Circulating cells and cytokines in arteriogenesis
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Collateral artery growth alters endothelial glycocalyx conformation and is attenuated after perturbation of the endothelial surface coating

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ABSTRACT

Objective: The endothelial glycocalyx is a network of proteoglycans and glycoproteins lining the luminal wall of blood vessels which participates in mechanotransduction and modulates adhesion of circulating cells. Several disease states that result in a disturbed glycocalyx (e.g. diabetes, hypercholesteremia) are correlated with a reduced ability to form collateral arteries, but the functional importance of this endothelial surface layer for collateral artery growth remained unknown. This study now investigated the effects of glycocalyx perturbation on collateral artery growth.

Methods and Results: Forty-three Rabbits underwent unilateral ligation of the femoral artery and sham operation of the contralateral side. Glycocalyx dimensions in growing collateral arteries were evaluated by electron microscopy and revealed a decrease in glycocalyx diameter compared to quiescent anastomoses. To assess the functional importance of the glycocalyx for arteriogenesis, the glycocalyx was perturbed by local infusion of hyaluronidase into the collateral circulation. Microsphere-based perfusion measurements showed a significant reduction in conductance of the collateral circulation seven days after femoral artery ligation in animals receiving hyaluronidase infusion (ml/min/100mmHg: Hyaluronidase: 27.5±3.5; Controls (inactive enzyme): 47.1±3.83; p<0.001) and a lower percentage of actively proliferating vascular smooth muscle cells (VSMCs) in collateral arteries. mRNA levels of eNOS and TGF-beta1 were significantly reduced following hyaluronidase infusion, whereas perivascular macrophage infiltration around collateral arteries was not affected.

Conclusion: We describe a differential diameter and morphology of the endothelial glycocalyx in growing collateral arteries and a significant attenuation of collateral artery growth following glycocalyx perturbation. This may be due to an impaired endothelial shear stress sensing and a decreased expression of shear-regulated pro-arteriogenic genes. The endothelial glycocalyx represents a novel entity influencing adaptive arteriogenesis and a potential target structure to modulate neovascularization.
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1. INTRODUCTION

The growth of collateral arteries (arteriogenesis) following the stenosis or occlusion of a major blood vessel is an important protective mechanism in patients with vascular occlusive diseases. Arteriogenesis is driven by the pressure gradient between pre- and poststenotic perfusion territories, resulting in an increased flow via small arterial and arteriolar anastomoses. Triggered by this increase in shear stress, distinct transcription factors, cytokines and adhesion molecules are expressed by the collateral endothelial cells and result in the infiltration of circulating cells [1]. Among the signaling events involved in the subsequent arterial remodeling, both nitric oxide [2, 3] as well as transforming growth factor beta-1 [4] dependent mechanisms have been shown to be of particular importance.

However, especially under hyperglycemic [5] and hypercholesterolemic conditions [6], the ability to develop functional collateral arteries is impaired and the collateral circulation remains insufficient. Interestingly, the same cardiovascular risk factors are also associated with a perturbation of the endothelial glycocalyx [7-9].

This network of negatively charged proteoglycans, glycoproteins and glycosaminoglycans lines the luminal wall of all blood vessels. Hyaluronic acid and heparan sulphate proteoglycans constitute its major components [10]. As the first phase of contact between the bloodstream and the vessel wall, the endothelial surface coating is an important mediator of the vascular microenvironment on different functional levels [11]. Local glycocalyx dimensions differ in the vascular tree [9] dependent on shear patterns and inflammatory status and are correlated with functional differences in leukocyte-endothelial interaction and endothelial shear stress sensing.

Both these processes are of eminent importance for the growth of collateral arteries [12, 13], but the structural morphology of the glycocalyx in growing collateral arteries, its reaction upon collateral vessel recruitment or its functional importance for arteriogenesis have not yet been investigated.

