Stretch-Induced Activation of the Transcription Factor Activator Protein-1 Controls Monocyte Chemoattractant Protein-1 Expression During Arteriogenesis

Elena Demicheva, Markus Hecker, Thomas Korff

Abstract—Cerebral, coronary, and peripheral artery diseases combined represent the most frequent cause of death in developed nations. The underlying progressive occlusion of large conductance arteries can partially be compensated for by transformation of preexisting collateral arterioles to small artery bypasses, a process referred to as arteriogenesis. Because biomechanical forces have been implicated in the initiation of arteriogenesis, we have investigated the mechanosensitive expression of a pivotal proarteriogenic molecule, monocyte chemoattractant protein (MCP)-1, which governs the recruitment of circulating monocytes to the wall of the remodeling collateral arterioles. Using a new ear artery ligation model and the classic hindlimb ischemia model in mice, we noted that MCP-1 expression is significantly increased in collateral arterioles undergoing arteriogenesis already 24 hours after its onset. By mimicking proarteriogenic perfusion conditions in small mouse arteries, we observed that MCP-1 expression is predominantly upregulated in the smooth muscle cells, which solely sense changes in circumferential wall tension or stretch. Subsequent analyses of cultured endothelial and smooth muscle cells confirmed that cyclic stretch but not shear stress upregulates MCP-1 expression in these cells. Blockade of the mechanosensitive transcription factor activator protein-1 by using a specific decoy oligodeoxynucleotide abolished this stretch-induced MCP-1 expression. Likewise, topical administration of the decoy oligodeoxynucleotide to the mouse ear abrogated arteriogenesis through downregulation of MCP-1 expression and monocyte recruitment. Collectively, these findings point toward a stretch-induced activator protein-1–mediated rise in MCP-1 expression in vascular smooth muscle cells as a critical determinant for the initiation of arteriogenesis. (Circ Res. 2008;103:477-484.)

Key Words: arteriogenesis ■ cyclic stretch ■ collateral arterioles ■ MCP-1 ■ activator protein-1

The combination of atherosclerosis in cerebral, coronary, and peripheral arteries and its sequela represent the single most important cause of death in the industrialized world. Thrombotic occlusion of these large conductance arteries often causes severe ischemia in the affected tissues. Based on the clinical relevance of the disease, significant efforts have been made to offset its consequences by stimulating the growth of new blood vessels into the ischemic area.1 However, angiogenesis per se is not sufficient to fully restore blood supply to the affected tissues.2,3 Spontaneous enlargement of collateral arterioles to small arteries bypassing the occluded main artery, an adaptive remodeling process referred to as arteriogenesis, on the other hand, seems to be much more efficient in compensating for the consequences of atherosclerosis.4,5 Morphologically, collateral arteriolar enlargement is associated with a corkscrew-like appearance that is the consequence of a growth in length between 2 fixed points.6 On the molecular level, arteriogenesis is characterized by the upregulation of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and chemokines like monocyte chemoattractant protein (MCP)-1 in collateral arterioles, which, in turn, leads to the recruitment of mononuclear leukocytes,7,8 a prerequisite for the subsequent structural changes of the vessel wall. Despite a growing number of putative proarteriogenic factors9,10 and an enhanced understanding of their complex interactions,11 the mechanistic background of this remodeling process is still poorly understood.

Based on Poiseuille’s equation, progressive stenosis of the main artery will lead to an increase in resistance hence a significant drop in pressure distal to the site of occlusion. As a consequence, the pressure difference between both ends of the collateral arterioles is enhanced, resulting in increased flow9,12 and, consequently, a rise in both shear stress and circumferential wall tension,13,14 which may act in concert on the collateral blood vessels. There is accumulating evidence that these altered hemodynamics to which the arteriolar vessel wall is exposed to initiate arteriogenesis,14,15 Whereas laminar shear stress is thought to be responsible for the initial dilation of the collateral arterioles through activation of
endothelial cell nitric oxide synthase, the role of circumferential wall tension in the early phase of arteriogenesis is less well defined. In this context, the expression of several putative proarteriogenic gene products has been shown to be controlled by the transcription factor activator protein (AP)-1,17,18 which, in vascular cells, is activated through an increase in static or cyclic stretch.19

Against this background, we have investigated the mechanism by which the expression of MCP-1 in the vessel wall is controlled during the early stages of arteriogenesis.

