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The impact of biomechanical forces on the differentiation of vascular cells during arteriogenesis

Presented by

Diplom – Biochemist	Elena Demicheva
born in	Moscow, Russian Federation
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Referees: Prof. Dr. M. Hecker
Prof. Dr. P. Angel
Prof. Dr. D. Robinson
PD Dr. M. Mayer

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Abbreviations

AP-1	activator protein-1
bFGF	basic fibroblast growth factor
bp	base pair
CCL2	chemokine (C-C motif) ligand 2
CCL5	chemokine (C-C motif) ligand 5
CCR2	chemokine receptor 2
CWS	circumferential wall strain
cDNA	complementary desoxyribonucleic acid
DNA	desoxyribonucleic acid
DHE	dihydroethidium
D-MEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxyd
dNTP	dosexynucleotide triphosphate
dODN	decoy oligodeoxynucleotide
EDTA	ethylendinitrilo-N, N, N', N'-tetraacetate
eNOS	endothelial nitric oxide synthetase
FCS	fetal calf serum
FGF	fibroblast growth factor
FSS	fluid shear stress
GM-CSF	granulocyte-macrophage colony stimulating factor
HIF-1	hypoxia inducible factor-1
hSMCs	human smooth muscle cells
HUVECs	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule-1
IL-8	interleukin-8
LFA-1	lymphocyte function-associated antigen-1

Mac-1	macrophage-1 antigen
MAP	mitogen-activated protein
MCP-1	monocyte chemotactic protein-1
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
mRNA	messenger ribonucleic acid
mSMCs	mouse smooth muscle cells
NF-κB	nuclear factor-kappa B
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIGF	placenta growth factor
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean
STAT-1	signal transducer and activator of transcription-1
TGF-ß	transforming growth factor-ß
TIMP-1	tissue inhibitor of matrix-proteases
TNF-α	tumour necrosis factor-α
TRE	triphorbol acetate response elements
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor

1. Introduction

1.1. Clinical relevance of arteriogenesis vs. angiogenesis

Cardiac and peripheral arterial occlusive diseases are the leading cause of death in the Western world and often result in the development of ischemia in the affected tissues. The treatment of patients with occlusive vascular disease consists of lifestyle modifications and pharmacotherapy addressing the risk factors to minimize the risk for disease progression and mortality in myocardial infarction and stroke. In case of progressing peripheral artery disease, the patient might suffer from rest pain and ischemic ulceration and gangrene due to critical limb ischemia (Figure 1).

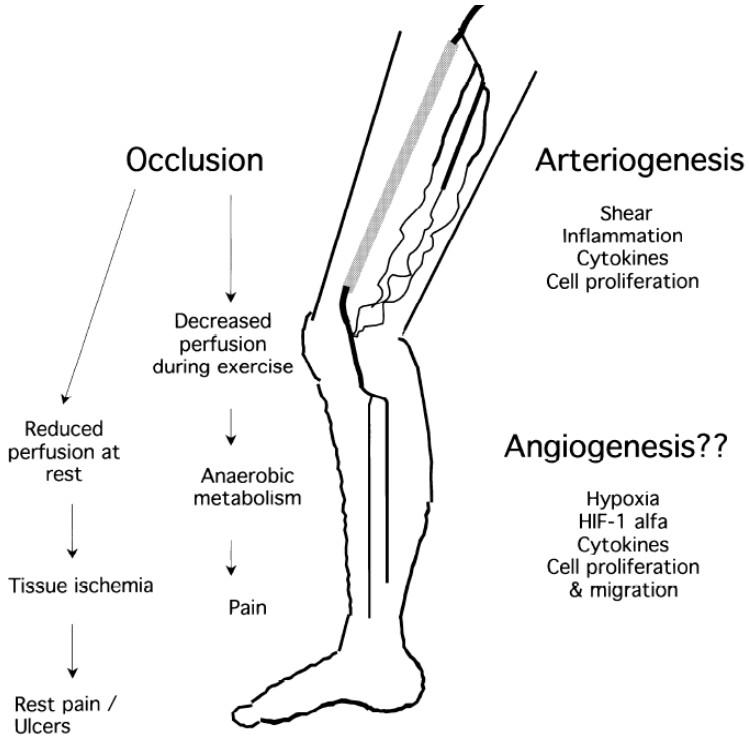

Figure 1. Schematic drawing of the pathogenesis in peripheral artery disease (taken from Palmer-Kazen, 2003). Symptoms of lower extremity peripheral artery disease include pain on walking—intermittent claudication, which is the earliest and most frequently present symptom.

Symptomatic invasive treatment consists of surgical or endovascular revascularization. Unfortunately, about 20% to 30% of patients with critical limb ischemia cannot be treated by any of these methods and amputation is often the only remaining option (Palmer-Kazen 2003).
However, it has been known for a long time that patients suffering from ischemic vascular diseases can develop collateral vessels bypassing the side of occlusion (Fulton 1969). Depending on the initial trigger, growth of blood vessels in adult organisms proceeds via two major processes, angiogenesis and arteriogenesis (Figure 1) (Semenza 2007). Stimulation and acceleration of these natural protective mechanisms have the potential to become an alternative therapeutic approach to improve the blood supply to ischemic tissue in case of occlusive vascular disease.

Angiogenesis is defined as the formation of new capillaries from existing vessels resulting in an improved new capillary network (Risau 1997). It is an important component of various normal and pathological conditions such as wound healing, fracture repair, folliculogenesis, ovulation, and pregnancy (Buschmann 2001). However, if not properly controlled, angiogenesis can also represent a significant pathogenic component of tumor growth and metastasis, rheumatic arthritis, and retinopathaties.
Angiogenesis consists of several distinct processes, which include endothelial cell migration and proliferation, extracellular proteolysis, endothelial cell differentiation, and vascular wall remodelling. In most cases angiogenesis is induced by local ischemia, where a decrease in local oxygen tension initiates the expression of hypoxia-inducible genes through activation of the transcription factor hypoxia inducible factor-1 (HIF-1). Besides the erythropoietin gene, regulating the production of erythrocytes in the bone marrow, several angiogenesis-related genes are under the control of HIF-1, among which VEGF (vascular endothelial growth factor) is the best studied. Synthesized in several splicing variants, VEGF is a potent mitogen for endothelial cells and of critical importance for physiological as well as pathological angiogenesis, e.g. in neoplastic tumors. VEGF acts like a pro-angiogenic factor by binding to two tyrosine-kinase receptors VEGFR1 and VEGFR2 on endothelial cells, whereas VEGFR3 induces lymphangiogenesis upon binding to

isoforms VEGF-C and VEGF–D. VEGF induces a complex response in endothelial cells including the expression of endothelial nitric oxide synthase (eNOS) and matrix metalloproteinases (MMPs) necessary for enhancing permeability of the basal membrane and the surrounding matrix (Tammela 2005).
As a result of angiogenesis, a dense capillary network is formed with the ability to support local tissue perfusion but especially to improve the diffusion of oxygen from the blood to the tissue. However, these newly formed capillary tubes lack vascular smooth muscle cells. Any developing new network of sprouting capillaries that is not surrounded by mural cells is fragile and probably unable to sustain proper circulation. Likewise, clinical studies revealed that angiogenesis per se is not sufficient to fully restore the blood supply to the affected tissues (Scholz 2001; Henry 2003; Heil 2006).

