

Endothelial glycocalyx dimensions are reduced in growing collateral arteries and modulate leucocyte adhesion in arteriogenesis

Sebastian Grundmann^{a, c, e, *}, Stephan H. Schirmer^{a, f}, Liesbeth H. P. Hekking^b, Jan Andries Post^b, Mihaela G. Ionita^c, Daphne de Groot^c, Niels van Royen^a, Bernard van den Berg^d, Hans Vink^d, Martin Moser^e, Christoph Bode^e, Dominique de Kleijn^g, Gerard Pasterkamp^c, Jan J. Piek^a, Imo E. Hoefer^c

^a Department of Cardiology, AMC, University of Amsterdam, The Netherlands

^b Department of Molecular Cell Biology, University of Utrecht, The Netherlands

^c Laboratory of Experimental Cardiology, UMC, University of Utrecht, The Netherlands

^d Department of Physiology, Cardiovascular Research Institute, Maastricht, The Netherlands

^e Department of Internal Medicine III, University Hospital Freiburg, Germany

^f Department of Internal Medicine III, University Hospital of the Saarland, Germany

^g Interuniversity Cardiology Institute of the Netherlands, Utrecht, The Netherlands

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Abstract

During collateral artery growth, monocytes adhere to the endothelium and secrete cytokines from the perivascular space promoting arteriogenesis. Recently, the endothelial glycocalyx has been shown to modulate leucocyte infiltration in atherogenic regions. The role of this endothelial surface coating in arteriogenesis, however, has not been investigated so far. We now report that local plasma levels of hyaluronic acid are specifically increased in collateral arterial blood of coronary artery disease patients and hypothesized that components of the endothelial glycocalyx are shed during arteriogenesis, resulting in decreased glycocalyx dimensions and an increased leucocyte extravasation. In a rabbit model of femoral artery ligation, electron microscopy revealed a decrease in glycocalyx dimensions in collateral arteries compared with quiescent anastomoses (67.5 ± 47.2 nm versus 101.0 ± 11.3 nm; $P < 0.001$). This decrease was correlated with a higher number of perivascular macrophages around collateral arteries. The additional glycocalyx perturbation by local hyaluronidase infusion almost completely removed the endothelial surface layer and temporarily stimulated leucocyte accumulation in the perivascular space. However, complete perturbation of the glycocalyx by hyaluronidase infusion resulted in a significant attenuation of collateral artery growth assessed by microsphere-based perfusion measurements (ml/min/100 mmHg: hyaluronidase: 27.5 ± 3.5 ; Controls: 47.1 ± 3.83 ; $P < 0.001$) and a lower percentage of actively proliferating vascular smooth muscle cells. A decreased expression of the shear-stress regulated pro-arteriogenic genes eNOS and TGF- β 1 suggests an impaired mechanotransduction as the underlying mechanisms. For the first time, we describe the role of the endothelial glycocalyx in collateral artery growth. Although complete abrogation led to attenuated arteriogenesis, shedding of glycocalyx components is observed during collateral artery growth.

Keywords: arteriogenesis • angiogenesis • arterial remodelling

*Correspondence to: Sebastian GRUNDMANN, M.D., Ph.D.,
Department of Internal Medicine III – Cardiology and Angiology,
University Hospital Freiburg, Hugstetter Straße 55,
79106 Freiburg, Germany.
Tel.: +49 (0) 761 270 7044
Fax: +49 (0) 761 270 7045
E-mail: sebastian.grundmann@uniklinik-freiburg.de

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 post-stenotic 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 adhesion and subsequent infiltration of circulating cells [1], especially leucocytes. Among these cell populations, infiltrating monocytes have been shown to play a major role as mediators of arterial remodelling and eventually an enlargement of the collateral artery.

Recently, the endothelial glycocalyx has been implicated in the mediation of leucocyte adhesion in atherosclerotic disease. This network of negatively charged proteoglycans, glycoproteins and glycosaminoglycans lines the luminal wall of all blood vessels. During atherogenesis, a disturbance of the glycocalyx by turbulent shear stress patterns results in a decrease of the glycocalyx width, allowing the approximation of circulating cells to the endothelium and the adhesion and infiltration of monocytes, resulting in atherosclerotic plaque formation [2]. In addition, the endothelial glycocalyx plays an important role in the shear stress sensing, mechanotransduction and NO-mediated vasodilation. Both leucocyte infiltration as well as shear stress sensing are of eminent importance for the growth of collateral arteries [3, 4], 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.

In this study, we aimed to screen for indirect parameters of local glycocalyx alterations in collateral arteries of coronary artery disease patients by measuring local plasma levels of glycocalyx components in the collateral circulation. We further investigated glycocalyx morphology and effects of glycocalyx modulation on arteriogenesis in a rabbit model of collateral artery growth.

Experimental procedures

Patient study

Patient selection. This study was approved by the institutional medical ethics committee. After giving informed consent, 40 Caucasian patients scheduled for percutaneous coronary intervention (PCI) of a high-grade stenosis ($n = 24$) or a total coronary occlusion ($n = 16$) were included. Exclusion criteria were previous myocardial infarction in the area of collateralization, previous cardiac surgery, severely depressed left ventricular function, diabetes mellitus, neoplastic disease and signs of inflammatory illness.

Collateral flow index (CFI). Forty patients with stable coronary artery disease underwent coronary angiography following intracoronary injection of 0.1 mg nitroglycerine. Quantitative coronary angiography (Medis, Leiden, The Netherlands) was performed to determine the percentage stenosis using perpendicular images.

