vein was measured from three longitudinal sections for each AVF using digital microscopy analysis software (Diskus, version 4.30.625, Hilgers, Königswinter, Germany). Furthermore, vascular calcification was evaluated using von Kossa’s staining as described by Westenfeld et al. [12]. For smooth muscle α-actin staining, the primary antibody was clone 1A4 (Dako, Glostrup, Denmark). Slides were washed in PBS and incubated with a rat tissue-adsorbed, biotinylated secondary antimouse antibody (Vector laboratories, Burlingame, CA, USA).

**Results**

**Surgical procedure**

AVFs were created in all rats without any procedure-related complications. The mean surgical time was 57 min (range: 45–73). All animals survived the surgery and lived until the harvesting time points. The patency rate was 93% at the time of harvesting. One AVF thrombosis was detected on Day 7 by ultrasound examination. Immediate surgical exposure confirmed an AVF thrombosis due to an infected, false anastomotic aneurysm. During the observation period,
there were no signs of peripheral ischaemia resulting from steal or oedema caused by venous congestion.

Sonomorphologic characterization of AVF maturation

Ultrasound investigation was possible in all rats. The mean time of investigation was 12 min (range: 8–15 min). Figure 2 shows a representative ultrasound B-mode image of neighbouring femoral artery and AVF (a) and respective PW Doppler flow velocity tracings (b and c). The diameter of the fistula vein was 0.17 ± 0.01 mm at baseline and significantly increased on Day 7 (0.27 ± 0.03 mm, \( P = 0.02 \); Figure 3). The maximum increase was stable after 2 weeks (0.35 ± 0.03 mm, \( P = 0.01 \)), whereas the contralateral control femoral vein diameter remained unchanged throughout the study period (0.15 ± 0.05 mm). Similarly, the flow velocity significantly increased in AVFs with maximal velocities reached after 2 weeks and maintained at these velocities thereafter (baseline: 18 ± 4 cm/s; 2 weeks: 34 ± 2 cm/s; \( P = 0.03 \)). These results were confirmed with a flow probe showing that the fistula volume doubled within 6 weeks (baseline: 21 ± 11 ml/min, 6 weeks: 42 ± 19 ml/min, \( P = 0.4 \)). The mean internal diameter of the afferent femoral artery was 0.12 ± 0.01 mm on Day 1 and rose gradually throughout the study period with maximal significant dilation at 12 weeks (0.20 ± 0.02 mm, \( P = 0.01 \)). Whereas the flow velocity of the femoral artery did not change throughout the first 6 weeks, there was a significant increase in the femoral artery flow velocity at 12 weeks (baseline: 3.4 ± 0.4 cm/s, 12 weeks: 8.2 ± 2.8 cm/s, \( P = 0.02 \)). A pronounced thickening of the intima–media complex was observed in the fistula vein. Whereas intima–media thickness of the afferent femoral artery (0.005 ± 0.002 mm) and contralateral femoral artery (0.006 ± 0.003 mm) remained unchanged throughout the study period, it increased significantly in the fistula vein, plateauing after 3 weeks (baseline: 0.003 ± 0.001 mm, 3 weeks: 0.014 ± 0.002 mm, \( P = 0.02 \)). The mean heart rate (baseline: 240 ± 46 bpm, 6 weeks: 233 ± 69 bpm, \( P = 0.89 \)), mean arterial pressure (baseline: 79 ± 9 mmHg, 6 weeks: 66 ± 15 mmHg, \( P = 0.31 \)) and cardiac output (baseline: 108 ± 30 ml/min, 6 weeks: 140 ± 40 ml/min, \( P = 0.40 \)) remained unaffected.

Histological and morphometrical characterization

The harvested femoral veins of the sham group showed a normal composition of the intima and media with one cell layer of endothelium and few SMCs, respectively (HE, Figure 4A). Endothelial and media cell loss was apparent on Day 1 after surgery at the site of the anastomosis (HE, Figure 5). Corresponding to the ultrasound findings, a continuous increase in intima–media thickness was
There was a progressive hyper trophy in the intima–media thickness from Week 1 (C) to Week 6 (D) to Week 20 (E). SMC proliferation (F) and collagen deposition (G) obviously contributed to vein wall alteration.

observed at the site of the anastomosis from Day 1 to Day 42. Between the period from Week 6 to Week 20, only a mild increase in intima–media thickness was noted and this peaked on Day 140 (HE, Figure 4E). Wall thickening was inhomogeneously distributed along the fistula veins. However, this pronounced wall thickening gradually decreased from the site of the anastomosis downstream (HE, Figure 1). The intima–media thickness was caused by both intimal and medial thickening. During fistula maturation, cells invading the intima that stained positive for α-actin were observed, which indicate that these were SMC (α-actin, Figure 4F). EVG stain showed that AVF maturation was associated with significant collagen deposition (EVG, Figure 4G). At Week 12, a severe venous valvular calcification in the fistula vein was observed (von Kossa, Figure 6).
Discussion

The AVF model presented in the current study is characterized by a structural and functional response of fistula veins that strongly resembles clinical findings. For this purpose, a limiting of the microsurgical created anastomosis (length 1.5 mm) is essential to prevent a calibre mismatch and a relevant steal syndrome.

In our study, we could show that high-resolution ultrasound is a feasible and reliable tool for noninvasive longitudinal monitoring in a rodent model of venous arterIALIZATION. Preoperative ultrasound imaging has been proven effective prior and after AVF creation in patients with end-stage renal disease [13]. This noninvasive investigation of morphological and haemodynamic parameters has been shown to predict dialysis access function. The expected flow parameters after 6 weeks in human fistula veins are characterized by a low resistance biphasic shape, high peak systolic velocities (PSV) and end-diastolic velocities in about one-half/thirds of PSV [14].