In contrast, the growth and remodelling of pre-existing collateral arteries bypassing an arterial occlusion much more efficiently compensate for the consequences of peripheral artery disease (Schaper 1971; Schaper 2001; Unthank 2004). This adaptive remodelling process is referred to as arteriogenesis. In contrast to angiogenesis, arteriogenesis is not driven by the methabolic demand of the tissue and is initiated independently from hypoxia. The region in which bypassing collaterals grow is much more proximal to the hypoxic zone (Figure 2) (Schaper 2003), and the remodelling process is initiated by physical forces which appear within the collateral arteriole after a marked increase in blood flow (Heil 2006).

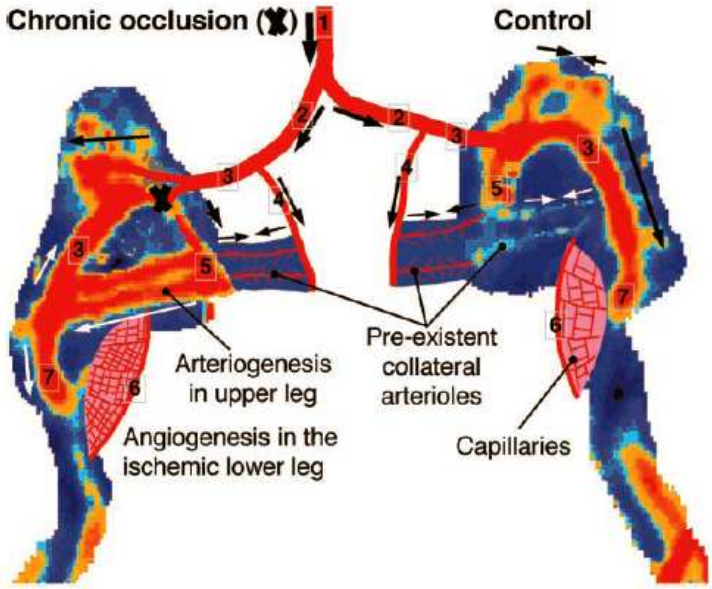

Figure 2. Collateral growth occurs in pre-existent arterioles (taken from Schaper, 2003). Laser Doppler image of collateral blood flow in an anesthetized mouse with exposed upper thigh skeletal muscles with chronic occlusion (7 days) of the left femoral artery. Shown are 2 preexistent arterioles that exhibit a faintly visible flow signal but a very strong signal on the occluded side, ie, the effect of growth after 7 days of occlusion. 1 indicates aorta; 2, A. iliaca; 3, A. femoralis; 4, A. pudenda externa; 5, A. profunda femoris; 6, A. tibialis posterior; and 7, A. saphena.

A variety of factors have been identified that are able to therapeutically stimulate arteriogenesis. While some of them reached clinical testing in pilot studies, others had to be abandoned in the preclinical phase due to significant side effects. For example, granulocyte-monocyte colony-stimulating factor (GM-CSF) is having positive effect on arteriogenesis via circulating cells. Although the mechanisms by which GM-CSF stimulates arteriogenesis remain partially unclear, both intravascular (Buschmann 2001) as well as subcutaneous (Schneeloch 2004) applications of GM-CSF have been shown to effectively stimulate arteriogenesis in animal model experiments. In the clinical setting, however, the equipotency of these administration routes remains questionable. In a pilot trial in patients with extensive coronary artery disease, *Seiler et al.* demonstrated a significant positive effect on coronary artery flow of a single intracoronary bolus of GM-CSF followed by a two week subcutaneous treatment period (Seiler 2001). In the START trial, *van Royen et al.* applied a similar treatment in patients with moderate to severe

intermittent claudication. However, in this setting of peripheral artery disease no beneficial effect on the primary end point walking was observed (van Royen 2005).
Another cytokine in preclinical testing for the enhancement of collateral artery growth is fibroblast growth factor (FGF). Infusion of this pluripotent cytokine into developing collateral circulation results in a strong stimulation of arteriogenesis (van Royen 2002). Exogenous application of FGF2 also results in a significant stimulation of collateral artery growth. After recombinant FGF2 was found to be safe and potentially efficacious in pilot studies, *Lederman et al*. reported in the TRAFFIC study of patients with intermediate claudication a significant improvement in the peak walking time 90 days after the first protein infusion (Lederman 2002). However, while the investigators observed a trend towards symptomatic improvement after 90 days, this effect diminished 180 days after the protein infusion and no beneficial effect on myocardial perfusion or exercise tolerance was detected.
Local infusion of one of the most potent pro-arteriogenic cytokines – monocyte chemotactic protein-1 (MCP-1) – into the collateral circulation following femoral artery ligation in the rabbit has been shown to significantly enhance collateral artery conductance compared to a control group only receiving vehicle (Ito 1997). Unfortunately, since the efficacy of MCP-1 infusion was shown to depend on monocyte chemoattraction, *van Royen et al*. demonstrated that local infusion of MCP-1 in apolipoprotein E-deficient mice results indeed in systemic monocyte activation, increased neointima formation and change in plague composition towards an unstable phenotype in pre-existing lesions (van Royen 2003).

It is difficult at this time point to rate the pro-arteriogenic compounds with regard to their chances of future success as most substances are in early phases of investigation. Moreover, the eligible patient population for angiogenic/arteriogenic therapies needs to be better identified, as previous trials often were focused on patients with severe coronary artery disease, where local application of drugs is difficult. The limited success of all clinical studies so far demonstrate that besides compound selection the issues of dosage, drug delivery and choice of valid endpoints remain to be solved.

1.2. Physical forces regulating arteriogenesis

Morphologically, collateral arteriolar enlargement is associated with a corkscrew-like appearance that is thought to be the consequence of a growth in length between two fixed points (Schaper 1971; Heil 2004). In the re-entry region they join up with the distal part of the occluded artery at nonphysiological angles, which adds to their resistance against flow.

Based on Poiseuille´s equation

$$\Delta P = \frac{8Q\mu L}{\pi R^3}$$

(Q = volumetric flow rate; μ = viscosity; L = length; R = radius), occlusion of the main artery will lead to an increase in resistance and to a significant drop in pressure distal to the stenosis. The resulting pressure gradient enhances blood flow through the vascular connections like the pre-existing collateral anastamoses between the high-pressure region proximal to the occlusion and the low-pressure region distal to the occlusion (Figure 3).

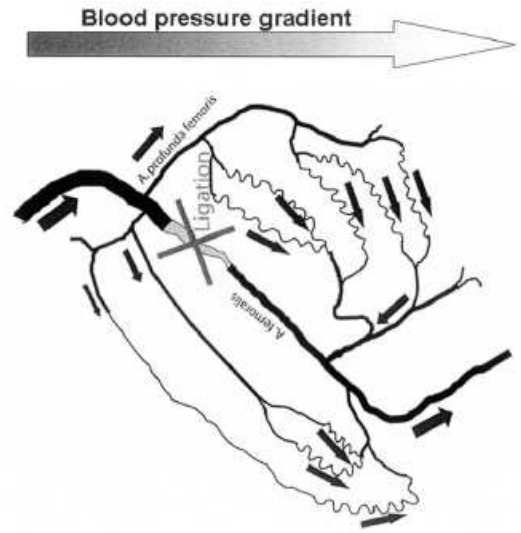

Figure 3. Scheme of the ischemic hindlimb model (taken from Heil, 2007). The femoral artery is ligated distally to the bifurcation of the A. profunda femoris causing a decrease in blood pressures distal to the side of ligation. Blood flow is redistributed along the blood pressure gradient, thereby recruiting pre-existing collateral anastomoses.