A 0.014'' pressure guide wire (BrightWire, Volcano, Rancho Cordova, CA) was used for intracoronary pressure measurements. During a 1-min. balloon inflation, the pressure distal to the coronary occlusion (wedge pressure, P_w) as well as aortic (P_{Ao}) pressure were determined. CFI was calculated as $(P_w - 5 \text{ mmHg}) / (P_{Ao} - 5 \text{ mmHg})$ as described previously [5].

Intracoronary blood plasma collection. For intracoronary blood sampling, a 7F (inner diameter 0.051'') proximal embolic protection device (Proxis[®]-catheter, St. Jude Medical, St. Paul, MN) was advanced through the guiding catheter and into the treated coronary artery which received collateral blood. Inflating a low-pressure (0.67 atm) balloon at the end of the catheter temporarily stops antegrade blood flow in the artery. Subsequently, gentle suction was applied manually to the proximal end of the catheter. The first 3 ml aspirated were discarded to prevent contamination with contrast medium. Approximately 10–15 ml blood were then aspirated within 1–2 min. from the retrogradely filled vessel and thus directly from the collateral circulation. After deflation of the balloon, the catheter was drawn back into the guiding catheter, where the same procedure (balloon inflation, aspiration of blood) was repeated to obtain a control sample from the systemic circulation. Blood was transferred into citrate tubes and centrifuged at 1550g for 30 min. Plasma was taken off carefully, aliquoted, snap frozen in liquid nitrogen and stored at -80°C .

ELISA. A commercially available ELISA (Echelon Inc., Salt Lake City, UT) was used for the detection of hyaluronic acid (HA) according to the manufacturer's instructions. Briefly, plasma samples were incubated with HA binding protein and transferred to a detection plate for competitive binding with pre-coated HA. An enzyme-linked antibody (alkaline phosphatase) detected the amount of HA binding protein on the plate, which was inversely proportional to the concentration in the samples. Using p-nitrophenyl phosphate as a substrate, absorption was measured at 405 nm using an EL808 spectrophotometer (BioTek, Winooski, VT). All ELISA results were normalized for plasma albumin levels.

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 ($3.0 \pm 0.2 \text{ kg}$) and age underwent unilateral ligation of the right femoral artery as previously described and sham operation of the contralateral side under general anaesthesia with ketamine and xylazine [6]. Forty animals received a single intra-arterial bolus infusion of 20 mg filtered hyaluronidase (bovine testes, fraction IV-S, activity 750–1500 U/mg, Sigma, St. Louis, MO) or heat-inactivated enzyme (90°C for 15 min.) into the proximal stump of the femoral artery (20 animals per group). The bolus infusion was followed by implantation of an osmotic minipump (alzet, durect corp., Cupertino, CA), 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 (0.1 mg/hr 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 width.

Electron microscopic visualization of the endothelial glycocalyx. Following induction of anaesthesia, the abdominal aorta was antegradely cannulated caudal to the renal branches at day 1 following femoral artery ligation for *in situ* perfusion staining of the endothelial glycocalyx as previously described for mice [7]. The protocol was adapted for the rabbit species and because 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 5 IU/ml heparin at a controlled flow rate of 30 ml/min. The abdominal *vena cava* was opened to create an outflow and perfusion was continued for 10 min. 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 15 mmol/l MgCl₂ at 20 ml/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) for 30 min. at 20 ml/min. A detailed description of the staining solutions is published as an on-line supplement by van den Berg *et al.* [7].

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 2 hrs at room temperature, dehydrated in alcohol and embedded in epon. For orientation, semi-thin 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). Width of the endothelial glycocalyx was measured at TEM magnifications of 93,000 \times 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 [2].

Haemodynamic 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 [6]. 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 labelled 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.

Histological assessment of vascular proliferation and perivascular cell infiltration. At 3 and 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, Torrance, CA). Five micrometres frozen sections were fixed in ice-cold acetone and incubated with a cross-reactive mouse anti-rat antibody against Ki67 (Clone MIB-5, Dako, Glostrup, Denmark). Macrophages were stained with a cross-reactive antibody against CD68 (mouse anti-human, Clone EBM11, Dako).

A Cy3-labelled anti-mouse antibody (Amersham Biosciences, Uppsala, 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.

Quantitative RT-PCR. In animals per groups, 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 [8]. Primers used were TGF- β 1 (forward: 5'-cttctccaccaactactgcttc-3'; reverse: 5'-gtccaggctccagatgtagg-3'), eNOS (forward: 5'- agcctcactcc-tgtctcca-3'; reverse: 5'-gtggccttcaactctcttgc-3'), Ki67 (forward: 5'-caacttgggtgattccatta-3'; reverse: 5'-ttaggaggcaagttttcatc-3'), VCAM-1 (forward: 5'- gaacactcttactctgtgcacagc-3'; reverse: 5'-ccatcctcatagcaattaagtg-gag-3'), Integrin subunit β 1 (forward: 5'-gaatgtcaccacccgtagc-3'; reverse: 5'-tcggaagtctgaagtcacc-3') and ABRA-1 (forward: 5'-tgccatagcatctctcttc-3'; reverse: 5'-ggacatgtgcttcattatctgc -3'),

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

Statistical analysis. Data are presented as mean \pm standard deviation. Statistical analysis was performed with Prism 4 for Windows. A Wilcoxon signed-rank test was used for the paired analysis of patient blood samples. Since the RT-PCR results were not normally distributed, a Mann-Whitney test was applied. A t-test was used for all other comparisons. *P*-values <0.05 were considered to be statistically significant.