Materials and Methods
An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Animal Models
All animal studies were performed by permission of the Regional Council Karlsruhe and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Male NMRI mice (at least 24 weeks old) were anesthetized with isoflurane, and the femoral artery was ligated just distal to the origin of the deep femoral artery. On day 1 or 7 after surgery, the mice were euthanized and the left ventricle of the heart was cannulated and perfused for 2 minutes at 100 mm Hg with Ringer solution containing 0.1% adenosine plus 0.05% BSA (wt/vol) at 37°C followed by zinc fixative containing a colored pigment (HKS Gouache 318; Schmincke, Erkrath, Germany) that cannot pass the capillary system. The hindlimbs and ears were dissected and processed for RNA and histological analysis. In contrast to the ear, where arteriogenic arterioles were easily identified by their typical corkscrew-like morphology, growing collaterals in the hindlimb follow a constant course on the surface of the adductor muscles facilitating their identification in transparent tissue and histological preparations.6

For ligation of the mouse ear artery, we adapted an animal experimental model originally developed by North and Sanders (1958).20 Male NMRI mice (at least 24 weeks old) were anesthetized as described above. The mouse ear is usually supplied by 3, occasionally 4, neurovascular bundles, each consisting of an artery, a vein, and a large axillary. Three to 4 consecutive orders of vessels with decreasing diameters originate from the major Y-shaped vascular branches, which then drain into the capillaries. For ligation, first order arterioles in the center of the ear were selected. Seven days after surgery, the mice were euthanized and the vasculature was perfused through the left ventricle of the heart, as described above (Figure 1 in the online data supplement).

Visualization of the Arterial System
Perfused mouse hindlimb and ear specimens were postfixed in zinc fixative (18 hours) and dehydrated using a series of alcohol and isopropanol following standard protocols. Tissues were then incubated in a mixture of benzyl alcohol and benzyl benzoate (1:1, vol/vol) having the same refractive index of the tissue for at least 18 hours. This procedure induces transparency of the tissue and allows detailed analysis of the pigment-loaded arterial system. The diameter of the collateral arterioles was measured by using the morphological analysis software Cell ‘R from Olympus (Hamburg, Germany) in at least 3 different sites of an individual arteriole.

Perfusion of Isolated Mouse Arteries
Animals were euthanized, and the intestine was dissected. Second order branches of the mesenteric artery were extracted from fat tissue and inserted into the chamber of the myograph (Culture Myograph, DMT, Copenhagen, Denmark). The chambers were placed in an incubator at 37°C and 5% CO2, and the arteries were continuously perfused for 6 hours with DMEM (Invitrogen) containing 15% FCS at a longitudinal pressure gradient of 20 mm Hg with a flow of about 0.07 mL/min or at a proarteriogenic pressure gradient of 50 mm Hg with a flow of about 0.17 mL/min (supplemental Figure II).

Based on these values, shear stress (SS) and circumferential wall tension (CWT) was roughly calculated by factoring in wall thickness, vessel diameter, medium viscosity, and transmural pressure (control condition: SS≈2.17 Pa, CWT≈6480 Pa; proarteriogenic condition: SS≈2.66 Pa, CWT≈14 665 Pa). Note that even a slight increase in vessel diameter by flow dependent dilation on proarteriogenic conditions counteracts a more pronounced increase in shear stress. The functional and cellular integrity of the segments was routinely checked by their vasodilator response to acetylcholine (5 μmol/L) and by immunohistochemical staining for the endothelial cell marker CD31.