Not surprisingly, physical forces previously have been identified to be instrumental for arteriogenesis (Pipp 2004). An increased blood flow directly augments fluid shear stress (FSS) which is sensed by the endothelial cells of the arteriole. Hence, one can assume that the altered blood flow after an arterial occlusion leads to increased FSS which in turn is the driving force for arteriogenesis (Unthank 1996). However, in physical terms FSS with a range of 10-100 dyn/cm^2 is a much weaker force acting on the arterial wall than circumferential wall strain (CWS)

$$S_t = \frac{P_{tm} R_i}{w}$$

(P_{tm} – pressure difference, R_i – inner radius, w – vessel wall thickness), which is 10^3-10^4 times higher in this case (Schaper 2003). On the other hand, even a small increase in the radius of the collateral arterioles leads to a decrease in FSS because of the cubic relationship

$$\tau = \frac{4\mu Q}{R^3 \pi}$$

and hence, the FSS-related growth of the vessels ends prematurely (Schaper 2003). Moreover, proliferation of smooth muscle cells (SMCs) – critical for the remodelling of the vessel wall - is activated by an increase in pressure-related forces like CWS which is due to the volume-mediated distension of the vessel wall, but structurally weakened by matrix degradation.

Taken together, collective evidence suggests that these two forces act together to alter the hemodynamics which govern arteriogenic remodelling (Scheel 1979; Pipp 2004). This is consistent with the earlier proposed hypothesis by *Price et al.* that the maintenance of mean CWS values and the pressure-shear stress relationship are operative design principles for collateral arteriole development during physiological growth (Price 2002).

1.3. Molecular mechanisms of arteriogenesis

Presently it is not well understood how the stimulus of an increased biomechanical load is transmitted from the vascular cell membrane to the nucleus. The initial step of arteriogenesis – the elevation of shear stress - is thought to primarily affect the endothelial cells (ECs). Although the nature and location of the

mechanotransducer of shear stress are still controversial, it has been observed that vascular ECs lose volume control and swell due to the changes in the open probability of chloride channels (Ali 2002).

Further, endothelial cells react by activating eNOS (Nishida 1992) and paradoxically with an increased expression of cytokines, such as MCP-1, GM-CSF and tumour necrosis factor-α (TNF-α) (Wahlberg 2003). Expression of selectins, intercellular adhesion molecules (ICAM-1 and -2) and vascular cell adhesion molecules (VCAM-1) is not only increased but they are also clustered in focal adhesion complexes (Scholz 2000). An increase in MCP-1 expression together with transforming growth factor-ß (TGF-ß) leads to the adhesion of monocytes (Hoefer 2004; Lee 2004). And their binding to the collateral surface is mediated by integrin receptors such as macrophage-1 antigen (Mac-1) and lymphocyte function-associated antigen-1 (LFA-1). These heterodimeric molecules are counterparts of ICAM-1, -2 and VCAM-1 and their expression on monocytes can be up-regulated by growth factors (*e.g.* VEGF, PlGF, TGF-ß) and chemokines like MCP-1 (Heil 2000; van Royen 2002; Pipp 2003). This pro-inflammatory differentiation of endothelial cells, which, however, cannot be the direct consequence of an increase in shear stress, finally leads to augmentation of the adhesion and recruitment of monocytes (Scholz 2000).

Adhesion of monocytes that become activated during arteriogenesis is a prerequisite for the subsequent changes in the structure of the vessel wall (Schaper 1976). However, no radial growth of the arteriole can occur without a controlled reorganization of the original vessel architecture. After transformation to macrophages, monocytes produce fibronectin, proteoglycans, and proteases, which remodel the extracellular matrix. These inflammatory cells also produce large amounts of vascular growth factors, essentially from the fibroblast growth factor (FGF) family, namely FGF-2 (basic FGF). One of the effects of FGF-2 binding to its receptor is the stimulation of endothelial and smooth muscle cell proliferation (Wahlberg 2003). Consequently, an active remodelling process takes place where the basic matrix is digested by matrix metalloproteinases (MMP-2, MMP-9) (Cai 2000). These proteases are up-regulated in the SMCs that simultaneously down-regulate the tissue inhibitor of matrix-proteases (TIMP-1).

Taken together, the described events are thought to increase mobility of the SMCs, which partially migrate into the intima, divide *in situ* or die by apoptosis (Scholz 2001), resulting in radial growth of the vessel wall.

1.3.1. Chemokines and arteriogenesis

Chemokines (chemotactic cytokines) are important in leukocyte trafficking and influence a diverse array of normal and pathophysiologic processes including arteriogenesis, angiogenesis, and skeletal muscle regeneration (Charo 2004). Increasing evidence suggest that MCP-1–dependent recruitment of monocytes/macrophages is a rate limiting step in arteriogenesis and reperfusion of ischemic tissue. MCP-1 (also known as chemokine ligand 2 - CCL2) belongs to the largest family of CC chemokines, known to attract mononuclear cells and first found at sites of chronic inflammation.

Hindlimb ischemia studies using $CCL2^{-/-}$ mice have consistently documented a decreased restoration of perfusion (Voskuil 2004; Shireman 2007). Interestingly, several studies showed that knock-out of the MCP-1 receptor (CC chemokine receptor 2 - CCR2) also dramatically reduced arteriogenesis in adult animals (Heil 2004), whereas few other studies reported the opposite - similar perfusion in CCR2 deficient mice compared to control mice (Tang 2004). Perhaps, MCP-1 functions through receptors other than CCR2, and the loss of MCP-1 signalling via these undefined receptors may be responsible for the delayed restoration of perfusion in $CCR2^{-/-}$ mice. Moreover, $CCR2^{-/-}$ mice exhibit increased CCL2 in tissue compared with control mice in response to hindlimb ischemia (Contreras-Shannon 2007) which may allow binding to alternative receptors not normally activated at physiological levels of MCP-1 (Shireman 2007).

Furthermore, it has been reported earlier that the infusion of MCP-1 leads to an increased monocyte accumulation in the walls of collateral arteries and an increase in collateral artery formation in the hindlimbs of MCP-1—treated animals compared with controls 7 days post femoral artery ligation (Ito 1997).

Although these data suggest that chemokines such as MCP-1 are important in collateral artery formation, involvement in other processes occurring simultaneously in the adductor muscle cannot be excluded (Lee 2004).