Results

Patient study

Intracoronary blood samples were obtained from 40 patients with stable coronary artery disease undergoing coronary angiography. Patients with a non-total occlusion were grouped into collateral responders (CFI > 0.21, *n* = 12) and non-responders (CFI \leq 0.21, *n* = 12). Patient characteristics are listed in Table 1.

Measurements in blood samples obtained from the coronary collateral circulation showed significantly higher hyaluronic acid plasma levels compared with samples obtained from the systemic circulation (Fig. 1). There was no significant correlation between the individual increase in hyaluronic acid plasma levels and CFI measurements. No differences between collateral-systemic gradients of hyaluronic acid were detected between non-responders, responders and patients with a chronic total occlusion. Correlating hyaluronic acid plasma levels and baseline characteristics, we found that the ratio of collateral/systemic hyaluronic acid plasma levels was significantly greater in patients with a family history of coronary artery disease (*P* = 0.033) and with hypertension (*P* = 0.035).

Animal study

Endothelial glycocalyx morphology is altered in proliferating collateral arteries. Attempting to follow up our findings from a heterogenous real-world patient population under experimental conditions, we subjected New Zealand white rabbits to unilateral

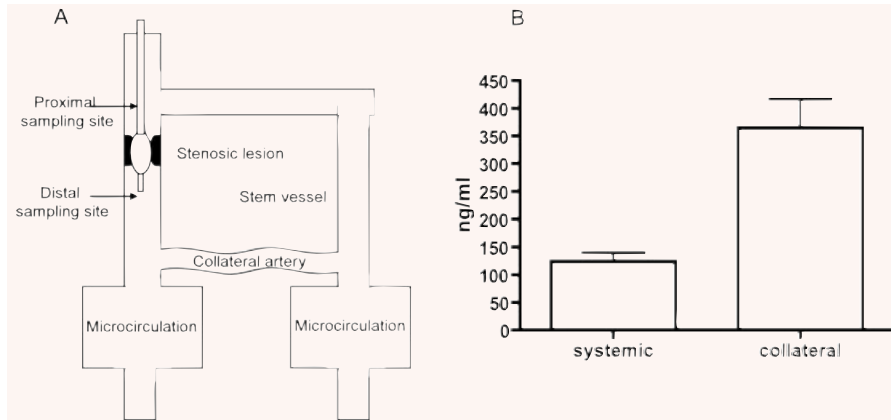


Fig. 1 Schematic drawing of experimental set-up of collateral flow index measurements and blood sample aspiration during coronary angiography in coronary artery disease patients (A). Blood samples aspirated via the collateral network contained significantly higher levels of hyaluronic acid than samples from the proximal site (B).

Table 1 Patient characteristics

	Non-responders (CFI \leq 0.21, $n = 12$)	Responders (CFI $>$ 0.21, $n = 12$)	CTOs ($n = 16$)	P-value
Age – years	62.8 \pm 13.5	61.4 \pm 11.0	61.9 \pm 11.8	0.95
Male sex – no. (%)	9 (75)	9 (75)	12 (58.3)	0.57
Body mass index (BMI)	27.2 \pm 2.6	27.0 \pm 2.8	26.4 \pm 4.9	0.82
Body surface area (BSA)	2.0 \pm 0.2	2.0 \pm 0.1	1.9 \pm 0.5	0.53
Hypertension – no. (%)	7 (58.3)	8 (66.7)	10 (62.5)	0.92
Hypercholesterolemia – no. (%)	5 (41.7)	7 (58.3)	5 (31.3)	0.36
Family history of CAD – no. (%)	10 (83.3)	7 (58.3)	8 (50.0)	0.19
Current smoker – no. (%)	4 (33.3)	1 (8.3)	3 (18.8)	0.42
Ex smoker – no. (%)	6 (50)	7 (58.3)	11 (68.8)	0.42
β -blockers – no. (%)	11 (91.7)	9 (75.0)	15 (93.8)	0.29
Statins – no. (%)	10 (83.3)	11 (91.7)	16 (100)	0.25
Aspirin – no. (%)	11 (91.7)	11 (91.7)	16 (100)	0.50
Clopidogrel – no. (%)	6 (50)	11 (91.7)	11 (68.8)	0.08
Calcium antagonists – no. (%)	5 (41.7)	3 (25.0)	3 (18.8)	0.42
Nitrates – no. (%)	6 (50.0)	5 (41.7)	9 (56.3)	0.75
ACE-inhibitors/ARBs – no. (%)	4 (33.3)	4 (33.3)	8 (50)	0.57
C-reactive protein – mg/dl	1.75 [0.7; 6.3]	1.4 [0.8; 3.5]	1.5 [1.0;3.3]	0.75
Haemoglobin – mmol/l	8.63 \pm 0.61	8.71 \pm 0.41	8.83 \pm 0.70	0.68
Glucose – mmol/l	6.0 \pm 1.1	6.0 \pm 2.1	5.9 \pm 0.8	0.97
Total cholesterol – mmol/l	4.0 \pm 0.9	3.8 \pm 0.8	3.8 \pm 0.8	0.77
LDL-cholesterol – mmol/l	1.2 \pm 0.4	1.1 \pm 0.2	1.3 \pm 0.3	0.32
HDL-cholesterol – mmol/l	2.1 \pm 0.7	2.0 \pm 0.8	2.0 \pm 0.7	0.85