Decoy Oligonucleotide Technique
Double-stranded decoy oligonucleotides (dODNs) were prepared from complementary single-stranded phosphorothioate-bonded oligodeoxynucleotides (ODNs) obtained from IBA (Göttingen, Germany) by melting at 95°C for 5 minutes followed by a cool-down phase of 3 to 4 hours at ambient temperature. The efficiency of the hybridization reaction was verified with 2.5% agarose gel electrophoresis and usually found to exceed 95%. The sequences of the forward strands were (+ indicates phosphorothioate-bonded bases): AP-1 decoy ODN, 5'-G*T*G*CTGACTCAG*C*A*C-3'; mutant control ODN, 5'-G*T*G*CTCCTTAG*C*A*C-3'. Cultured mouse smooth muscle cells (mSMCs) and isolated mouse arteries were transfected with the naked ODNs (10 μmol/L), ie, without using any cationic lipid or liposomal complex for 4 hours at 37°C. For local application in vivo, the hybridized ODNs were mixed with Unguentum emulsificans to yield a final concentration of 0.25% (wt/vol), and 20 mg of this mixture was homogeneously spread across the surface of the mouse ear 1 day before, directly after the ligation, and then every second day thereafter until the animals were euthanized on day 7.

Statistical Analysis
All results are expressed as means±SEM. Differences between 2 matched experimental groups (Figure 1 through 4A) were analyzed by unpaired Student t test, with a probability value of <0.05 considered statistically significant. Differences among 3 or more experimental groups (Figures 4B through 7) were analyzed by 1-way ANOVA, followed by a Bonferroni post hoc test for selected pairs of groups, with a probability value of <0.05 considered statistically significant.

Results
Adaptive Remodeling of Collateral Arterioles in Mouse Hindlimbs and Ears Is Accompanied by an Increase in MCP-1 Expression
To analyze arteriogenesis we used 2 mouse models. In the hindlimb ischemia model, ligation of the femoral artery leads to an increase in the diameter of collateral arterioles 7 days postocclusion (Figure 1A, 1C, and 1E). This was accompanied by an increase in the expression of MCP-1, which was already observed 24 hours after the onset of arteriogenesis (Figure 1G). Likewise, ligation of the first order ear artery resulted in an increased diameter of collateral arterioles accompanied by a corkscrew-like morphology (Figure 1B, 1D, and 1F) and an increase in MCP-1 expression (Figure 1H). Similarly, the expression of ICAM-1, another well-known gene product associated with arteriogenesis, was also upregulated in the remodeling collateral arterioles in both animal experimental models (Figure 1I and 1J).

Proarteriogenic Perfusion Conditions Upregulate MCP-1 Expression in Vascular Smooth Muscle Cells In Situ
Next, we established an in situ model to mimic the changes in arteriolar perfusion that occur after occlusion of the femoral
artery (supplemental Figure II). This model is mainly based on the fact that mean arterial pressure distal to the site of occlusion drops, thereby increasing the pressure difference between both ends of the collateral arterioles. As a result of the experimentally set pressure gradient (50 mm Hg versus 20 mm Hg), flow increased proportionally to the difference in pressure from 0.07 mL/min to 0.17 mL/min in the isolated perfused second order branches of the mouse mesenteric artery (Figure 2A and 2B; note that the integrity of the endothelial cell monolayer is not perturbed under these conditions). Immunohistochemical analyses revealed an increased abundance of MCP-1 in the media of these segments on perfusion under proarteriogenic conditions for 6 hours (Figure 2C and 2D). MCP-1 and ICAM-1 mRNA expression in these vessels was additionally analyzed 24 hours after ligation of the femoral artery (Figure 2E). To identify the source of MCP-1, we mechanically removed the endothelial cell layer immediately after perfusion. According to real-time PCR analysis, this intervention did not affect the expression of MCP-1 in arterial segments after proarteriogenic perfusion (Figure 2E). Because of the highly reproducible and, thus, predictable remodeling of defined collateral arterioles in the mouse hindlimb, expression of MCP-1 and ICAM-1 in these vessels was additionally analyzed 24 hours after ligation of the femoral artery (Figure 2E).
relative change in MCP-1 expression under proarteriogenic flow conditions (Figure 2E), suggesting that the smooth muscle cells of the isolated arteries were the main source of MCP-1.