During our longitudinal rat ultrasound study, we frequently observed similarities to the findings in patients. Fistula vein dilatation occurs rapidly after AVF creation. Human studies by Wong et al. [15] and Corpataux et al. [16] demonstrated that forearm fistula veins are dilated by 125% and 179% within a period of 12 weeks after surgery, respectively. Analogously, we observed an ∼200% increase in fistula vein diameter. In contrast, this dilatation in rats was detectable 2 weeks after surgery.

Additional to vein dilatation, we observed an increase in fistula volume, which is in accordance with Corpataux et al.'s human findings in six forearm fistulas. In this study, it was shown that the venous limb of an AVF is subjected to high volume flow but low blood-pressure environment and that the venous wall remodels to an eccentric hypertrophy (constant wall thickness combined with dilatation) due to higher wall shear stress [16]. In contrast to these findings, we observed a significant increase of intima–media thickness in our ultrasound investigations. The reason for the discrepancy remains unclear and needs further investigation. However, the molecular mechanisms that cause venous dilatation are still not completely understood. It is very likely that dilatation of the fistula vein results from increased biomechanical forces such as increased blood pressure and wall shear stress.

Increased levels of matrix metalloproteinase (MMP) are a well-known phenomenon in afferent arteries proximal to AVFs [17]. An increased expression of MMP 2 and 9 was observed in veins that were subjected to arterialization [7,9], too. This increased MMP activity in arterialized vein walls may lead to structural changes in the extracellular matrix composition (e.g. elastin and collagen fibres) that could explain the diameter increase.

In addition to dilatation, we noticed a significant increase in intima–media thickness in fistula veins in the B-mode images. Intima–media thickness in human AVFs is a frequent phenomenon in the fistula vein [17], and mostly intima–media thickness is located in the anastomotic area as shown in angiographically evaluated AVF occlusions [18]. In our longitudinal sections, we could clearly verify intima–media thickness in the fistula vein. Importantly, it was most pronounced immediately downstream of the anastomosis and in the anastomosis itself. Our data are consistent with those of Chan et al. who showed varying degrees of intima–media thickness and thickening of the venous wall in a rat AVF femoral loop model [7]. Furthermore, our structural analysis showed that neointimal hyperplasia formation was composed by proliferating SMC and collagen matrix deposition. Similar findings were described by Lin et al. in an AVF rat model, where a microsurgical anastomosis was created between the lateral and the ventral artery of the rat tail [8].

There is increasing evidence that neointimal hyperplasia formation in arterialized veins is triggered by endothelial cell dysfunction or damage. Using a mouse model for vein grafting, Xu et al. demonstrated that early cell death due to apoptosis and necrosis occurs shortly after vein arterIALIZATION and is followed by endothelial progenitor cell repair [19]. In our study, we could demonstrate an endothelial and media cell loss predominantly at the site of the anastomosis as early as 1 day after surgery. Since neointimal hyperplasia was most prominent in the perianastomotic area, our observations suggest that early endothelial cell death may represent a key event for a pathological remodelling of the proximal fistula vein. In addition to alterations of the arterialized veins, we could also observe an ∼40% increase in diameter of the afferent femoral vein within 3 weeks after fistula creation, which is in accordance with human dialysis access findings [16]. Nevertheless, the underlying physiological mechanisms that induce arterial dilatation are still not completely understood. There is evidence, however, that longitudinal wall shear stress and circumferential deformation also represent substantial mechanical factors that influence vascular dilatation and remodelling [20].

These structural alterations in both arteries and veins of the AVF were accompanied by changes in fistula haemodynamics that could be detected by high-resolution ultrasound, flow measurements and cardiac output assessment. Although a strong correlation between shunt volume and cardiac output exists [21], the increasing fistula volume in our study was only associated with a mild increase of cardiac output within 6 weeks after AVF surgery. Therefore, increased fistula flow in our AVF model primarily resulted from reduced venous resistance. The significance of this finding, however, might be restricted by this relatively short observation period of 6 weeks. Thus, it cannot be excluded that fistula flow increases beyond that observation time and causes a further increase in cardiac output.

Finally, we could observe severe calcification of venous valves in fistula veins 12 weeks following surgery. Although vascular calcification predominantly affects arterial vessels, calcification of arterialized vein grafts and of AVFs in haemodialysis patients is a well-known phenomenon, although the underlying pathomechanisms have not been completely elucidated [22]. Vascular calcification does not merely consist in passive deposition of calcium phosphate crystals; it is now clear that it is a well-organized process that involves cell activity and specific protein synthesis [23]. Interestingly, systemic effects of uraemia or other metabolic disorders can be excluded in our study. Therefore, it is likely that calcification occurred as a pathological response of the fistula vein to the arterialization. This
finding suggests a role for local calcification regulators and needs further investigation. In addition, future research has to focus on a possible association between factors leading to venous calcification and fistula vein thrombosis/neointimal hyperplasia.

In summary, our rodent model proved excellent comparability to the clinical findings in patients with AVF. The morphological and functional response of fistula veins was characterized by a continuous increase in intima–media thickness and mean flow velocity. The vessel diameter rose in both, the femoral artery and femoral vein. Importantly, assessment of these parameters by high-resolution ultrasound proved to be a practicable and reliable tool for longitudinal in vivo monitoring of AVF function and structure. On the basis of this model, noninvasive in vivo monitoring of the fistulas will allow investigating systemic effects, such as chronic renal failure on fistula maturation and haemodynamics. Moreover, it will also be possible to evaluate new therapeutic approaches that prevent AVF failure.

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References


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