We therefore hypothesized that perturbation of the glycocalyx attenuates arteriogenesis by reducing the expression of shear-regulated pro-arteriogenic genes. In addition, we studied the local dimensions of the endothelial glycocalyx in newly recruited collateral arteries and peri-vascular leukocyte accumulation.
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2. EXPERIMENTAL PROCEDURES

2.1. Animal Experiments

This study conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). After securing the appropriate institutional approvals, 43 New Zealand White rabbits of comparable weight (3.0±0.2 kg) and age underwent unilateral ligation of the right femoral artery as previously described and sham operation of the contralateral side under general anesthesia with ketamine and xylazine [14]. Forty animals received a single intra-arterial bolus infusion of 20mg filtered hyaluronidase (bovine testes, fraction IV-S, activity 750-1500U/mg, Sigma, St. Louis, MO) or heat-inactivated enzyme (90°C for 15min) into the proximal stump of the femoral artery (20 animals per group). The bolus infusion was followed by implantation of an osmotic minipump connected to an infusion catheter. The tip of the catheter was positioned distal to the branches of the arteria circumflexa femoris and the arteria profunda femoris, pointing upstream in order to deliver the substances (0.1mg/h active or inactive hyaluronidase) continuously and during first-pass into the collateral circulation. Three animals underwent femoral artery ligation only and served for the electron microscopic studies of glycocalyx diameter.

2.2. Electron microscopic visualization of the endothelial glycocalyx

Following induction of anesthesia, the abdominal aorta was antegrade cannulated caudal to the renal branches at day one following femoral artery ligation for in situ perfusion staining of the endothelial glycocalyx as previously described for mice [15]. The protocol was adapted for the rabbit species and since flow rate is critical for glycocalyx preservation, perfusion was monitored continuously with an in-line flow probe installed in the perfusion system (Transonic Systems, Ithaca, NY). Perfusion was started with HEPES-buffered salt solution containing 0.1% BSA and 5IU/ml heparin at a controlled flow rate of 30ml/min. The abdominal vena cava was opened to create an outflow and perfusion was continued for 10min to remove blood in the distal extremities. The animal was euthanized with an overdose of thiopental and perfusion was changed to a phosphate-buffered fixative containing 15mmol/l MgCl2 at 20ml/min for 2 min. Finally the hind limb was perfused with fixative solution containing 0.05% alcian blue 8GX and 0.01% acridine orange (both Sigma, St. Louis, MO) for 30min at 20ml/min. A detailed description of the staining solutions is published as an online supplement by van den Berg et al. [15].

After perfusion, the vastus intermedius quadriceps muscle that contains two distinct collateral arteries spanning from the circumflex femoral artery to the arteria genualis was dissected and fixed overnight in 4% paraformaldehyde. The following day, samples were post-fixed in 1% osmium tetroxide and 1% lanthanum nitrate in water for 2h at room temperature, dehydrated in alcohol and embedded in epon. For orientation, semithin sections were stained with toluidine blue. Thin sections (60 nm) were stained in a saturated
solution of uranyl acetate in 70% methanol followed by Reynold’s lead citrate. The sections were examined using a Technai 12 (FEI Co., Eindhoven, the Netherlands) equipped with a side-mounted Megaview II camera (SIS, Muenster, Germany). Pictures were analyzed using analySIS software 3.0 (Soft-Imaging, Muenster, Germany). Diameter of the endothelial glycocalyx was measured at TEM magnifications of 93000x and was defined as the distance of the stained structures between the luminal membrane of the endothelial cell and their luminal boundary as previously described [9].

2.3. Hemodynamic measurements of collateral conductance

Assessment of the collateral conductance as the functional parameter of arteriogenesis was performed in six animals per group as previously described at 7 days following femoral artery ligation [14]. In short, a roller pump driven shunt was established between the carotid artery and the abdominal aorta, which allowed perfusion of the hind limb vasculature at selected pressure levels. Central-peripheral pressure differences between the abdominal aorta and the saphenous arteries were recorded on a computerized system. Maximal vasodilation was induced by adenosine infusion into the shunt system and at each pressure level, differently labeled fluorescent microspheres were injected into the shunt system. Following tissue harvest and processing, the microspheres were quantified in a flow cytometer and collateral conductance was derived from the slope of the pressure-flow relation, allowing a precise determination of the functional capacity of the developing collateral circulation.

