Wall Shear Stress Regulates the Proliferation and Migration of Vascular Smooth Muscle Cells Depending on a TGF-β1 Manner

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Abstract

Background: Venous intimal hyperplasia (VIH) is the main cause of arteriovenous fistula (AVF) dysfunction. Hemodynamic forces have an important role in VIH. The proliferation and migration of vascular smooth muscle cells (VSMCs) play a crucial role in the development of VIH and TGF-β1 just has the biological function of inducing proliferation and migration of VSMCs. We use parallel plate flow chamber system to simulate different shear stress and investigate whether shear stress regulate VSMCs proliferation and migration through TGF-β1

Methods: Shear stress (SS) was simulated with an ECs/VSMCs cocultured parallel plate flow chamber system. The coculture system was established by plating cells on the two sides of polyethylene terephthalate membrane. The EC side was subjected to different shear stress (Low-SS, Normal-SS and Oscillating-SS), whereas the opposite VSMCs side was maintained under static conditions. Computational fluid dynamics were applied to three-dimensional models of ECs/VSMCs cocultured flow chamber system to estimate the velocity and WSS. The expression of TGF-β1 were analyzed by immunofluorescence assay. VSMCs proliferation and migration assay was performed with the BrdU kit and Transwell system.

Results: The expression of TGF-β1 was significantly up-regulated following application of Low-SS and Oscillating-SS, and the distribution of TGF-β1 was transferred to the cell membrane, compared with the static group. The migration and proliferation of cocultured VSMCs were significantly up-regulated after Low-SS and Oscillating-SS. Conclusion: Our results suggest that Low-SS and Oscillating-SS exerts atherosclerotic influences on the ECs and VSMCs in a TGF-β1-dependent process. TGF-β1 increases the proliferation and migration of VSMC and is thought to be a pro-atherogenic effect, which can be used as a new therapeutic target for the treatment of AVF dysfunction. The formation and
development of VIH in AVF may be a local hyperplasia process by shear stress-TGF-β1 regulation, which provides new insights into the mechanisms of neointimal hyperplasia.

**Background**

The Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines recommend that arteriovenous fistula is the preferred vascular access for end-stage renal disease (ESRD) patients[1]. However, arteriovenous fistula (AVF) dysfunction caused by stenosis or occlusion of anastomosis is one of the most common clinical problem which is difficult to prevent[2]. The surgery is often used for treating AVF dysfunction, but it increases the pain and economic burden of patients[3]. The molecular mechanism leading to AVF dysfunction is complex and there are no effective methods available for treatment and prevention.

The stenosis or occlusion of AVF is a long-term pathological process, which is chartered with venous intimal hyperplasia (VIH)[4, 5]. Its pathophysiological manifestations include: endothelial cells (ECs) dysfunction, migration and proliferation of vascular smooth muscle cells (VSMCs), inflammatory response, oxidative stress and so on[6, 7]. Recent studies suggest that the above factors cannot fully explain why the location of neointimal hyperplasia is always close to the anastomosis of AVF[8]. The intimal hyperplasia often occurred in the inner venous wall near the anastomosis and hemodynamic factors has an important role. The hemodynamics of these locations is characterized by low shear stress and disordered flow[9]. Altered wall shear stress (WSS) is associated with several cardiovascular and cerebrovascular diseases, like atherosclerosis and cerebral infarction[10, 11]. Hemodynamic abnormalities are one of the important causes of vascular disease. Studying the relationship between hemodynamics, especially shear stress and vascular remodeling, has potential theoretical and practical value for elucidating the pathogenesis of vascular disease and exploring new treatments for
The transforming growth factor beta (TGFβ) family is a class of cytokines with a variety of biological functions, including regulating apoptosis, cell proliferation and differentiation, wound healing, embryo development, tissue remodeling, tissue fibrosis and tumor through autocrine and paracrine modes[12]. There are six structurally related molecules (TGFβ1-6) at least, of which TGF-β1 has the strongest activity and the highest content. It is widely distributed and has a variety of biological functions and participates in various pathophysiological processes, which not only promotes the proliferation and migration of mesenchymal cells such as fibroblasts, but also promotes the excessive accumulation of extracellular matrix (ECM) such as collagen[13-16]. The proliferation and migration of VSMCs play a crucial role in the development of venous intimal hyperplasia[8, 17], and TGF-β1 just has the biological function of inducing proliferation and migration of VSMCs. In this study, we use ECs/VSMCs cocultured flow chamber system to simulate different shear stress and investigate whether shear stress regulate VSMCs proliferation and migration through TGF-β1 to mediate intimal hyperplasia, which provides new insights for the understanding of the molecular mechanisms of VIH.