1.3.2. Transcriptional regulation of MCP-1

Multiple signalling mechanisms have been reported to be involved in MCP-1 gene expression in vascular cells (Shyy 1993; Roebuck 1999; Goebeler 2001) including but not limited to the activation of phosphatidylinositol-3-OH kinase, Akt/protein kinase B, phospholipase C, p60src-Ras, protein kinase C, and tyrosine kinases in response to a variety of stimuli like cytokines, mitogens or mechanical strain. These may ultimately be transmitted to the nucleus through the activation of mitogen-activated protein (MAP) kinases and transcription factors such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) (Beyaert 1996; Whitmarsh 1996; Cho 2002). These transcription factors have also been shown to contribute to the hemodynamic forces-induced gene expression (Wung 1997; Takemura 2004; Wu 2007), and particularly to the cytokine-dependent induction of MCP-1 in human ECs (Martin 1997).

The promoter region of the human MCP-1 gene contains putative consensus binding sites for a variety of transcription factors (Ueda 1994). Likewise, the triphorbol acetate response element (TRE), which is recognised by the AP-1 heterodimer c-Jun and c-Fos, and/or NF-κB consensus sequence upstream of the transcription start site are involved in the control of MCP-1 gene expression in various cell types in response to diverse extracellular stimuli (Ueda 1994; Martin 1997). It has been suggested that the differential activation and binding of inducible transcription factors such as AP-1 and NF-κB to the promoter regions of chemokine genes provides a critical regulatory mechanism by which the chemokine can be selectively expressed in a cell type and stimulus-specific manner (Roebuck 1999).

Several reports demonstrated that AP-1 is activated in vascular cells through an increase in static or cyclic stretch (Kumar 2003; Mitchell 2004). Accumulating evidence, however, suggest that reactive oxygen species (ROS) may act as second messengers in these cells exposed to the stretch stimulus (Satriano 1993; Lo 1996). Earlier studies by *Wung et al.* (Wung 1997) showed that MCP-1 expression is directly mediated by strain-induced ROS formation in vascular endothelial cells. For example, the antioxidant enzyme catalase, co-incubated with strained ECs, abolished strain-induced MCP-1 expression but had no effect on expression in static cells. Moreover, cyclic strain as well as H_2O_2 treatment induced AP-1 DNA binding activity, and this could be attenuated by antioxidant pretreatment of strained ECs.

These observations suggest that cyclic strain-induced MCP-1 expression involves ROS-dependent activation of the AP-1 binding sites in the MCP-1 promoter region.

1.4. Aims of the study

The induction of the expression of MCP-1 during the onset of arteriogenesis seems to be a key trigger in the remodelling process of collateral arterioles occurring after occlusion of the main artery. Thus, the first aim of this study was to analyze the arteriogenic remodelling process by employing two different *in vivo* mouse models – hindlimb ischemia and ear artery ligation models and in particular to analyze at which time point MCP-1 expression is up-regulated in the collateral arterioles following artery occlusion.

The second aim was to establish a perfusion model *in situ* which allowed studying the impact of different biomechanical forces on gene expression *in situ*. In this context, MCP-1 expression should be induced by pro-arteriogenic flow conditions and the cellular source of MCP-1 identified.

The third aim was to analyse in detail how application of shear stress and cyclic stretch – a surrogate of circumferential wall strain – individually affects expression of MCP-1 in vascular both endothelial and smooth muscle cells *in vitro*.

Based on these results, the final approach was aimed at characterising the transcriptional mechanism leading to MCP-1 expression during the onset of arteriogenesis.

If successful, this study should provide new insights into the mechanism of the arteriogenic remodelling process and the role of MCP-1 expression during its early phase. The investigation would then contribute to a better understanding of the initiation of arteriogenesis and possibly pave a way towards an induction of this remodelling process as a new alternative treatment for occlusive vascular diseases.

2. Materials

2.1. Antibodies

All antibodies used for protein detection are listed in the Table 2.1. In case of using the DakoCytomation EnVision™+System-HRP (DAB) Kit, antibodies specifically for this kit were used according to the manufacturer's instructions.

Table 2.1. Antibodies used for protein detection

Name	Source
Anti α-smooth muscle actin mouse monoclonal (clone 1A4)	Dianova (Hamburg, Germany)
Anti CD31 rat polyclonal (clone MEC 13.3)	Santa Cruz Biotechnology®, Inc. (Heidelberg, Germany)
Anti MCP-1 rabbit polyclonal	AbCam® (Cambridge, UK)
Anti F4/80 rat polyclonal	Dianova
Anti-rat biotinylated	DakoCytomation (Hamburg, Germany)

2.2. Cell culture

All medium and substances used for cell culture are listed in the Table 2.2.

Table 2.2. Medium and substances for cell culture

Medium and substances	Source
M199	Invitrogen™ (Karlsruhe, Germany)
SMC growth media	Promocell® (Karlsruhe, Germany)
D-MEM	Invitrogen™
Suplemental Mix	Promocell®
0.05% Trypsin/ 0.2% EDTA	Invitrogen™

Penicillin	Invitrogen™
Streptomycin	Invitrogen™
Fungizone	Invitrogen™
FCS	Invitrogen™
Hank's BSS	PAA (Cölbe, Germany)
Dispase	Boehringer (Mannheim, Germany)
Gelatine	Sigma-Aldrich™ (Steinheim, Germany)
Collagenase	Sigma-Aldrich™
Polyvinylpyrrolidone	Sigma-Aldrich™

2.3. Bacteria and plasmid vectors

Bacterial strains and plasmid vectors used for cloning and maintenance of plasmids constructs are listed in Table 2.3

Table 2.3. Bacterial E. coli strains and plasmid vectors

A. Bacterial strain		
Strain	**Company**	**Genotype**
Top 10 F	Invitrogen™ (Karlsruhe, Germany)	F´{*lac*Iq Tn*10* (TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G
B. Plasmid vector		
Vector	**Company**	**Properties**
pCR®2.1-TOPO® 3.9 Kb	Invitrogen™	pUC origin, Ampicillin & Kanamycin resistance genes, lacZα reporter, T7 RNA polymerase promoter, TA cloning site.

2.4. Primers

Primers used for real-time PCR gene expression analyses are listed in Table 2.4.

Table 2.4. Primers for real-time PCR

Gene product	Expected fragment length (bp)	Annealing temperature (˚C)	Primer (forward/reverse)
Mouse RPL32	63	60	GGGAGCAACAAGAAAACCAA ATTGTGGACCAGGAACTTGC
Mouse CD31	96	55	GAGCCCAATCACGTTTCAGTTT TCCTTCCTGCTTCTTGCTAGCT
Mouse MCP-1	64	60	TTCCTCCACCACCATGCAG CCAGCCGGCAACTGTGA
Mouse ICAM-1	49	60	ATCTCAGGCCGCAAGGG CGAAAGTCCGGAGGCTCC

2.5. Decoy oligodeoxynucleotides

Decoy oligodeoxynucleotides used for decoy experiments are listed in table 2.5. Sequence specific decoys were manufactured by IBA (Göttingen, Germany).

Table 2.5. Decoy oligodeoxynucleotides

Name of decoy	Sequence (forward/reverse) ("*" indicates phosphorothioate-bonded bases)
AP-1	G*T*G*CTGACTCAG*C*A*C G*T*G*CTGAGTCAG*C*A*C
Mutated AP-1	G*T*G*CTTACTTAG*C*A*C G*T*G*CTAAGTAAG*C*A*C
STAT-1	C*A*T*GTTATGCATATTCCTGTAA*G*T*G C*A*C*TTACAGGAATATGCATAAC*A*T*G
Mutated STAT-1	C*A*T*GTTATGCAGACCGTAGTAA*G*T*G C*A*C*TTACTACGGTCTGCATAAC*A*T*G

2.6. Kits

All kits used are outlined in Table 2.6.