2.4. Histological assessment of vascular proliferation and perivascular cell infiltration

At 7 days following femoral artery occlusion, tissue samples from the quadriceps and adductor muscles were harvested from six animals per group, snap frozen in liquid nitrogen and subsequently embedded in tissue-tek (Sakura Finetek, Torrence, CA, U.S.A.). 5μm frozen sections were fixed in ice-cold acetone and incubated with a cross-reactive mouse anti-rat antibody against Ki67 (Clone MIB-5, Dako, Glastrup, Denmark). Macrophages were stained with a cross-reactive antibody against CD68 (mouse anti-human, Clone EBM11, Dako). A Cy3-labeled anti-mouse antibody (Amersham Biosciences, Upsalla, Sweden) was used as a secondary reagent. Vascular smooth muscle cells were visualized with a FITC-conjugated antibody against alpha-smooth muscle actin (Sigma) and nuclei were stained with Hoechst 33342 dye (Molecular Probes, Eugene, Oregon). Quantitative analyses of proliferation indices and macrophage accumulation were performed in a blinded manner.
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2.5. In vitro vascular smooth muscle cell proliferation assay

Vascular smooth muscle cells were cultured in standard medium in the presence of 10% fetal bovine serum (FBS), and hyaluronidase (0, 1, 10, 100 μg/ml) was added to the culture for 24h. Proliferation was measured using an assay based on BrdU incorporation (Roche, Mannheim, Germany) according to the manufacturer’s instructions. In short, 10 μM BrdU was added to each vial for four hours. After fixation and permeabilization, anti-BrdU antibody linked to peroxidase was added and cells were incubated for 90 minutes. Following the necessary washing steps, substrate solution to visualize the bound peroxidase was added and absorption was measured at 450 nm in a EL808 spectrophotometer (BioTek, Winooski, VT).

2.6. Quantitative RT-PCR

In 8 animals per group, the carotid artery was cannulated and the animals were exsanguinated at day 7 after femoral artery ligation. The abdominal aorta was cannulated and the hind limb vasculature was perfused with warm (37°C) liquid latex (Chicago Latex Products no. 563, Crystal Lake, IL) at a constant pressure of 80 mmHg to enable visual identification and the gentle excision of collateral arteries. Collateral arteries bypassing the site of femoral artery occlusion were carefully dissected from the quadriceps and the adductor muscles after hardening of the latex and snap frozen in liquid nitrogen. Total RNA was extracted from the isolated arteries of the occluded and the unoccluded hind limb using Tripure reagent (Roche, Basel, Switzerland) according to the manufacturer’s instructions, transcribed into cDNA and subjected to quantitative reverse transcriptase polymerase chain reaction (RT-PCR), as described before [16]. Primers used were TGF-beta1 (forward: 5’-cttcttccacaactagtcttc-3’; reverse: 5’-gtccaggctccagatgtagg-3’) and eNOS (forward: 5’- agcctcactcctgtcttcca-3’; reverse: 5’-gtggccttcactctcttgc-3’).

All mRNA expression levels were normalized for 18s rRNA (forward: 5’-tcaacacgggaaetcctc-3’; reverse: 5’-ataaatgcttccagcaac-3’) and expressed as a relative expression value.

3. Statistical Analysis

Data are presented as mean ± standard deviation. Intergroup comparisons were performed with SPSS (Version 12.0.1) using a Mann-Whitney U test for independent samples. P-values <0.05 were considered to be statistically significant.
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4. Results

4.1. Endothelial glycocalyx morphology is altered in proliferating collateral arteries

Visualization of the endothelial surface glycocalyx by in situ combined alcian blue and acridine orange staining and subsequent electron microscopy revealed a thinning of the glycocalyx diameter in recruited collateral arteries compared to quiescent anastomoses. At 24h following femoral artery ligation, the diameter of the endothelial surface coat was on average 67.5±47.2 nm in the newly recruited collateral arteries, whereas the glycocalyx in the corresponding non-recruited anastomoses from the sham-operated contra-lateral hind limb was significantly thicker (p<0.001) with an average diameter of 101.0±11.3 nm. In addition, the glycocalyx in growing collateral arteries showed an undulating luminal surface and a more heterogeneous distribution on the endothelial cell membrane (figure 1). A single intra-arterial bolus infusion of 20mg hyaluronidase 1h before begin of the staining procedure reduced average glycocalyx diameter on the collateral artery surface to 17.5±6.0nm, whereas infusion of inactive enzyme did not visibly affect the endothelial coat (diameter 58.7±41.1nm, p<0.001 for hyaluronidase vs. inactive enyzme) (figure 2).
Figure 1: Electron microscopic imaging of Alcian blue 8GX stained rabbit arteries from the quadriceps muscle at 39000x (A, B) and 93000x magnification (C, D). Mean glycocalyx diameter was significantly reduced in growing collateral arteries (B, D) compared to quiescent anastomoses from the unoccluded hind limb (A, C). The lumen of luminal endothelial cell caveolae also stained positive.
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Figure 2: Glycocalyx perturbation by hyaluronidase infusion. A single bolus infusion of 20mg hyaluronidase significantly reduced endothelial glycocalyx dimensions (A) compared to infusion of the heat-inactivated enzyme (B).