Results

Hemodynamic analysis of cocultured parallel plate flow chamber system

We applied computational fluid dynamics (CFD) to the three-dimensional model of the ECs/VSMCs cocultured flow chamber system (Fig.2 a) and analyzed velocity and WSS. For laminar shear stress, we analyzed low shear stress (LSS, 4 dyn/cm²) and normal shear stress (NSS, 12 dyn/cm²). The flow rate of the fluid has a positive correlation with WSS (Fig.2 b). Accordingly, the flow rates were 25 ml/min and 75 ml/min. For oscillatory shear stress (OSS, 0±4 dyn/cm²), we use a sinusoidal function with a frequency of 1 Hz and an
amplitude of 25 ml/min as the speed input condition to vary its flow rate over time. When the time is 0.25s, the WSS peak is 4 dyn/cm².

The velocity distribution on the Z = 0 section under LSS and NSS is respectively analyzed, as shown in Fig. 3 a and d. The velocity at the inlet is higher than the surrounding region, which is directly collided to the fluid. Although the speed is large at the entrance and exit, it returned to a steady state quickly. The distribution of WSS also conforms to this rule, which is proportional to the velocity. Fig. 3 b and e shows the velocity vector distribution of different cross section in the X-axis direction under LSS and NSS. Three cross-sectional positions were taken in this study (X = -20 mm, X = 0 mm and X = 20 mm). The velocity vector shows that the fluid moves along the X axis. The flow is symmetrical along the Z = 0 plane and the fluid are stable and steady between the parallel plates. Fig. 3 c and f is a cloud diagram of velocity distribution on the Y = 0 section under LSS and NSS. It represents that the central region has a substantially stable flow and uniform distribution, which shows the Poiseuille flow and laminar WSS in the parallel plate flow chamber.

**LSS and OSS up-regulated expression of TGF-β1 in ECs**

To simulate different hemodynamic forces in vivo, LSS group (4 dyn/cm²), NSS group (12 dyn/cm²) and OSS group (0±4 dyn/cm²) were designed. The duration of shear stress application was 12 hours in all groups.

Immunofluorescence assay (Fig. 4) showed that there was no significant difference on TGF-β1 expression in ECs between the NSS group and static group. Compared with the static group, the fluorescence intensity of TGF-β1 in the LSS group and OSS group was stronger (P<0.05). We found that not only the expression of TGF-β1 was significantly up-regulated following application of LSS and OSS, but also the TGF-β1 distribution tends to transfer to the cell membrane following the application of LSS or OSS. Furthermore, OSS increased TGF-β1 expression, however, there was no significant difference compared with
LSS and OSS induced migration and proliferation of cocultured VSMCs

Transwell analysis and proliferation assay showed that LSS and OSS significantly up-regulated the migration (Fig. 5 a and b) and proliferation (Fig. 5 c) of cocultured VSMCs, which are fully consistent with the results from the previous animal model study. However, there was no significant difference between LSS group and OSS group. NSS treatment had no significant effect on cocultured VSMC, compared to the static group.

Discussion

Although the composition of the blood environment is the same, VIH mainly occurs at the branch or turning of the vessel[10]. Shear stress has an important role in maintaining normal function of ECs and VSMCs. The blood flowing through the normal blood vessels forms laminar shear stress, which maintain ECs in a stable state and low level of inflammatory and oxidative stress. However, the laminar flow becomes turbulent when it goes through curved blood vessel. The turbulent flow makes ECs out of order, highly transformed, active inflammatory gene and increase oxidative stress. Abnormal turbulence results in low shear stress and damage ECs and VSMCs, which leads to intimal hyperplasia and vascular remodeling[18].