Cyclic Stretch Rather Than Shear Stress Affects MCP-1 Expression in Cultured Endothelial and Smooth Muscle Cells

As an increase in flow with the resulting distention of the vessel wall affects both shear stress and circumferential wall tension, we individually analyzed the impact of these 2 biomechanical forces on MCP-1 expression in cultured cells. On exposing human umbilical vein endothelial cells (HUVECs) and mSMCs for 6 hours to cyclic stretch (as a surrogate parameter for an increase in circumferential wall tension), MCP-1 expression was significantly upregulated in both cell types on the mRNA (Figure 3A) and protein level (Figure 3B). In contrast, an increase in shear stress, which, in vivo, directly affects only the endothelial cells, had no effect on MCP-1 expression in the cultured HUVECs both on the mRNA (Figure 3A) and protein level (Figure 3B) even on prolonged stimulation (Figure 3C). In endothelial cells adapted to a low level of shear stress, however, MCP-1 mRNA expression was clearly downregulated on a further increase in the level of shear stress (supplemental Figure III). To analyze possible paracrine effects of shear stress-stimulated endothelial cells on MCP-1 expression in human smooth muscle cells (HSMCs), the latter cells were exposed to supernatants from the former cells. Whereas MCP-1 protein levels were increased in the supernatant of stretch-stimulated HSMCs (Figure 3D), the conditioned medium from shear stress-stimulated HUVECs had no effect on MCP-1 expression in the HSMCs neither on the protein (Figure 3D) nor mRNA level (supplemental Figure III).

Expression of MCP-1 Induced by Proarteriogenic Perfusion Conditions Is Dependent on the Activation of AP-1

Among several putative mechanosensitive transcription factors, AP-1 has repeatedly been shown to contribute to stretch-induced gene expression in vascular cells, and this also seems to apply to the regulation of several proinflammatory gene products.17 Therefore, we analyzed the role of AP-1 in stretch-induced MCP-1 expression in the isolated perfused mouse arteries by using the dODN technique. Pretreatment with an appropriate AP-1 dODN completely abrogated the increase in MCP-1 expression in arteries exposed to proarteriogenic perfusion conditions (Figure 4A). Likewise, stretch-induced upregulation of MCP-1 expression in the cultured mSMCs was abolished following pretreatment with the AP-1 dODN both on the mRNA (Figure 4B) and protein level (Figure 4C). Treatment with a mutant control ODN, on the other hand, had no appreciable effect on the expression of MCP-1 neither in the isolated perfused arteries (Figure 4A) nor in the cultured mSMCs (Figure 4B and 4C).

Activation of AP-1 Is Crucial for MCP-1 Expression and Monocyte Recruitment During Arteriogenesis

Because the aforementioned data had identified AP-1 as a pivotal factor in stretch-induced MCP-1 expression in vascular smooth muscle cells in vitro and in situ, we sought to verify its role during arteriogenesis in vivo. To this end, mouse ears were treated with a topical formulation of the
AP-1 dODN or the corresponding mutant control ODN during the induction and early manifestation phase of the remodeling process. Topical administration resulted in an excellent penetration of the AP-1 dODN through the skin into the wall of the ear blood vessels within 24 hours after the first application (Figure 5A through 5D). Both the enlargement and increase in the number of collateral arterioles with corkscrew-like morphology was virtually blunted on treatment with the AP-1 dODN, whereas treatment with the mutant control ODN had no effect (Figure 6A through 6E). In addition, real-time PCR analysis confirmed that MCP-1, ICAM-1, and vascular cell adhesion molecule (VCAM)-1 expression in the collateral arterioles was diminished in AP-1 dODN-treated animals (Figure 6F through 6H). Treatment with the mutant control ODN, on the other hand, increased rather than decreased both ICAM-1 and VCAM-1 expression but did not affect the increase in MCP-1 mRNA abundance in the collateral arterioles undergoing arteriogenesis. Because MCP-1 is a powerful chemoattractant for circulating monocytes,21 we finally analyzed whether the recruitment of monocytes to the arteriogenic vessel wall is affected in AP-1 dODN-treated animals. In line with the changes in MCP-1 expression, we noted a marked decline in the number of F4/80-positive macrophages surrounding the arteriolar vessel wall following treatment with the AP-1 dODN but not the mutant control ODN (Figure 7).