4.2. Glycocalyx perturbation by hyaluronidase infusion significantly attenuated collateral artery growth

To assess the functional importance of the hyaluronic-acid containing glycocalyx for adaptive arteriogenesis, we measured the conductance (collateral flow/gradient of perfusion pressure) of the developing collateral circulation using fluorescent microspheres under maximal vasodilation. Continuous infusion of hyaluronidase into the stem vessels of the collateral circulation resulted in a significant impairment of arteriogenesis following femoral artery ligation compared to the infusion of inactivated enzyme as a control (ml/min/100mmHg: Hyaluronidase: 27.5±3.5; Controls: 47.1±3.83; p<0.001) (figure3).
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This attenuation of collateral artery growth was also reflected on a cellular level, where staining for actively proliferating vascular smooth muscle and endothelial cells revealed a significantly lower number of Ki67+ smooth muscle cells in the hyaluronidase treated group (%Ki67+VSMC: Hyaluronidase: 15.3±2.07; Controls: 19.1±1.28; p=0.018) (figure 4).

Figure 4: Hemodynamic assessment of collateral conductance. Microsphere based perfusion measurements demonstrated a significant reduction in conductance of collateral arteries at seven days following femoral artery ligation.

4.3. Hyaluronidase has no direct anti-proliferative effect on vascular smooth muscle cells

To investigate a potential direct inhibitory effect of hyaluronidase on vascular smooth muscle cell proliferation, we added different concentrations of hyaluronidase to cultured VSMCs. Incubation with hyaluronidase in vitro did not influence smooth muscle cell proliferation (BrdU incorporation [absorption units]: 0μg/ml hyaluronidase: 0.85±0.12; 1μg/ml: 0.85±0.09; 10μg/ml: 0.79±0.11; 100μg/ml: 0.84±0.18).
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Figure 4: Proliferation of vascular smooth muscle cells in collateral arteries (400x magnification). Using a double staining for the proliferation marker Ki67 (red) and alpha-smooth muscle actin (green), the percentage of Ki67 positive nuclei of all nuclei (blue) of vascular smooth muscle cells was calculated. Hyaluronidase infusion resulted in a significantly lower number of actively proliferating cells (A) in collateral arteries compared to control animals treated with inactive enzyme (B). Unrecruited anastomoses from the unoccluded hind limb show almost no smooth muscle cell proliferation in both the hyaluronidase (C) and the control group (D).
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4.4. Hyaluronidase infusion did not affect the number of perivascular macrophages around growing collateral arteries

Since the endothelial glycocalyx is also known to influence leukocyte adhesion, we quantified the number of perivascular macrophages as known mediators of collateral artery growth. Staining of frozen sections from the quadriceps muscle verified the accumulation of CD68+ positive cells in the perivascular space of growing collateral arteries (CD68+cells/mm²: Hyaluronidase: 27.6±16.5; Controls: 26.5±7.0), whereas only few cells were present around the quiescent anastomoses from the unoccluded hind limb (CD68+cells/mm²: Hyaluronidase:1.3±2.1; Controls: 1.6±2.3). No significant difference in number of perivascular macrophages around collateral arteries from the occluded hind limb was observed between the two treatment groups (figure 5).
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Figure 5: Macrophage accumulation around growing collateral arteries from the quadriceps muscle (400x magnification). A double staining for the macrophage marker CD68 (red) and alpha-smooth muscle actin showed an increased number of perivascular macrophages around growing collateral arteries (A, B) compared to unrecruited anastomoses from the contralateral hind limb (C, D). There was no significant difference in number of CD68+ cells between animals that received hyaluronidase (A, C) or inactivated enzyme (B, D).
4.5. Perturbation of the glycocalyx reduced the expression of shear-regulated and pro-arteriogenic genes in collateral arteries