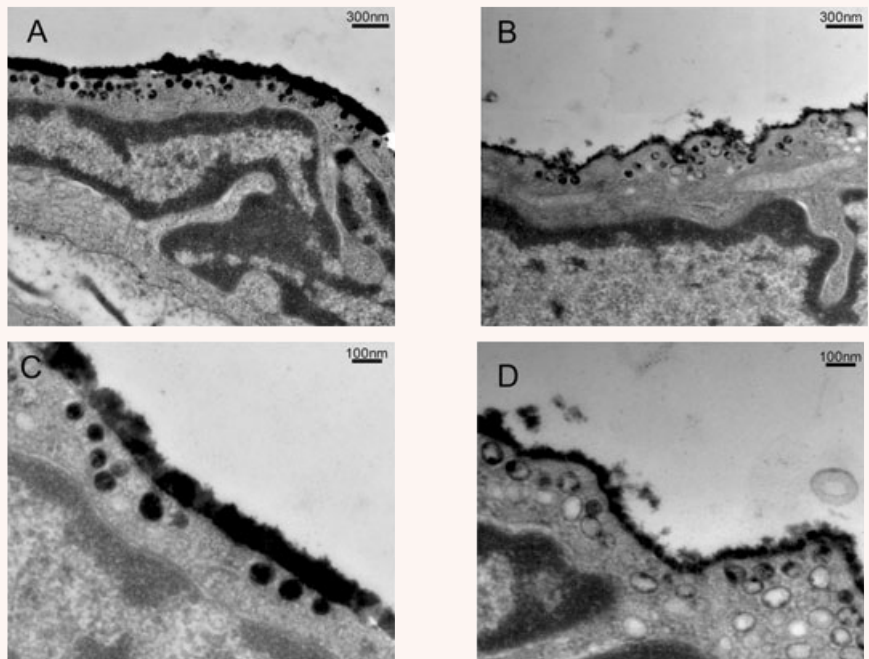
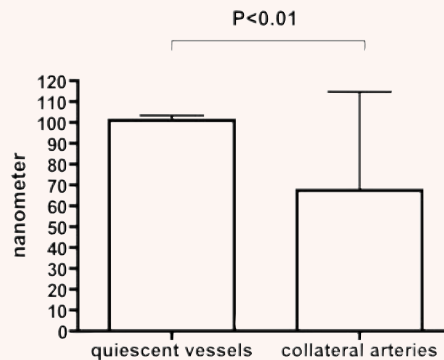


Fig. 2 Electron microscopic imaging of Alcian blue 8GX stained rabbit arteries from the quadriceps muscle at 39,000 \times (A, B) and 93,000 \times magnification (C, D). The glycocalyx on the luminal surface as well as in the endothelial cell caveolae stains dark and is reduced in growing collateral arteries (B, D) compared with quiescent anastomoses from the unoccluded hind limb (A, C).



femoral artery. At different time-points after induction of collateral artery growth, we directly visualized the endothelial surface glycocalyx by employing a protocol of combined *in situ* staining with alcian blue and acridine orange and subsequent electron microscopy. We found a significant thinning of the glycocalyx width in recruited collateral arteries compared with quiescent anastomoses (Fig. 2). At 24 hrs following femoral artery ligation, the width of the endothelial surface coat was on average 67.5 \pm 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 width of 101.0 \pm 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.

Because a decrease in glycocalyx width has previously shown to correlate with an increase in infiltration of monocytes to in developing atherosclerotic lesions, we quantified the number of perivascular macrophages around collateral arteries and quiescent anastomoses. Staining of frozen sections from the quadriceps muscle verified the accumulation of CD68+ positive cells in the perivascular space of growing collateral arteries, whereas only few cells were present around the arteries from the unoccluded hind limb (CD68+ cells/mm²: Collateral arteries: 26.5 \pm 7.0, quiescent anastomoses: 1.6 \pm 2.3).

Additional glycocalyx perturbation by hyaluronidase infusion stimulated perivascular monocyte infiltration, but significantly attenuated collateral artery growth. Because a partial shedding of endothelial glycocalyx in growing collateral arteries was correlated with an increased perivascular monocyte infiltration around collateral

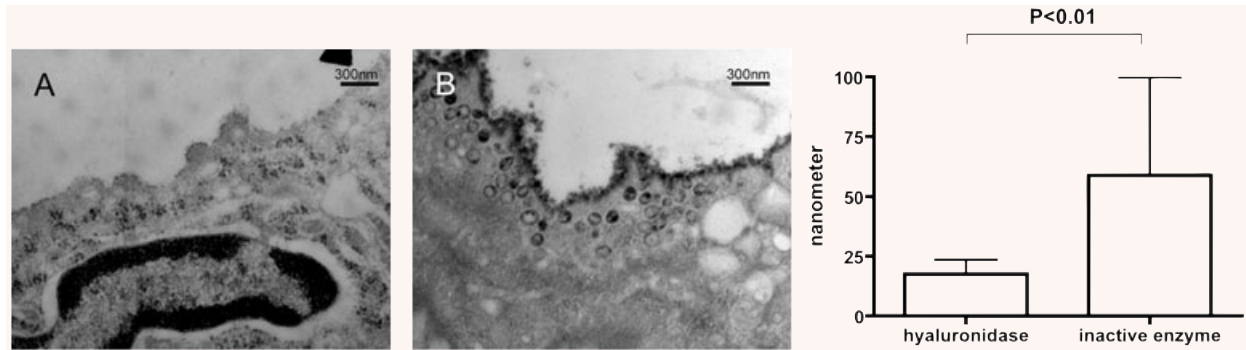


Fig. 3 Quantification of glycocalyx thickness in collateral arteries and quiescent anastomoses. Infusion of inactivated hyaluronidase had not significant effect on the glycocalyx width (B), whereas the active enzyme almost completely removed the endothelial surface layer (A).