TGF-β1 has the function of regulating the proliferation and migration of VSMCs and the ECM synthesis, which is not only involved in the development of fibrotic diseases such as cirrhosis, pulmonary fibrosis, hypertrophic cardiomyopathy and renal interstitial fibrosis[13, 19, 20], but also played an important role in proliferative diseases such as intimal hyperplasia [21, 22]. Wolf[23] reported that the expression of TGF-β1 mRNA
increased in the hyperplastic endometrium on the carotid balloon injury model of rabbit. However, the degree of intimal hyperplasia was significantly reduced following injection of specific antibodies against TGF-β1. The ratio of media to intima was also decreased, which confirm that TGF-β1 plays an important role in the process of intimal hyperplasia.

Wolff[24] found that intimal hyperplasia was not evident in mice given TGF-β1 inhibitor (SD–208) following vascular graft surgery, compared with control group. Heine[25] investigated the genetic predisposition of maintenance hemodialysis (MHD) patients and found that AVF patency was significantly associated with TGF-β1 genotype polymorphism. The MHD patients with high expression of TGF-β1 had a higher incidence of AVF dysfunction than patients with low TGF-β1 expression. Stracke[26] found that TGF-β1, TGF-β1 binding protein–1 (LTBP–1) and insulin-like growth factor–1 (IGF–1) expression were significantly increased in the intima hyperplasia of MHD patients with AVF dysfunction, compared with patients who established arteriovenous fistula for the first time. TGF-β1 may bind to LTBP–1 and then acts on the ECM to promote its synthesis and accumulation. However, there is few researches touching on the interactions of hemodynamic forces and TGF-β1.

In previous studies, we analyzed the WSS using CFD software in a three-dimensional model of arteriovenous fistula. We found a strong inverse relationship between WSS levels and neointimal hyperplasia in AVF[27]. As shown in Fig. 6a, the WSS were simulated in the three-dimensional model of ‘end to side’ AVF. The maximum and the minimum values of the WSS were shown in Fig. 6b along the cross-section circle of the anastomosis site. The WSS was inconsistent within the same cross section. The outer wall of AVF has the maximum WSS, which was directly collided by blood flow. And the inner wall of AVF has low level of WSS. Fig. 6c show the stream line of blood flow in the cross section of the anastomosis site. The outer wall underwent laminar flow, but the inner wall showed eddy
turbulence. Interestingly, different positions matching with various WSS patterns within the same venous cross section showed different pathological changes. Neointimal hyperplasia in the low and disturbed WSS regions is more evident than the high and laminar WSS regions. The intima-media thickening in the inner wall of AVF is more evident, as shown in Fig. 6d.

As the first barrier between blood and vessel walls, ECs constantly exposed to the shear stress of blood flow, converted the mechanical stimuli into intracellular signals and interacting with the underlying VSMCs[28]. Fig. 6e showed that positive immunostaining for alpha smooth muscle actin (α-SMA) was obvious in the both media and intima. Neointimal hyperplasia consisted of both ECs and VSMCs. VSMCs not only proliferate, but also migrate to the intima from media in the process of VIH formation. It represents that smooth muscle cell proliferation and migration play an important role in the process of intimal hyperplasia and TGF-β1 just has the function of inducing smooth muscle cell proliferation and migration. Previous studies showed that TGF-β1 immunostaining is particularly evident in the intimal hyperplasia region of AVF and has a positive relationship with the number of neointimal cells, as shown in Fig. 6f. TGF-β1 is thought to be produced by endothelial cells in the local intimal hyperplasia region of AVF.