**Discussion**

The adaptive remodeling of arterioles running in parallel to an occluded artery holds great promise for the treatment of a wide range of cardiovascular diseases.22,23 Therefore, several animal models have been developed to delineate factors that promote arteriogenesis.2,10 Although differing in the spatiotemporal progression of the remodeling process, the common feature of these models is that acute occlusion of a main feeding artery is used to increase blood flow in the collateral arterioles.12 As a result, arteriogenesis is biomechanically induced irrespective of the specific vascular entity.10

In this study, we adapted a mouse ear artery ligation model20 to analyze the arteriogenic remodeling of collateral arterioles, thereby exploiting the easy accessibility and simplicity of the nearly 2D architecture of the ear vasculature. Occlusion of a distinct first order ear artery upregulated the expression of ICAM-1 and MCP-1 in the remodeling arterioles, which could be identified by their corkscrew-like morphology after four to seven days. These findings correspond to those obtained with the classical mouse hindlimb ischemia model24 and resemble previously published observations related to vascular remodeling processes in the mouse ear.20

In addition, MCP-1 expression was significantly upregulated within 24 hours after occlusion of the main artery, suggesting that this cytokine may orchestrate rather early steps in the proarteriogenic cascade. Apparently, the release of this chemokine is a prerequisite for the recruitment of...
monocytes to the arteriolar vessel wall, which, in turn, initiate its transformation to a small conductance artery. Correspondingly, infusion of this chemokine into the proximal stump of an occluded femoral artery in rabbits markedly enhanced arteriogenesis, whereas this was diminished in MCP-1 or CC chemokine receptor 2-deficient mice. Despite its importance for the initiation of arteriogenesis, only little is known about the mechanisms that control the expression of MCP-1 in this context. Therefore, we subjected small second order branches of the mouse mesenteric artery to proarteriogenic flow conditions and identified the SMCs of the media as the main source of MCP-1. This finding is in
biomechanical determinants of arteriogenesis, fluid shear stress. Similarly, supernatants from shear stress–stimulated endothelial cells but not SMCs. To confirm this idea, we dissected the individual effects of these biomechanical forces because changes in fluid shear stress can only be sensed by endothelial cells but not SMCs. To confirm this idea, we dissected the individual effects of these biomechanical forces on the expression of MCP-1 in cultured cells.

Exposing cultured endothelial cells to shear stress resulted in a decrease rather than an increase in MCP-1 expression at both the mRNA and protein level. This finding corroborates previous studies demonstrating that endothelial cell MCP-1 expression is in fact downregulated in response to laminar shear stress. Similarly, supernatants from shear stress–stimulated endothelial cells did not upregulate the expression of MCP-1 in SMCs, a finding that is supported by previous publications which suggest that the release of nitric oxide (NO) from shear stress–stimulated endothelial cells inhibits rather than stimulates the expression of MCP-1 in vascular SMCs.

In contrast, exposure of the cultured cells to cyclic stretch resulted in a robust increase in MCP-1 expression both in the human endothelial cells and in the mouse and human SMCs, where the increase in MCP-1 protein in the supernatant was particularly striking. These findings reinforce the notion that cyclic or static stretch, as a result of an increase in circumferential wall tension, plays a decisive role in the expression of MCP-1 in the collateral arteriolar wall during the early phase of arteriogenesis. Conversely, they question the view of arteriogenesis as a predominantly shear stress–mediated remodeling process.

Although shear stress may not directly affect the expression of MCP-1, it nevertheless affects circumferential wall tension and, hence, stretch of vascular cells. During arteriogenesis, both biomechanical forces are interdependently linked to each other as a flow-dependent increase in shear stress in the collateral arterioles will subsequently lead to an NO-mediated dilation, which decreases the wall thickness and, thus, according to LaPlace’s law, would result in an increase in circumferential wall tension. In addition, the increased volume load and the NO-mediated reduction in contractility of the SMCs will further enhance tension in the vessel wall.