As the endothelial glycocalyx was previously shown to facilitate mechanotransduction of fluid shear stress in endothelial cells, we measured the expression of eNOS and TGF-beta1 as two shear stress regulated genes involved in arteriogenesis. Quantitative RT-PCR of isolated collateral arteries showed a small but significant reduction in expression of both eNOS (relative expression: Hyaluronidase: 0.31±0.57; Controls: 1.0±1.43; p=0.039) and TGF-beta1 (relative expression: Hyaluronidase: 0.41±0.32; Controls: 1.0±0.65; p=0.033) in animals that underwent continuous hyaluronidase infusion compared to control animals, figure 6).

Figure 6: mRNA expression levels of endothelial nitric oxide synthase (eNOS) and transforming growth factor beta1 (TGF-beta1) of isolated collateral arteries. Glycocalyx perturbation by hyaluronidase infusion resulted in a significant reduction of both eNOS and TGF-beta1 expression compared to arteries from the control group that received inactivated enzyme. Expression levels are normalized to 18s ribosomal RNA and expressed as relative values.
5. DISCUSSION

We describe a reduced endothelial glycocalyx diameter and altered glycocalyx surface pattern in growing collateral arteries following femoral artery occlusion in the rabbit. Glycocalyx perturbation by continuous local hyaluronidase infusion decreased the expression of pro-arteriogenic genes in collateral arteries and resulted in a significant attenuation of arteriogenesis. Perivascular macrophage accumulation was not affected by hyaluronidase treatment. This is the first study demonstrating a direct involvement of the endothelial glycocalyx in collateral artery growth.

5.1. Endothelial glycocalyx morphology in growing collateral arteries

Besides heparan sulphate proteoglycans, hyaluronic acid glycosaminoglycans are a main component of the glycocalyx [10]. Arterial as well as capillary and venous endothelial cells have been shown to be covered with this endothelial surface layer and dimensions of the glycocalyx have previously been shown to differ locally within the arterial tree: The disturbed flow pattern in the carotid bifurcation is correlated with a reduced glycocalyx diameter, potentially contributing to leukocyte adhesion and atherosclerotic lesion development [9]. In our study we now observe a similar phenomenon in newly recruited collateral arteries, with a reduced glycocalyx diameter and a more irregular surface pattern compared to quiescent anastomoses. Both ischemia as well as pro-inflammatory stimuli [17] have previously been shown to induce a shedding of the endothelial glycocalyx, decreasing the glycocalyx barrier for leukocyte adhesion and extravasation [18]. In the case of arteriogenesis, infiltration of circulating cells in the perivascular space is essential to mediate the remodeling process resulting in the increase in arterial diameter [13, 19]. Arteriogenesis is an inflammatory-like process [18] and the adaptive reduction of local glycocalyx diameter could facilitate the adhesion of circulating leukocytes to the collateral endothelium and subsequent transmigration in the perivascular space. Indeed, our results show an increased number of perivascular macrophages around growing collateral arteries compared to the quiescent anastomoses from the contralateral hind limb, in good correspondence with previous studies in different animal models of arteriogenesis [20, 21]. In vitro data also suggest a change in glycocalyx composition dependent on fluid shear stress pattern with a stimulation of hyaluronan incorporation by high laminar shear stress [22]. Since our merely morphological assessment of the collateral glycocalyx did not allow an analysis of glycocalyx composition this question remains to be answered by future investigations.
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5.2. Effects of glycocalyx perturbation on collateral artery growth

Following this description of altered glycocalyx dimensions in growing collateral arteries, we wanted to investigate the functional importance of the endothelial surface layer for adaptive arteriogenesis and studied collateral artery growth under conditions of a perturbed glycocalyx following hyaluronidase infusion. The systemic infusion of this degrading enzyme has been shown to significantly reduce glycocalyx diameter on the capillary endothelium in rats [15] and hamsters [23]. Our electron microscopical studies on rabbit collateral arteries verified this effect of hyaluronidase infusion and showed a severely reduced endothelial surface layer with electron microscopical images comparable to those of previous studies [15].