We investigate how shear stress influences ECs and the role they play in cocultured VSMCs using an ECs/VSMCs cocultured parallel plate flow chamber system. The parallel plate flow chamber system was established to simulate the effects of shear stress on ECs. Hemodynamic analysis was carried out on the three-dimensional model of flow chamber system by CFD technique. The shear stress distribution and blood flow patterns in the parallel plate flow chamber system was evaluated, which is the basis for exploring the molecular mechanisms of VIH. The CFD technology has advantages in the cardiovascular field. The application of CFD can analyze fluid pressure and range in time and space,
which is beyond clinical methods and provides a new perspective on the disease development. WSS has a significant role in atherosclerosis and in-stent restenosis. Furthermore, CFD can simulate the spatial distribution of WSS, which establish a relationship between hemodynamic forces and atherosclerosis and explain that the susceptible sites of atherosclerotic plaque are turning and branches of artery[29]. In this study, we found that the expression of TGF-β1 was significantly up-regulated following application of LSS and OSS, and the distribution of TGF-β1 was transferred to the cell membrane, compared with the static group. The migration and proliferation of cocultured VSMCs were significantly up-regulated after LSS and OSS. In our co-culture system, LSS and OSS are factors that pathologically induce vascular pathology by up-regulating VSMCs proliferation and migration. The possible effects of close communication between cocultured cells through humoral transmission. ECs and VSMCs were grown on opposite sides of a polyethylene terephthalate (PET) membrane. The shear stress is applied to the ECs side while the VSMCs remains static on the opposite side, which is closer to the physical environment of the human vessel wall. Although ECs and VSMCs can be prevented from direct contact in the co-culture, they act in a paracrine manner by producing a transferable humoral factor. Therefore, we assume that the increasing secretion of ECs-derived growth factors due to the shear stress is a regulator of VSMCs proliferation and migration. In our co-culture system, VSMCs and ECs are separated by very thin membranes and TGF-β1 released from ECs can reach VSMCs at very high concentrations for a limited time and distance, resulting in these significant results. In view of this, it can be speculated that ECs-mediated TGF-β1 induced by LSS and OSS may participate in the regulation of proliferation and migration of cocultured VSMCs by paracrine or autocrine manner.

Conclusions
In summary, our results suggest that LSS and OSS exerts atherosclerotic influences on the ECs and VSMCs in a TGF-β1-dependent process, which was consistent with our previous reports that LSS and OSS is a vascular wall virulence factor that induces neointimal hyperplasia in the animal model. TGF-β1 increases the proliferation and migration of VSMCs and is thought to be a pro-atherogenic effect, which can be used as a new therapeutic target for the treatment of AVF dysfunction. The formation and development of VIH in AVF may be a local hyperplasia process by shear stress-TGF-β1 regulation, which provides new insights into the mechanisms of neointimal hyperplasia.

Disadvantages

First, although we only focused on the relationship between shear stress and TGF-β1, which is a continuation of previous study, we could not eliminate the possibility that other growth factors might have been involved in the findings of this study. Second, we found that TGF-β1 could be alerted by shear stress in the current study. Further investigations of mechanochemical transduction mechanism are needed.

Methods

Cell culture

ECs and VSMCs were isolated from fresh human umbilical vein using explanted techniques as previously described[30]. ECs and VSMCs were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Gibco, New York, USA). The ECs phenotype of the cells was characterized by immunocytochemical staining for von willebrand factor. VSMCs showed typical spindle-shaped morphology and was characterized by immunocytochemical staining for alpha smooth muscle actin. In all experiments, cells between passage 2 and passage 7 and cell populations with over 95% purity were used.
ECs/VSMCs cocultured system

The ECs/VSMCs cocultured system (Fig. 1) was established by plating cells on both sides of a 10μm-thick porous polyethylene terephthalate (PET) membrane. ECs were first planted to the outside of the PET membrane at a density of 3x10^5 cells/cm^2 and grown over 5 to 6 hours. VSMCs were then seeded on the opposite side at a density of 1x10^5 cells/cm^2. ECs and VSMCs were grown to confluence within 1 to 2 days.

Parallel plate flow chamber system

To apply laminar flow onto the cultured ECs, we established a cocultured parallel plate flow chamber like the system designed by Nackman[31]. The shear stress intensity (τ) was calculated using the formula \( \tau = \frac{6\mu Q}{wh^2} \) (μ is the viscosity of the medium; Q is the flow rate; w is the width; h is the height). Subsequently, the cocultured ECs side was applied to the designed shear stress while the opposite VSMCs side was maintained under static conditions. The experiments were performed in a humidified incubator at 37°C and 5%CO₂ with a pH of 7.4.

Experimental protocol

(1) VSMC/EC, VSMCs cocultured with ECs under static conditions; (2) VSMC/EC+LSS, VSMCs cocultured with ECs and low shear stress (LSS) was applied to the ECs side; (3) VSMC/EC+NSS, VSMCs cocultured with ECs and normal shear stress (NSS) was applied to the ECs side; (4) VSMC/EC+OSS, VSMCs cocultured with ECs and oscillatory shear stress (OSS) was applied to the ECs side.