Table 2.6. Kits used throughout this project

Name	Source
A. Nucleic acid purification kits	
RNeasy® Mini Kit	Qiagen (Hilden, Germany)
QIAPrep® Spin Miniprep Kit	Qiagen
B. RT-PCR kits	
Sensiscript® Kit	Qiagen (Hilden, Germany)
Omniscript® Kit	Qiagen
QuantiTect SYBR Green® Kit	Qiagen
C. PCR cloning kits	
TOPO TA Cloning® kit	Invitrogen™ (Karlsruhe, Germany)
D. Protein detection kits	
EnVision™+System-HRP (DAB) Kit	DakoCytomation (Hamburg, Germany)

2.7. Analysis software and online tools

Table 2.7. Software and online tools

Program	Use	Reference
A. Software		
Cell^R	Imaging analysis	Olympus (Hamburg, Germany)
analySIS^D version 5.0	Imaging analysis	Olympus Soft Imaging Systems GmbH
InStat® version 3.06	Statistical analysis	Graph Pad Software Inc.
PhotoImpact XL	Imaging analysis	Ulead Systems, Inc.

Program	Use	Reference
B. Online tools		
BLAST	Finding similar sequences	http://www.ncbi.nlm.nih.gov/BLAST
Primer3	Primer design	http://primer3.sourceforge.net/

2.8. Buffers and stock solutions

2.8.1. Histological buffers and stock solutions

Ringer solution

154 mM NaCl
5.6 mM KCl
2.4 mM $CaCl_2$
6.0 mM $NaHCO_3$

Zinc fixative solution

0.1 M Tris-HCl pH 7.4
3.2 mM $Ca(CH_3COO)_2 \bullet H_2O$
22.8 mM $Zn(O_2CCH_3)_2(H_2O)_2$
35.9 mM $ZnCl_2$

Coloured pigment solution

8 g HKS Gouache 318 (Schmincke, Germany)
50 ml zinc fixative solution

Coloured particles are dissolved by mechanical mixing and homogenized by using ultrasound.

2.8.2. Cell biology buffers and stock solutions

Dispase solution

5 g lyophilised extract
1.6 l dH_2O

Solution is sterile filtrated.

2.8.3. Molecular biology solutions

TEN Buffer

100 mM NaCl
10 mM Tris pH 7.5
1mM EDTA

LB (Luria-Bertani) Medium

To 950 ml dH_2O add:
Bacto-tryptone 10 g
Bacto yeast extract 5 g
NaCl 10 g

Solutes are dissolved and pH is adjusted to 7.0 with 5 M NaOH. The volume is adjusted then to 1L with dH_2O and the solution is sterilised by autoclaving for 20 min at 15 lb/square inch.

LB agar

15 g agarose
1l LB-medium

Sterilisation as described above.

3. Methods

3.1. *In vivo* and *in situ* models

All animal studies were performed with permission of the Regional Council Karlsruhe and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.1.1. Hindlimb ischemia model

At least 24 weeks old male NMRI mice were anesthetized with isoflurane and the femoral artery was ligated just distal to the origin of the deep femoral artery using a 6-0 surgery silk suture The wound was closed and animals were allowed to recover. On day 1 or 7 after surgery, the mice were euthanized and the left ventricle of the heart was cannulated and perfused for 2 min at 90-100 mm Hg with Ringer solution containing 0.1% adenosine and 0.05% BSA (w/v) at 37°C followed by zinc fixative containing a colored pigment that cannot pass the capillary system. The hindlimbs were then dissected and processed for RNA and histological analyses.

3.1.2. Ear artery ligation model

For ligation of the mouse ear artery at least 24 weeks old male NMRI mice were anesthetized as described for the hindlimb ischemia model. The mouse ear is usually supplied by three, occasionally four neurovascular bundles, each consisting of an artery, a large vein and a nerve. Three to four consecutive orders of vessels with decreasing diameters originate from the major Y-shaped vascular branches which then drain into the capillaries (Barker 1989). First order arteries in the center of the ear were ligated by using 6-0 surgery silk sutures. Seven days after surgery, the mice were euthanized and the vasculature perfused through the left ventricle of the heart as described above.

3.1.3. 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: 1 hour incubation in 70%, 85% and 95% ethanol each followed by 1 hour incubation in isopropanol. Tissues were then incubated in a mixture of benzyl alcohol and benzyl benzoate (1:1, v/v) 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 (Barker 1989) (Figure 4). The diameter of the collateral arterioles was measured by using the morphological analysis software Cell^R from Olympus at at least 3 different sites of an individual arteriole.

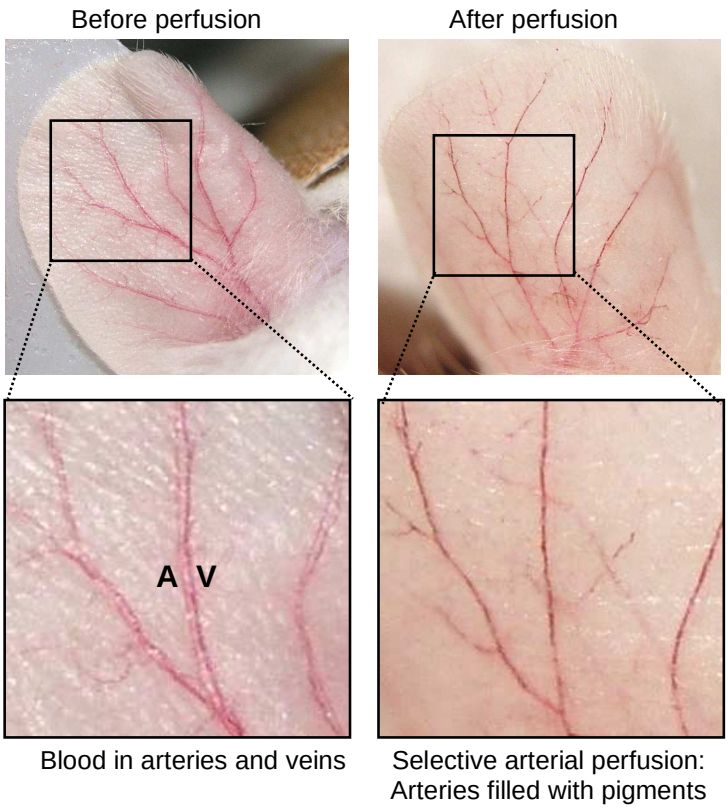

Figure 4. Visualization of the arterial vasculature in the ear artery ligation model. The arterial system of the mouse can selectively be visualized by perfusion with pigmented particles that cannot pass the capillary system.

3.1.4. Perfusion of isolated branches of mouse mesenteric artery

Animals were sacrificed and the ileum together with branches of superior mesenteric artery and vein were dissected. Second order branches of the mesenteric artery were excised and inserted into the perfusion chambers of the Culture Myograph system (DMT, Copenhagen, Denmark). The chambers were placed in an incubator at 37°C and 5% CO_2, and the branches of the arteries were continuously perfused for 6 hours with D-MEM medium containing 15% FCS at a longitudinal pressure gradient of 20 mm Hg with a flow of ~0.07 mL/min or at a pro-arteriogenic pressure gradient of 50 mm Hg with a flow of ~0.17 mL/min (Figure 5).