arteries and the glycocalyx has previously been shown to directly modulate leucocyte adhesion and infiltration [9], we hypothesized that an additional perturbation of the endothelial surface coating could result in a further enhancement of monocyte infiltration and a stimulation of collateral artery growth. We therefore infused the enzyme hyaluronidase locally into the stem vessels of the collateral circulation to induce a continuous deduction of hyaluronic acid from the glycocalyx and thereby a further perturbation of the endothelial surface coating. The effects of hyaluronidase infusion were visualized in electron microscopy and infusion of the heat-inactivated enzyme served as a control. Indeed, a single intra-arterial bolus infusion of 20 mg hyaluronidase 1 hr before begin of the *in situ* staining procedure reduced average glycocalyx width on the collateral artery surface to 17.5 ± 6.0 nm, whereas infusion of the inactive enzyme did not visibly affect the endothelial coat (width 58.7 ± 41.1 nm, $P < 0.001$ for hyaluronidase versus inactive enzyme) (Fig. 3).

To assess a potential stimulating effect of glycocalyx degradation on monocyte infiltration, we performed histological analyses of the perivascular macrophage accumulation at 3 and 7 days following femoral artery ligation (Fig. 4).

Glycocalyx perturbation resulted in a significantly higher number of perivascular CD68+ macrophages at day 3, compared with control animals treated with heat-inactivated hyaluronidase (CD68+ cells/mm²: hyaluronidase: 18.9 ± 4.1 ; controls: 7.3 ± 2.9). At 7 days, the perivascular macrophage accumulation had further increased in both groups and no significant difference was detectable any more (CD68+ cells/mm²: hyaluronidase: 27.6 ± 16.5 ; Controls: 26.5 ± 7.0).

Because the number of perivascular macrophages at day 3 after femoral artery ligation has been shown to correlate with the restoration of hindlimb perfusion [10], we measured the conductance (collateral flow/gradient of perfusion pressure) of the developing collateral circulation using fluorescent microspheres.

However, despite the increase in perivascular macrophages achieved by this treatment, the continuous infusion of hyaluronidase into the stem vessels of the collateral circulation

resulted not in a stimulation but in a significant impairment of arteriogenesis following femoral artery ligation (ml/min./100 mmHg: hyaluronidase: 27.5 ± 3.5 ; Controls: 47.1 ± 3.83 ; $P < 0.001$). This attenuation of collateral artery growth was also reflected on a cellular level, where histological 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 ; $P = 0.018$) (Fig. 5). This increased number of Ki67+ cells was also reflected by a lower expression of Ki67-mRNA in qRT-PCR analysis of isolated collateral arteries following glycocalyx perturbation (Ki67 mRNA [relative expression]: hyaluronidase: 0.21 ± 0.29 ; Controls: 1.0 ± 0.99 ; $P = 0.027$).

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 (see supplementary data section).

Perturbation of the glycocalyx reduced the expression of shear-regulated and pro-arteriogenic genes in collateral arteries. As the endothelial glycocalyx was previously shown not only to influence leucocyte adhesion but also to facilitate mechanotransduction of fluid shear stress in endothelial cells, we measured the expression of eNOS and TGF- β 1 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- β 1 (relative expression: hyaluronidase: 0.41 ± 0.32 ; Controls: 1.0 ± 0.65 ; $P = 0.033$) in animals that underwent continuous hyaluronidase infusion compared with control animals. There were no significant differences in the expression of integrin β 1 (relative expression: hyaluronidase: 0.64 ± 1.40 ; Controls: 1.0 ± 1.99 ; $P = 0.68$), VCAM-1 (relative expression: hyaluronidase: 1.06 ± 1.43 ; Controls: 1.0 ± 2.79 ; $P = 0.97$) or the recently described