Establishment of three-dimensional model of parallel plate flow chamber system

The key geometric parameters of the physical model of the parallel plate flow chamber system are as follows: the inlet is a round tube with 3 mm in diameter and 30 mm in length; the center is a cuboid with 90 mm in length, 50 mm in width and 11 mm in height;
the exit geometry is as the same as the entrance; there is a rectangular buffer tank with a
length of 40mm, a width of 5mm and a height of 7mm at the distance of 5mm to the edge
on both sides. Use the SolidWorks software (Version 2017, USA) to establish a three-
dimensional model of the flow chamber system according to the above parameters.

Settings of calculation model

The three-dimensional model was imported into ANSYS Workbench software (Version 14.0,
USA) and then meshed with Mesh module. We select the Multizone Sweep Meshing Method
and set the Mapped Mesh Type into Hexa (hexahedron). Compared with the traditional
meshing method, partition of the geometry is not required, which improve stability and
accuracy of calculation. In this study, we focus on wall shear stress (WSS) of the bottom
surface, the mesh is encrypted in order to perform more accurate calculations. The grid is
encrypted with 10 layers to capture changes of WSS accurately. The resulting grid is
shown in Fig. 2a.

The inlet of the circular tube pointing to the negative x-axis is set as the velocity entrance
and the circular tube in the forward direction of x-axis is the velocity exit in the model.
We assume that the flow at the outlet has been fully developed and has no effect on the
upstream flow and the boundary condition is set as the free outflow boundary condition.
The remaining flow field boundary is set as the solid wall boundary and the non-slip
boundary condition is satisfied between the solid wall and the fluid.

Simulation and analysis of velocity
and WSS

Following the setting of boundary conditions, the Reynolds number Re≈339 at the
entrance and the Reynolds number Re≈57 between the two parallel plates can be
calculated, which could be regarded as laminar flow in the entire calculation domain and
no turbulence is generated. The calculation model could be regarded as the laminar flow model. The solver uses the RANS model (the Reynolds Average Navier-Stokes Equation):

The spatial discrete format adopts the second-order upwind difference format, the pressure velocity coupling adopts the SIMPLEC algorithm. The velocity and WSS was then calculated according to the above settings.

**Fluorescent immunostaining**

Following application of different shear stress, the VSMCs is separated. ECs was fixed with 4% paraformaldehyde for 20 minutes at room temperature after the PET membrane was dissected. The fixed cells were blocked with 5% normal goat serum for 1 hour at room temperature and then incubated with primary antibody, TGF-β1 (Santa Cruz Biotechnology, CA, USA; sc–146, 1:50), overnight at 4 °C. Next day, the cells were incubated with secondary antibodies, Alexa Fluor 647 (Abcam, Cambridge, USA; ab150079, 1:200), for 1 hour at room temperature. The cells were further stained with 4’,6-diamidino–2-phenylindole (DAPI) for 20 minutes at room temperature. Finally, the coverslip is mounted on a glass slide. Fluorescence images were captured using a confocal laser scanning microscope (Nikon A1R, Tokyo, Japan). Quantification of fluorescence intensity was calculated with ImageJ software (Version 1.50, Bethesda, MA, USA). Each experiment was performed in quadruplicate.

*Preparation and transfection of lentiviral vectors*

A cDNA-carrying lentiviral vector (LV/GFP; ViGene Biosciences) with a modified green fluorescent protein (GFP) was prepared. VSMCs were grown to 50% to 70% confluency in an antibiotic-free normal growth medium supplemented with 10% FBS. Then, VSMCs were transfected with LV/GFP expressing enhanced GFP according to the manufacturer’s
instructions. Fluorescence microscopy analysis showed efficient viral vector transfection. After 3 to 4 days of transient and stable transfection, the VSMC was prepared for further experiments.

Transwell migration analysis

VSMCs migration was performed by the Transwell system (Costar, Corning Inc., NY, USA), which allowed cells to migrate through a PET membrane with a pore size of 8-μm. The cells inside the upper well gently were removed with a cotton swab. The number of migrated cells was counted under a microscope (Olympus, Japan). Ten randomly selected fields per well were evaluated for each membrane and each experiment was repeated five times.