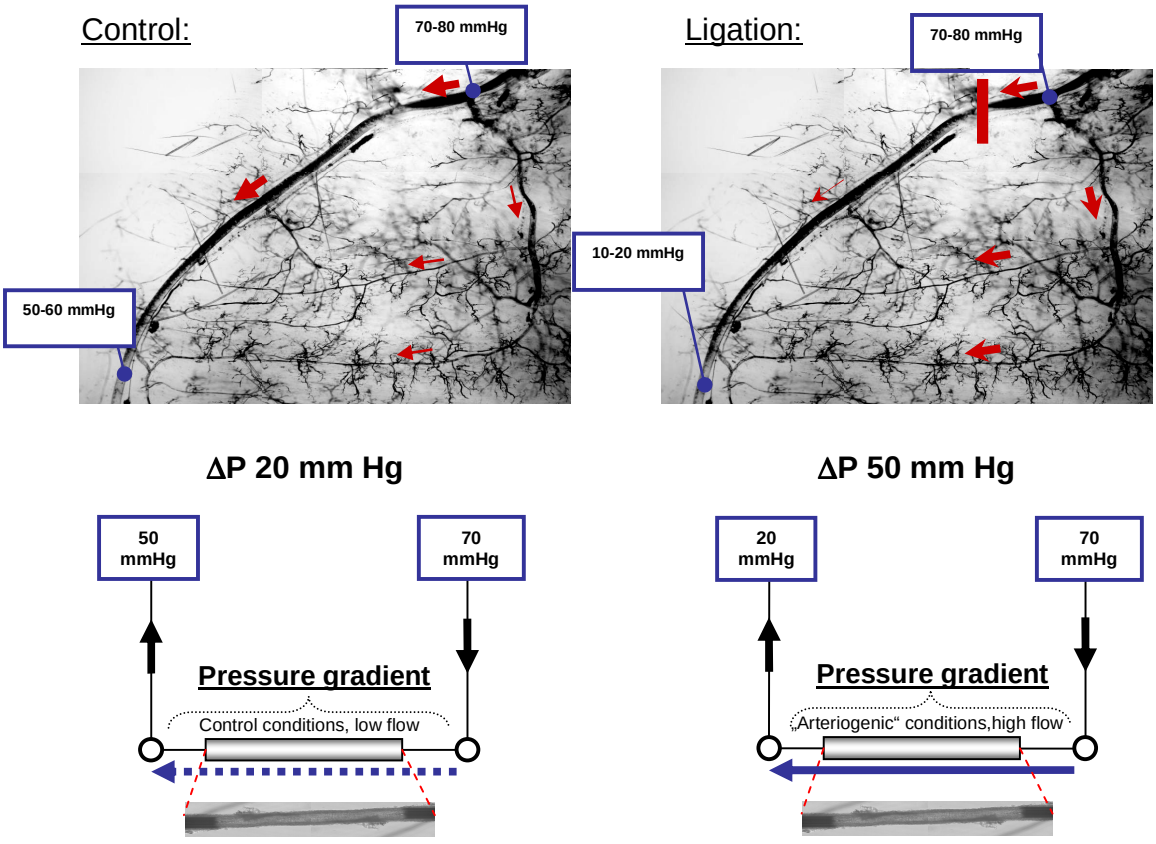

Figure 5. The *in situ* perfusion model mimics the hemodynamic situation of the collateral arterioles after occlusion of the main artery. Perfusion of isolated second order branches of the mouse mesenteric artery was performed under control conditions (ΔP=20 mm Hg, flow ~0.07 mL/min) and under pro-arteriogenic conditions (ΔP=50 mm Hg, flow ~0.17 mL/min), resembling the increased flow in the collateral arterioles following occlusion of the femoral artery.

The functional and cellular integrity of the segments was routinely checked by their vasodilator response to acetylcholine (5 μM) and by immunohistochemical staining for the endothelial cell marker CD31 (Figure 6).

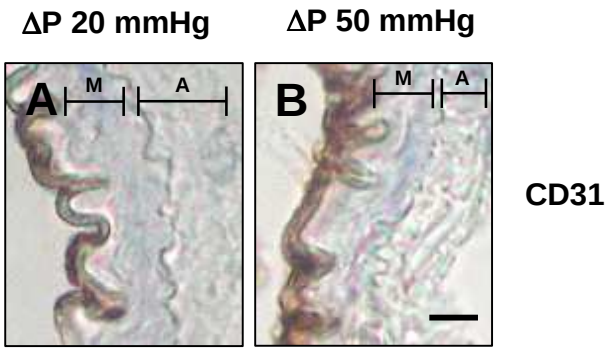

Figure 6. Analysis of CD31 expression in isolated perfused small mouse arteries. Representative immunohistochemical staining (brown colour) of the endothelial cell marker CD31 in second order branches of the mesenteric artery verified the integrity of the endothelial cell monolayer after perfusion (6 hours) under control (ΔP = 20 mmHg, low flow, A) and pro-arteriogenic (ΔP = 50 mmHg, high flow, B) conditions (M – media; A – adventitia; scale bar=10 μm).

3.2. Cell biology methods

3.2.1. Culture of human umbilical vein endothelial cells (HUVECs)

Human umbilical vein endothelial cells were isolated from freshly collected umbilical cords with the consent of parents. The procedure has been approved by the Local Ethical Committee (document number 336/2005). Umbilical veins were flushed with Hank's buffer solution, filled with 10 ml dispase solution (3,1 g/l) and incubated for 30 min at 37°C. Veins were then flushed with 40 ml M199 medium which was collected in a 50-ml tube, the cell-containing medium was centrifuged at 1000 rpm for 5 min. The pellet was resuspended in M199 medium containing 20% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.25 μg/ml Fungizone® antimycotic, and supplemented with endothelial cell growth supplement. The cells were routinely cultured on plastic dishes coated with 2% gelatine or collagen type I coated BioFlex elastomers. Culture medium was changed every third day.

3.2.2. Culture of mouse smooth muscle cells

Mouse smooth muscle cells were isolated from branches of the mouse mesenteric artery. The branches of the artery were dissected, cut into to fragments, washed several times with Hank's BSS solution and transferred to a 40 mm Petri dish containing 250 µl of 1% collagenase solution and 1.4 ml mixture (1:1) of smooth muscle cell medium with D-MEM and 2,5% supplemental mix.

Petri dishes with the fragments of mesenteric arteries were placed in an incubator at 37°C with 5% CO_2 to allow hydrolysis of the extracellular matrix overnight (14-16 hours). Isolated cells were then centrifuged for 5 min at 1000 rpm and were re-suspended in 2 ml mixture (1:1) of smooth muscle cell medium with D-MEM and 2,5% supplemental mix. Cells were allowed to adhere and the medium was changed every 4 days thereafter. After passage one the smooth muscle cells were cultured in D-MEM containing 15% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 0.25 µg/ml Fungizone® antimycotic. The phenotype of the cells was confirmed by anti-mouse α-smooth muscle actin immunofluorescent staining (Figure 7).