Interestingly, this perturbation of the endothelial glycocalyx resulted in a significant attenuation of collateral artery growth compared to the control group receiving inactivated enzyme. While natural arteriogenesis is correlated with the spontaneous reduction of mean glycocalyx diameter in growing collateral arteries, the further derogation had an inhibitory effect. The number of perivascular macrophages was not different between the two treatment groups, making effects on leukocyte adhesion an unlikely explanation for this observation. However, besides modulating endothelial-leukocyte interactions, the endothelial glycocalyx has previously been shown to be an important mediator of shear stress-sensing and flow-mediated vascular reactions[24]. Both heparan sulphate proteoglycans and hyaluronic acid glycosaminoglycans are involved in endothelial cell mechanotransduction: Pre-treatment of bovine aortic endothelial cells with heparinidase III for 2h completely inhibited the induction of nitric oxide (NO) in response to steady and oscillatory shear stress [25]. In isolated canine femoral arteries, hyaluronidase treatment reduced flow induced NO-production by 80% [26]. Acetylcholine-induced production of NO remained unaffected by glycocalyx perturbation, indicating that hyaluronic acid is essential for shear-stimulated, but not for agonist induced NO-production. Since arteriogenesis is largely driven by increased fluid shear stress following collateral artery recruitment [27], impairment in endothelial shear stress sensing could explain the observed attenuation of arteriogenesis following glycocalyx perturbation. The shear stress regulated genes eNOS and TGF-beta1 have previously been shown to be upregulated in growing collateral arteries and to be functionally important for the arteriogenic response following femoral artery occlusion [2-4]. Using quantitative RT-PCR, we now found a significantly reduced expression of both eNOS and TGF-beta1 in collateral arteries from hyaluronidase treated animals. Since fluid shear stress and the changes in collateral gene expression induced by this mechanic stimulus are an important driving force of arteriogenesis [12], these findings could explain the attenuated collateral artery growth following glycocalyx perturbation. A recent study in the hamster window chamber model also described an impaired capillary perfusion following acute hyaluronidase infusion [28]. In our own model, continuous infusion of high-dose adenosine into the perfusion system ensures maximal vasodilation at the time of hemodynamic measurements and excludes potential differences in vasomotor tonus as a confounding factor. Hyaluronidase effects on endothelial shear stress sensing could however be a common explanatory mechanism for both flow dependent vascular relaxation and shear stress mediated collateral artery growth.
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5.3. Clinical implications

While representing an important structural component of all blood vessels in the human body, the endothelial glycocalyx has been little explored regarding its role in vascular pathology and adaptation. Only in the last few years increasing evidence indicated a crucial role of the endothelial glycocalyx in atherosclerotic plaque formation, ischemia/reperfusion injury [15] and diabetic vasculopathy [8]. In type 1 diabetic patients, total endovascular glycocalyx volume was reduced to about 50% of healthy volunteers [7], and in an atherosclerotic mouse model reduced glycocalyx dimensions precede the development of atherosclerotic lesions [9]. Interestingly, both diabetes [29] as well as hypercholesterolemia [6] are also correlated with a severe impairment of collateral artery growth. Although several explanations have been postulated [5], the mechanisms for this negative effect remain largely unknown. Our study now directly demonstrates a functional relevance of glycocalyx integrity for arteriogenesis and warrants further investigations whether glycocalyx perturbation may be a causal factor for impaired collateral artery growth in the clinical setting.

5.4. Study limitations

Our electron microscopical studies verified a significantly reduced glycocalyx dimension and displayed a decreased expression of shear-stress regulated pro-arteriogenic genes in collateral arteries. However, our loss of function approach does not exclude other effects of hyaluronidase infusion that might negatively affect arteriogenesis independent of glycocalyx perturbation. Since the endothelial glycocalyx is quickly regenerated after discontinuation of the hyaluronidase infusion, no rescue experiment by therapeutic glycocalyx reconstitution could be performed in our model.

6. Conclusion

These results implicate the endothelial glycocalyx as a novel player in the regulation of adaptive arteriogenesis by influencing the expression of shear-stress regulated pro-arteriogenic genes in collateral arteries. The endothelial surface represents a new target for the therapeutic stimulation of collateral artery growth. Further studies in disease models with a morbidity-induced glycocalyx perturbation (e.g. diabetic animals) are warranted to determine the potential effects of glycocalyx reconstitution on collateral artery growth.
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7. REFERENCES


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