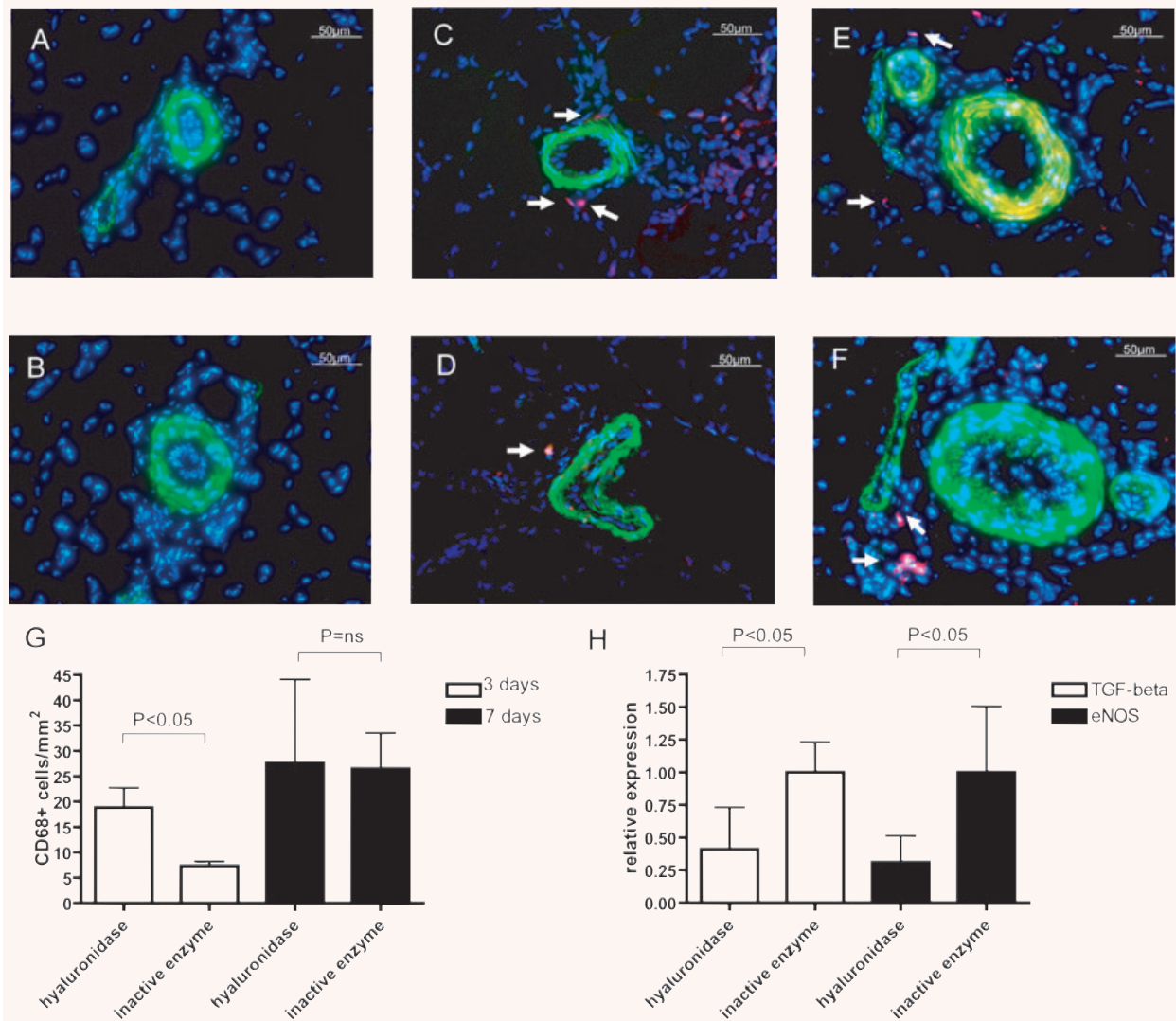


Fig. 4 Macrophage accumulation around growing collateral arteries from the quadriceps muscle (400× magnification) shown in a double staining for the macrophage marker CD68 (red) and alpha-smooth muscle actin (green). Macrophages were not regularly present in the perivascular space of unrecruited anastomoses (A, B). Following femoral artery ligation, macrophages are accumulating around growing collateral arteries at day 3 after the induction of arteriogenesis, with a significantly higher number of infiltrating cells in animals treated with hyaluronidase (C), compared to animals treated with the inactivated enzyme (D). At day 7, this difference between hyaluronidase treated (E) and control animals (F) was no longer statistically significant (G). Glycocalyx perturbation resulted in a significant reduction of the expression of the shear stress-regulated pro-arteriogenic genes eNOS and TGF-β1 (H).

pro-arteriogenic transcription factor ABRA-1 (relative expression: hyaluronidase: 2.07 ± 7.03 ; Controls: 1.0 ± 2.22 ; $P = 0.58$).

Discussion

We provide indirect evidence for the local shedding of hyaluronic acid from the endothelial glycocalyx in the coronary collateral cir-

ulation of coronary artery disease patients and describe a reduced endothelial glycocalyx width and altered glycocalyx surface pattern in growing collateral arteries following femoral artery occlusion in the rabbit. This decrease in glycocalyx width is correlated with the accumulation of perivascular macrophages around growing collateral arteries and further glycocalyx degradation by continuous local hyaluronidase infusion resulted in a temporary stimulation of monocyte infiltration. However, this approach of glycocalyx perturbation resulted not in a further stimulation, but in