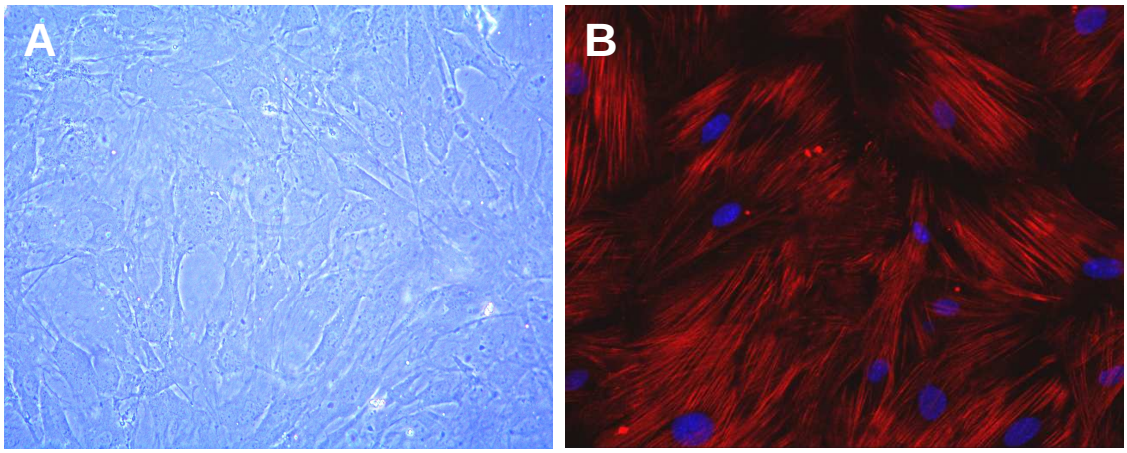

Figure 7. Analysis of the phenotype of cultured mouse smooth muscle cells. Representative immunofluorescent staining for the smooth muscle cell marker α-smooth muscle actin (B; red fluorescence - α-SMA; blue - nuclei) in the cultured smooth muscle cells (A) isolated from mouse mesenteric artery.

3.2.3. Plating and passage of smooth muscle cells (enzymatic hydrolysis)

Confluent cultures of smooth muscle cells were washed with serum free D-MEM medium, incubated with 0.05% trypsin/0.02% EDTA solution for 5 min at 37°C, re-suspended in M199 and spun at 1000 rpm for 5 min. Thereafter, the supernatant was carefully removed and mSMCs were re-suspended in D-MEM with 15% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 0.25 µg/ml Fungizone® antimycotic. For the experiments described, cells of passage 3 to 4 were used throughout.

3.2.4. Application of shear stress and cyclic stretch

Confluent cultured human vascular endothelial cells were exposed to laminar shear stress (30 dyn/cm^2) by using a cone-and-plate viscometer (Cattaruzza 2004) (Figure 8). Shear stress stimulated and static control monolayers were cultured in the same medium for each experiment. One hour prior to the experiment the medium was changed to M199 containing 20% FCS and 6% polyvinylpyrrolidone to increase the viscosity of the medium.

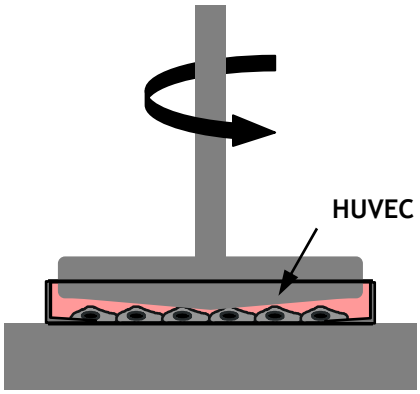

Figure 8. Scheme of the cone-and-plate viscometer. The essential components consist of a teflon cone with an angle of 0.5° rotating over a stationary base plate, which supports either 60 mm or 100 mm diameter polystyrene plates. The entire apparatus is maintained at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

Application of cyclic stretch onto human vascular endothelial cells and human and mouse smooth muscle cells was performed by using a microprocessor controlled vacuum pump (FX-3000 FlexerCell Strain Unit, Flexcell, Hillsborough, NC). Both

cell types were cultured on BioFlex collagen type I 6-well plates (Figure 9), for primary cultured HUVECs the plates were coated with gelatin (2 mg/ml gelatin in 0.1 M HCl for 30 min at 37°C). Stretching was performed with 15% cyclic elongation at frequency of 0.5 Hz, which corresponds to the physiological maximum deformation in the human carotid artery (Perktold 1995).

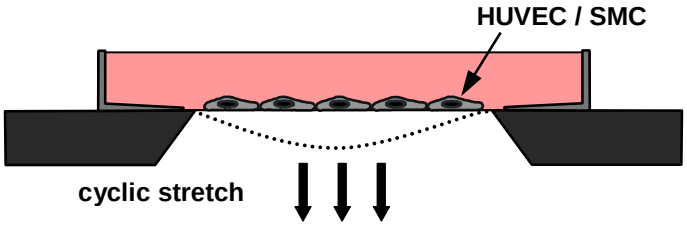

Figure 9. Scheme of the BioFlex collagen type I elastomer. BioFlex culture 6-well plates are fixed in the BioFlex baseplate and maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere. The computer-regulated bioreactor applies cyclic tensile stain to the cells by using regulated vacuum pressure to deform the flexible-bottomed culture plates producing up to 30% substrate elongation.

3.2.5. Stimulation of cultured cells with Idebenone or glucose oxidase

Cells were stimulated with glucose oxidase reagent (final concentration 12 mU/ml, Sigma-Aldrich) or with Idebenone reagent (final concentration 3 µM, Takeda Pharma AG). For one well of a 6-well plate 2 µl of glucose oxidase in dH_2O or Idebenone in DMSO was added to a medium volume of 2 ml. Cells were kept at 37°C and 5% CO_2 at static conditions or exposed to cyclic stretch for 6 hours.

3.3. Molecular biology methods

3.3.1. Isolation of total RNA

Total RNA was isolated from cultured cells, individually excised collateral arterioles and isolated branches of mouse mesenteric arteries using the RNeasy® kit according to the manufacturer's instructions. An aliquot of 13 µl of RNA was used to make single-stranded (ss) cDNA for RT-PCR reactions (3.3.2.).

3.3.2. RT-PCR

Conventional RT-PCR was performed by using Oligo$(dT)_{15}$ primers (Promega, Mannheim, Germany) and the Sensiscript® kit for material from the isolated mouse arteries or the Omniscript® kit for material from the cultured cells and the dissected mouse arterioles according to the manufacturer's instructions.

3.3.3. Measurement of RNA/cDNA concentration

Measurement of RNA and cDNA concentrations was performed by using the NanoDrop® ND-1000 spectrophotometer. For quantitative real-time PCR analysis 80 pg - 1 ng of cDNA was used (3.3.4.).

3.3.4. Quantitative real-time PCR

Real-time PCR was carried out in a LightCycler instrument (Roche Diagnostics, Penzberg, Germany) by using the QuantiTect SYBR Green® kit according to the manufacturer's instructions. Standard cDNA probes for quantitative analysis were generated by using the TOPO TA Cloning® kit (3.3.5.1.). As an internal standard the ribosomal protein L32 (RPL32) or the endothelial cell marker platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) were chosen.