Proliferation assay

The proliferation assay was measured using a colorimetric BrdU kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. The medium was supplemented with BrdU labeling solution (final concentration: 10 M BrdU) and reintubate for 2 h at 37°C. The samples were fixed with fixing solution for 30 min at room temperature to denature the DNA, incubated with anti-BrdU-POD working solution for 90 min at room temperature, and then incubated with substrate solution until color development is enough for photometric detection. The samples were added stop solution and read the plate using a spectrophotometric microtiter plate reader set at 450 nm.

Statistical Analysis.

Each experiment was performed at least in quadruplicate and values were shown as mean ± SD. **P < 0.001 indicate statistically significant.

Declarations
Ethics approval and consent to participate

Our study conformed with the Ethics Committee of Tianjin Medical University (Tianjin, China).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; Lan Jia, Lihua Wang, Fang Wei and Aili Jiang wrote the manuscript, Lan Jia, Zhi Lu and Bo Wang conducted the cell experiments, Haiyan Chen and Haibo Yu contributed to perform hemodynamic analysis.
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Figures

Figure 1

The model of ECs/VSMCs cocultured system. ECs and VSMCs are on the two sides of a porous polyethylene terephthalate membrane. The EC side of the coculture was applied to the designated shear stress, whereas the opposite VSMCs side was maintained under static conditions.
a. The three-dimensional model of ECs/VSMCs cocultured parallel plate flow chamber system was meshed with Multizone Sweep Meshing Method. The grid of bottom surface is encrypted. b. The flow rate of the fluid has a positive correlation with WSS. The corresponding flow rates were 25 ml/min and 75 ml/min for LSS (4 dyn/cm²) and NSS (12 dyn/cm²) respectively.
Figure a and d shows the velocity distribution on the Z=0 section under LSS (4 dyn/cm²) and NSS (12 dyn/cm²) respectively. Figure b and e shows the velocity vector distribution of different cross section in the X-axis direction (X=-20 mm, X=0 mm and X=20 mm) under LSS (4 dyn/cm²) and NSS (12 dyn/cm²) respectively. Figure c and f shows a cloud diagram of velocity distribution on the Y=0 section under LSS (4 dyn/cm²) and NSS (12 dyn/cm²) respectively.

|   | TGF-β1 | DAPI | Merge |
|---|--------|------|-------|
| Static | ![Static TGF-β1](image1) | ![Static DAPI](image2) | ![Static Merge](image3) |
| LSS    | ![LSS TGF-β1](image4) | ![LSS DAPI](image5) | ![LSS Merge](image6) |
Figure 4

a. Immunofluorescence assay shows expression of TGF-β1 in ECs treated with different shear stress for 12 hours. LSS and OSS up-regulated the expression of TGF-β1. The TGF-β1 distribution tends to transfer to the cell membrane following the application of LSS or OSS (yellow arrow points). b. Values were expressed as mean ± SD for each condition from four independent experiments (**P < 0.001
vs. static).
The migration (a and b) and proliferation (c) of cocultured VSMCs were significantly up-regulated following ECs treated with LSS and OSS, compared to the static group. Values were expressed as mean ± SD for each condition from five independent experiments (**P < 0.001 vs. static).
a. The simulation of WSS in the three-dimensional model of ‘end to side’ AVF. The specimens were taken according to the a-b vertical plane, which was near the distal end of anastomosis. b. The maximum and minimum WSS values along the circle of the venous cross sections near the distal end of anastomosis. c. The streamline diagram of flow velocity along the Y=0 plane in the venous cross-sections near the distal end of anastomosis. The inner wall has recirculation flow and eddy turbulence. d. The 100× magnified hematoxylin and eosin stained pictures of venous inner wall near the distal end of anastomosis, which showed evident neointimal hyperplasia. e. The 100× magnified α-SMA stained pictures of venous inner wall near the distal end of anastomosis, which showed that immunostaining for α-SMA was significantly evident in the both media and intima. f. The 400× magnified TGF-β1 stained pictures of venous inner wall near the distal end of anastomosis, which has a positive relationship with the number of neointimal cells.