Further experiments revealed that the stretch-induced increase in MCP-1 expression both in the isolated perfused arteries as well as in the cultured SMCs of the mouse could be abolished by blocking the transcription factor AP-1 with a specific dODN. AP-1 has been shown to regulate the expression of many stress response genes, including those associated with a proinflammatory phenotype of endothelial cells or SMCs. In most cases, activation of this transcription factor is triggered by an increased formation of reactive oxygen species (ROS), which is another hallmark of vascular stress responses. Concordantly, we found that ROS formation is strongly increased in the cultured SMCs on exposure to cyclic stretch as well as in the isolated arteries perfused under proarteriogenic conditions (supplemental Figure IV). We also could verify that ROS in fact upregulate MCP-1 expression in cultured SMCs of the mouse and treatment with the ROS scavenger idebenone inhibits the increase in MCP-1 expression in arteries perfused under proarteriogenic conditions (supplemental Figure IV). Finally, we detected an increased formation of ROS in the collateral arterioles of the mouse hindlimb undergoing arteriogenesis (supplemental Figure V).

Collectively, these data suggest that in the early phase of arteriogenesis an increase in circumferential wall tension; hence, stretch triggers an increase in ROS formation in the SMCs of the arteriolar vessel wall, which, in turn, leads to the translocation of AP-1 to the nucleus, where it transactivates the MCP-1 gene. If this assumption is correct, then blocking of AP-1 activity in vivo should preclude the onset of arteriogenesis. To this end, the ear artery ligation model proved highly valuable because of the fact that it allowed administration of the AP-1 dODN in a dermal formulation. The resulting neutralization of AP-1 not only abrogated the arteriogenic response but also completely prevented the increase in MCP-1 expression, as well as monocyte diapedesis in the collateral arterioles, as evidenced by the marked decrease in the number of macrophages surrounding the vessel wall. Furthermore, a decreased expression of VCAM-1 and ICAM-1, 2 other prototypic AP-1 target genes, was observed. Even though MCP-1 is only 1 among many AP-1 target genes, these findings corroborate the pivotal role of MCP-1 dependent monocyte recruitment in arteriogenesis.

Moreover, they establish AP-1 activation as a rate-limiting step in MCP-1 expression in an as yet unrecognized context and point toward stretch-induced AP-1 dependent changes in gene expression in general as a decisive step in the initiation of arteriogenesis. Because such AP-1–dependent changes in gene
expression also play a role in hypertension-induced arterial remodeling or restenosis following angioplasty,13,36 systematically comparing the initial steps of these adaptive remodeling processes with that of arteriogenesis may ultimately lead to identification of the main trigger of arteriogenesis and thus spur the development of a proarteriogenic therapy.

In summary, the present findings establish the ear artery ligation model of the mouse as a new experimental tool to investigate adaptive arteriolar remodeling processes in vivo. They further suggest that (1) the expression of MCP-1 in vascular SMCs is triggered by an increase in circumferential wall tension; (2) this change in gene expression is controlled by AP-1; and (3) this mechanism is rate-limiting for the recruitment of monocytes and thus the remodeling of collateral arterioles during arteriogenesis. By enhancing the existing mechanistic knowledge in this field, our observations may contribute to the understanding of a well-orchestrated and self-limiting arterial remodeling process that eventually may be exogenously induced or reinforced for therapeutic purposes.

Acknowledgments
We acknowledge the excellent technical assistance of Gudrun Scheib, Lorena Urda, and Ender Serbest.

Sources of Funding
This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB/TR23, project C5).

Disclosures
None.

References
Stretch-Induced Activation of the Transcription Factor Activator Protein-1 Controls Monocyte Chemoattractant Protein-1 Expression During Arteriogenesis
Elena Demicheva, Markus Hecker and Thomas Korff

Circ Res. 2008;103:477-484; originally published online July 31, 2008;
doi: 10.1161/CIRCRESAHA.108.177782

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/103/5/477

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/07/31/CIRCRESAHA.108.177782.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Expanded Materials and Methods

Antibodies

The polyclonal rat anti-mouse CD31 antibody (clone MEC 13.3) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The polyclonal rabbit anti-mouse MCP-1 antibody was from AbCam (Cambridge, UK) and the polyclonal rat anti-mouse F4/80 antibody was from Dianova (Hamburg, Germany).