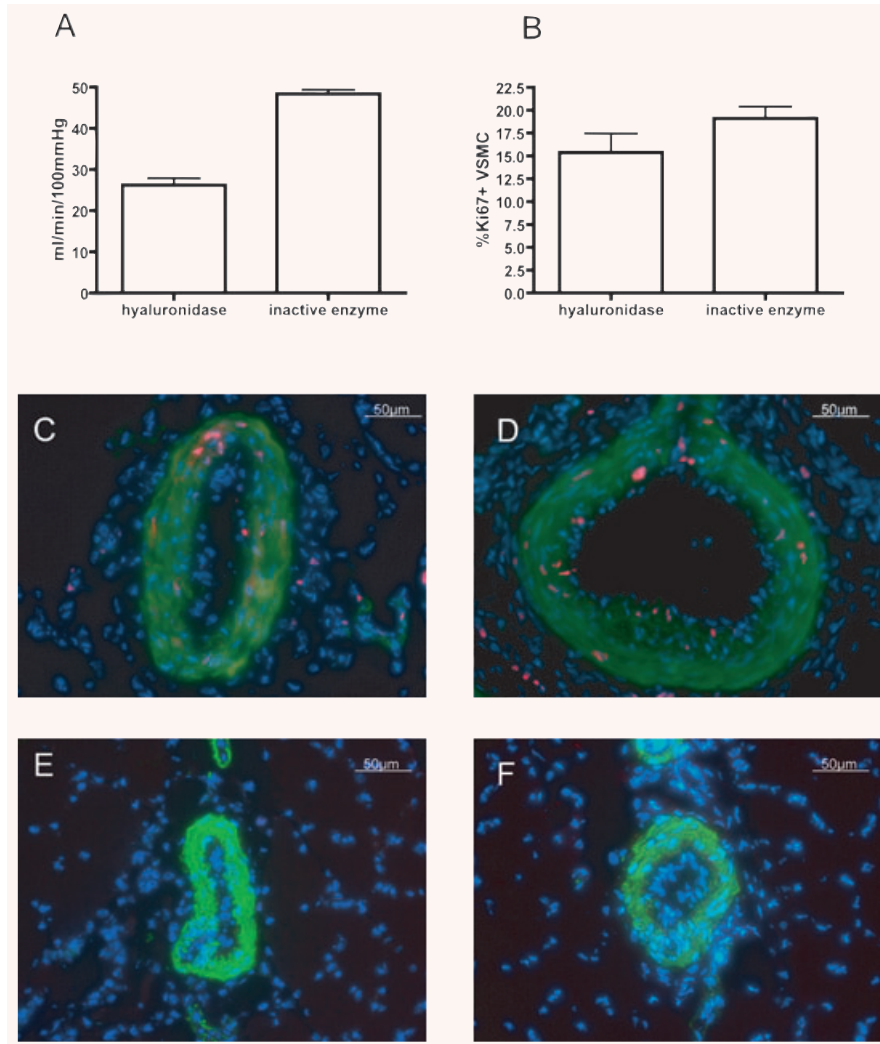


Fig. 5 (A) Haemodynamic assessment of collateral conductance. Microsphere-based perfusion measurements demonstrated a significant reduction in the conductance of collateral arteries at 7 days following femoral artery ligation in the hyaluronidase-treated group compared with the control animals. (B) Proliferation of vascular smooth muscle cells in collateral arteries (400 \times 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 (C) in collateral arteries compared with control animals treated with inactive enzyme (D). Unrecruited anastomoses from the unoccluded hind limb show almost no smooth muscle cell proliferation in both the hyaluronidase (E) and the control group (F).

a strong attenuation of collateral artery growth. This might be due to a negative effect on endothelial shear stress sensing and mechanotransduction, as suggested by the decreased expression of shear stress-regulated pro-arteriogenic genes in collateral arteries following artificial glycocalyx perturbation. This is the first study implicating an involvement of the endothelial glycocalyx in collateral artery growth.

Arteriogenesis and angiogenesis

The growth of functional collateral arteries (arteriogenesis) differs in several aspects from the sprouting process of capillaries (angiogenesis). The term *arteriogenesis* refers to the enlargement of pre-existing arterial and arteriolar blood vessels upon the occlusion or stenosis of a major artery in the same perfusion territory [11]. Although hypoxia is the main stimulus for angiogenesis,

arteriogenesis is relatively independent of local oxygen tension and driven by a change of biomechanical forces due to the increase in the pre- and post-stenotic pressure gradient, resulting in a shift of blood flow towards the yet unrecruited anastomoses. Meanwhile there is compelling evidence that fluid shear stress is indeed an important stimulus for arterial enlargement [12]. Although angiogenesis and arteriogenesis differ in these aspects, they also share several mechanistic principles, such as the infiltration of circulating cells. The different forms of vascular growth have recently been reviewed [13].

Endothelial glycocalyx morphology in growing collateral arteries

Besides heparan sulphate proteoglycans, hyaluronic acid glycosaminoglycans are a main component of the glycocalyx [14].

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 width, potentially contributing to leucocyte adhesion and atherosclerotic lesion development [2]. In our study, we now observe a similar phenomenon in newly recruited collateral arteries, with a reduced glycocalyx width and a more irregular surface pattern compared with quiescent anastomoses. Both ischaemia as well as pro-inflammatory stimuli [15] have previously been shown to induce a shedding of the endothelial glycocalyx, decreasing the glycocalyx barrier for leucocyte adhesion and extravasation [16]. In the case of arteriogenesis, infiltration of circulating cells in the perivascular space is essential to mediate the remodelling process resulting in the increase in arterial diameter [4, 17]. Arteriogenesis is an inflammatory-like process [16] and the adaptive reduction of local glycocalyx width could facilitate the adhesion of circulating leucocytes 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 with the quiescent anastomoses from the contralateral hind limb, in good correspondence with previous studies in different animal models of arteriogenesis [18, 19]. In addition, further glycocalyx perturbation resulted in an increase in the number of perivascular macrophages at 3 days after femoral artery ligation. *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 [20]. 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.

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 width on the capillary endothelium in rats [7] and hamsters [21]. 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 [7].