Histological analysis

Immunohistochemical and immunofluorescence staining for MCP-1 and CD31 was performed on 4-5 µm thick paraffin or frozen sections by using the polyclonal rabbit anti-mouse MCP-1 and rat anti-mouse CD31 antibody in combination with an enhanced detection method (Envision™, DAKO, Hamburg, Germany) and 3,3-diaminobenzidine (DAB) as a substrate. The polyclonal rat anti-mouse F4/80 antibody together with a biotinylated rabbit anti-rat antibody and Streptavidin-RPE (DAKO) was employed for the detection of macrophages. Nuclei were visualized with Mayer’s hemalaun or the Hoechst dye 33258 counter staining.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords and cultured in M199 medium (Invitrogen, Karlsruhe, Germany) containing 20% fetal bovine serum (Life Technologies, Karlsruhe, Germany), 50 U/mL penicillin, 50 µg/mL streptomycin and 5 mmol/L HEPES, and supplemented with endothelial cell growth supplement (PromoCell, Heidelberg, Germany). Murine smooth muscle cells (mSMCs) were isolated from branches of the mouse mesenteric artery and cultured in D-MEM (Invitrogen) supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin and 15% fetal bovine serum. The phenotype of these cells was confirmed by anti-mouse smooth muscle α-actin immunofluorescence staining. All cell types were cultured on plastic dishes or collagen type I linked BioFlex elastomers (Flexcell, Hillsborough, NC) coated with gelatin following standard protocols or the manufacturer’s instructions, respectively. Confluent primary cultured HUVECs were subjected to laminar shear stress (30 dyn/cm²) in a
cone-and-plate viscometer as described previously\textsuperscript{1}. Application of cyclic stretch (15\%, 0.5 Hz) was performed by using a microprocessor controlled vacuum pump (FX-3000 FlexerCell Strain Unit, Flexcell) as previously described\textsuperscript{2}.

**RNA isolation, RT-PCR and real-time PCR analysis**

Total RNA was isolated from individually excised collateral arterioles using the RNeasy kit from Qiagen (Hilden, Germany). Conventional RT-PCR was performed by using Oligo(dT)\textsubscript{15} primers (Promega, Mannheim, Germany) and the Sensiscript kit (Qiagen) for material from the isolated mouse arteries or the Omniscript kit (Qiagen) for material from the cultured cells and the dissected mouse arterioles. The following gene specific primers were used: Mouse MCP-1 forward, 5'-TTCCTCCACCACCAGCAG-3'; mouse MCP-1 reverse, 5'-CAGGCC-GGCAACTGTGA-3' (GenBank accession number NM_011333); mouse RPL32 forward, 5'-GGGAGCAACAAGAAAACCAA-3', mouse RPL32 reverse, 5'-ATTGTGGACCAGGAACTT-GC-3' (GenBank accession number XR_031977); mouse ICAM-1 forward 5'-ATCTCAGGC-GAAGGG-3'; mouse ICAM-1 reverse 5'-CGAAAGTCCGGAGGCTCC-3' (GenBank accession number NM_010493). Real-time PCR was carried out in a LightCycler instrument (Roche Diagnostics, Penzberg, Germany) by using the QuantiTect Sybr Green PCR detection kit from Qiagen. Standard cDNA probes for quantitative analysis were generated by using the TOPO TA Cloning kit (Invitrogen).

**ELISA**

The concentration of MCP-1 protein in the supernatant of the cultured mSMCs and HUVECs was determined by using an appropriate ELISA kit (R&D Systems, Wiesbaden, Germany) accordingly to the manufacturer's instructions. For detection of human and mouse MCP-1, samples were diluted 1:10 and 1:5, respectively. For both ELISAs, MCP-1 levels attained under control conditions were set to 100%.
References


Supplemental figure legends

Online Figure I Visualization of the arterial vasculature in the ear artery ligation model. The arterial system of the mouse can be selectively visualized by perfusion through the left ventricle of the heart with pigmented particles that cannot pass the capillary system.

Online Figure II The *in situ* perfusion model mimics the hemodynamic situation in the collateral arterioles after occlusion of the main artery. Perfusion of isolated second order branches of the mouse mesenteric artery was performed under control conditions (ΔP=20 mm Hg, flow ~0.07 mL/min) and under pro-arteriogenic conditions (ΔP=50 mm Hg, flow ~0.17 mL/min), resembling the increased flow in the collateral arterioles following occlusion of the femoral artery.

Online Figure III Analysis of MCP-1 expression in cultured endothelial cells in response to different levels of shear stress. HUVECs were exposed to shear stress (3 dyn/cm²) for 24 hours. Thereafter shear stress was increased to 12 dyn/cm² for up to 36 hours resulting in a constant decrease in MCP-1 mRNA in the cultured endothelial cells (A, **p<0.01 vs. control [ANOVA-Dunnett], n=6; the level of MCP-1 expression under control conditions (exposure to low shear stress for 24 h) was set to 100%). Similarly, MCP-1 mRNA expression in human smooth muscle cells (HSMC) is not affected by the treatment with supernatants from shear stress-stimulated (6 h) endothelial cells for 3 hours. (B, n=3; the level of MCP-1 expression under control conditions was set to 100%).

Online Figure IV Reactive oxygen species (ROS) formation in stretched mSMC and isolated perfused mesenteric artery segments of the mouse. Detection of ROS by dihydroethidium in stretch-stimulated mSMCs (A, control; B, cyclic stretch: 15% elongation at 0.5 Hz for 6 hours; scale bar=50 µm). Expression of MCP-1 mRNA in these cells was increased after glucose oxidase treatment (12 mU/mL) which elevates the level of hydrogen peroxide in the medium (C, real-time PCR analysis, *p<0.05 vs. control, n=3 with MCP-1 mRNA under control conditions set to 100%).
ROS formation was also detected in the isolated arterial segments after 6 hours of perfusion (C, perfusion under control conditions; D, perfusion under pro-arteriogenic conditions; scale bar=50 µm). Treatment of perfused arteries with the ROS scavenger Idebenone (3 µM) abolished the increase in MCP-1 mRNA expression upon pro-arteriogenic (ΔP: 50 mmHg) conditions (E).

**Online Figure V** Reactive oxygen species (ROS) formation in collateral arterioles after ligation of the femoral artery (mouse hindlimb ischemia model). Detection of ROS by dihydroethidium (red fluorescence), combined with BS I lectin-FITC labeling of the vessel wall (green fluorescence) in collateral arterioles of the mouse hindlimb 3 days after ligation of the femoral artery (A-C, sham; D-F, ligation; scale bar=50 µm).
Before perfusion

Blood in arteries and veins

After perfusion

Selective arterial perfusion:
Arteries filled with pigments

Demicheva et al. (2008)
Online Figure I
Demicheva et al. (2008)
Online Figure II

Control:

```
Control conditions, low flow
70-80 mmHg
```

ΔP 20 mm Hg

```
50 mmHg
```

Ligation:

```
Arteriogenic conditions, high flow
```

ΔP 50 mm Hg

```
20 mmHg
```

Pressure gradient

```
70 mmHg
```

```
10-20 mmHg
```

```
70-80 mmHg
```

```
50-60 mmHg
```

```
10-20 mmHg
```
Figure III

(A) Shear stress

- + + + 3 dyn/cm² (24 h)
- + + + 12 dyn/cm²

3h 18h 36h
HUVEC

(B) Indirect shear stress

- + EC-supernatant

MCP-1/RPL [%]

Demicheva et al. (2008)
Online Figure III
Demicheva et al. (2008)
Online Figure IV

Mouse SMC

A control  

B stretched

ΔP 50 mm Hg

C

Glucose oxidase–+  

MCP-1/RPL [%]

0 50 100 150

DE

Fl  

F

ΔP 20 mm Hg  

ΔP 50 mm Hg

MCP-1/RPL [%]

0 20 40 60 80 100

*  

20 50 ΔP (mm Hg)
Demicheva et al. (2008)
Online Figure V

DHE staining of a collateral arteriole - sham

A
BS I Lectin - FITC

B
DHE

C
Merge

DHE staining of a collateral arteriole - 3 days after femoral artery occlusion

D
BS I Lectin - FITC

E
DHE

F
Merge