Interestingly, this perturbation of the endothelial glycocalyx resulted in a significant attenuation of collateral artery growth compared with the control group receiving inactivated enzyme. Although natural arteriogenesis is correlated with the spontaneous reduction of mean glycocalyx width in growing collateral arteries, the further derogation had an inhibitory effect. The number of perivascular macrophages as pro-arteriogenic mediators was

higher in the hyaluronidase-treated group, making the effects on leucocyte adhesion an unlikely explanation for this observation. However, besides modulating endothelial-leucocyte interactions, the endothelial glycocalyx has previously been shown to be an important mediator of shear stress sensing and of flow-mediated vascular reactions [22]. Both heparan sulphate proteoglycans and hyaluronidic acid glycosaminoglycans are involved in endothelial cell mechanotransduction: Pre-treatment of bovine aortic endothelial cells with heparinidase III for 2 hrs completely inhibited the induction of NO in response to steady and oscillatory shear stress [23]. In isolated canine femoral arteries, hyaluronidase treatment reduced flow induced NO production by 80% [24]. 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. Because arteriogenesis is largely driven by increased fluid shear stress following collateral artery recruitment [12], impairment in endothelial shear stress sensing could explain the observed attenuation of arteriogenesis following glycocalyx perturbation. The shear stress-regulated genes eNOS and TGF- β 1 have previously been shown to be up-regulated in growing collateral arteries and to be functionally important for the arteriogenic response following femoral artery occlusion [25–27]. Using quantitative RT-PCR, we now found a significantly reduced expression of both eNOS and TGF- β 1 in collateral arteries from hyaluronidase-treated animals. Because fluid shear stress and the changes in collateral gene expression induced by this mechanic stimulus are an important driving force of arteriogenesis [3], 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 haemodynamic 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.

CFI measurements and glycocalyx shedding

The invasive assessment of CFI using intracoronary pressure measurements is currently considered the most accurate and sensitive method of evaluating collateral circulation in patients [29] and recent investigations have tried to link circulating cellular markers to different degrees of collateralization [5, 30]. Therefore, one might expect that the degree of glycocalyx shedding in individual patients positively correlates with their CFI measurements. However, this was not the case in the present study. CFI in patients is often assessed irrespective of the phase in time of collateral artery development, that is, it mostly remains unknown in patients how long coronary collateral arteries have been growing, or if growth has already come to a halt. Time course of collateral artery

growth may strongly influence glycocalyx shedding, which might occur to large extent in rapidly growing arteries (such as in our animal model after acute occlusion) and diminish in a more stable plateau phase of collateralization. Also, intracoronary measurements of hyaluronic acid might not be sensitive enough to pick up small differences between different degrees of collateralization.

Another important issue is in how far the intracoronary blood samples truly represent the collateral-derived blood only. In all patients, the first 3 ml of blood withdrawn were discarded, so that inaccuracies due to other fluids still in the catheter are avoided. Furthermore, equilibrium of collateral perfusion will have built up, which is also indicated by stable values of pressure-derived CFI after the first minute of balloon occlusion of the original artery. In patients with chronic total occlusion, blood distal to the occlusion is dependent on collateral flow only, so that the equilibrium has already been formed.

Stroes and co-workers recently employed an interesting technique of measuring intravascular distribution volumes as an indirect but sensitive marker of glycocalyx volumes in patients [31]. However, this technique primarily measures capillary glycocalyx volume rather than glycocalyx volumes of larger vessels such as collateral arteries and was therefore not suitable for our study.

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, ischaemia/reperfusion injury [7] and diabetic vasculopathy [32]. In type 1 diabetic patients, total endovascular glycocalyx volume was reduced to about 50% of healthy volunteers [33], and in an atherosclerotic mouse model reduced glycocalyx dimensions precede the development of atherosclerotic lesions [2]. Interestingly, both diabetes [34] as well as hypercholesterolemia [35] are also correlated with a severe impairment of collateral artery growth. Although several explanations have been postulated [36], 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.

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 definitely exclude other effects of hyaluronidase infusion that might nega-

tively affect arteriogenesis independent of glycocalyx perturbation. However, a direct inhibitory effect of hyaluronidase on cultured vascular smooth muscle cell proliferation was not detected. Because 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.

Conclusion

These results implicate the endothelial glycocalyx as a novel player in the regulation of adaptive arteriogenesis and the expression of shear-stress regulated pro-arteriogenic genes in collateral arteries. However, the direct link between glycocalyx perturbation and endothelial shear stress sensing in collateral artery growth yet remains to be made. As one of the basic structural elements of the vascular wall, the function of this endothelial surface lining is not limited to mechanotransduction. Besides the modulation of leucocyte adhesion and pro-arteriogenic gene expression investigated in our study, the binding and presentation of growth factors and cytokines on the endothelial surface could be an additional mechanistic factor in this context. The modulatory function of hyaluronic acid on inflammation and proliferation has recently been reviewed by our group [37].

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.

Supporting Information

Supplementary data 1: *In vitro* vascular smooth muscle cell proliferation assay

Methods: Vascular smooth muscle cells were culture in standard medium in the presence of 10% foetal bovine serum (FBS), and hyaluronidase (0, 1, 10, 100 $\mu\text{g/ml}$) was added to the culture for 24 hrs. 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 4 hrs. After fixation and permeabilization, anti-BrdU antibody linked to peroxidase was added and cells were incubated for 90 min. Following the necessary washing steps, substrate solution to visualize the bound peroxidase was added and absorption to visualize the bound peroxidase was added and absorption was measured at 450 nm in an EL808 spectrophotometer (BioTek, Winooski, VT).

Results: 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]: 0 μg/ml hyaluronidase: 0.85 ± 0.12; 1 μg/ml: 0.85 ± 0.09; 10 μg/ml: 0.79 ± 0.11; 100 μg/ml: 0.84 ± 0.18).

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