3.3.5. TOPO cloning

Amplified DNA fragments of the desired molecules were cloned into the pCR® TOPO 2.1 vector (3.9 Kb) using the TOPO TA Cloning® Kit according to the manufacturer's instructions. Plasmids containing the inserts were amplified further (3.3.5.1.).

3.3.5.1. Transformation of competent bacteria

pCR® TOPO vector with insert was mixed with 20 µl of Top10F^{TM} competent cells in a 1.5 ml Eppendorf tube and incubated on ice for 30 min. Thereafter, cells were submitted to the heat shock at 42°C for 40 s, and placed back on ice. Two hundred and fifty µl of SOC medium was added to the cells and the suspension incubated at 37°C for 1 h with shaking at 300 rpm. Sterile LB-agar medium with 20 µg/ml of

ampicillin in Petri dishes was pre-warmed to 37°C and 25 µl and/or 75 µl of transformed competent cells were plated onto the dishes. Plates were then incubated at 37°C overnight to allow bacterial colonies to grow.

3.3.5.2. Plasmid mini-cultures and plasmid purification

Transformed colonies of Top10F™ competent cells were picked by using a sterile pipette tip and added to 3 ml of LB Broth (with appropriate antibiotic) in 15 ml falcon tubes. Caps of the falcon tubes were perforated to allow air to circulate through the tube. These cultures were then incubated at 37°C with shaking overnight.

Plasmids grown in minicultures were purified using the QIAPrep® Spin Miniprep Kit according to the manufacturer's instructions.

3.3.6. Decoy oligodeoxynucleotide technique

3.3.6.1. Hybridization of decoy oligodeoxynucleotides

Equal volumes of single-stranded phosphorothioate-bonded decoy oligodeoxynucleotides (dODNs) were mixed and melted at 95°C for 5 min in a water bath. The dODNs were then allowed to cool down to approximately 30°C in the water bath. The efficiency of the hybridization reaction was verified with 2.5% agarose gel electrophoresis in comparison with the single stranded oligodeoxynucletides and usually found to exceed 95% (Figure 10).

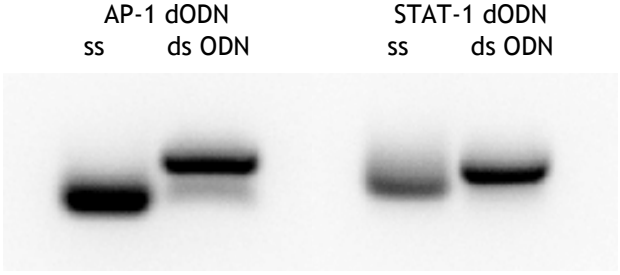

Figure 10. Hybridization of dODNs. Exemplary agarose gel depicting single-stranded (ss) vs. hybridised double-stranded (ds) oligodeoxynucleotide.

3.3.6.2. Administration of the decoy oligodeoxynucleotides

The transcription factor decoys are molecules that mimic the binding sites for transcription factor proteins, and compete with promoter regions to absorb this binding activity in the cell nucleus (Figure 11).

Cultured mSMCs and isolated mouse arteries were transfected with naked ODNs (10 µmol/L), i.e. without using any cationic lipid or liposomal complex for 4 hours at 37°C.

For local application *in vivo*, hybridized ODNs were mixed with *Unguentum emulsificans* to yield a final concentration of 0.25% (w/v) and 20 mg of this mixture was homogeneously spread across the surface of the mouse ear one day before, directly after occlusion of an artery, and then every second day thereafter until euthanization of the animals on day 7.

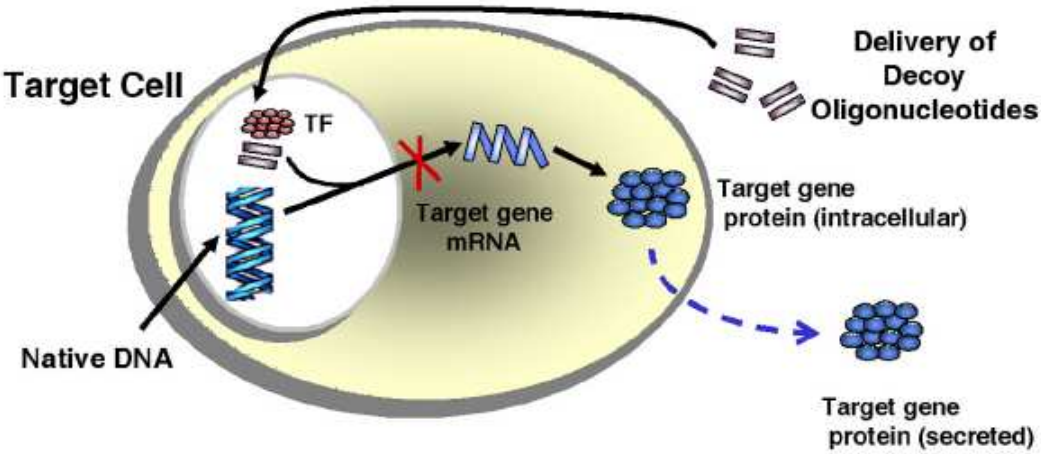

Figure 11. Transcription factor-decoy strategy (taken from Mann, 2005). Transcription factor decoys mimic the sequence specific binding sites for transcription factor proteins that are found in the promoter regions of target genes. Delivery of the decoy to the cell leads to binding of the transcription factor and prevention of transactivation (or suppression) of the target gene.

3.4. Protein biochemistry methods

3.4.1. Immunohistological analysis

Immunohistochemical and immunofluorescence staining for MCP-1, CD31 or F4/80 was performed on 5-µm 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; or the polyclonal rat anti-mouse F4/80 antibody with biotinylated rabbit anti-rat antibody and Streptavidin-RPE (DAKO) according to the manufacturers instructions. Nuclei were visualized by Mayer´s hemalaum counter stain or Hoechst dye 33258. MCP-1 staining intensity was determined by using the Cell^R software analyzing at least two different sections per experimental group and animal. Exposure times during digital imaging were kept constant.

3.4.2. Dihydroethidium staining

Detection of reactive oxygen species (ROS) by using dehydroethidium (DHE), which is rapidly oxidized by ROS into its derivative ethidium bromide, was performed with cells cultured on elastomers or with freshly prepared 5-7 µm frozen sections. Dihydroethidium was added onto the sections or into the cell culture medium at a final concentration of 5 µM in PBS. After 30 min of incubation at 37°C, sections or cells were washed with PBS and covered with coverslips. Fluorescence intensity was determined as described above.

3.4.3. *Bandeiraea* lectin staining

To visualize blood vessels in whole mount preparations of mouse tissues, 200 µl of biotinylated *Bandeiraea* (BS-I) lectin labelled with FITC was injected intravenously into the tail vein of anesthetized mice and allowed to circulate for 3-5 min. Thereafter, animals were sacrificed and perfused through the left ventricle of the heart with Ringer solution followed by 1% paraformaldehyde (PFA) solution. Arterioles from mouse hindlimbs were dissected from adductor, medial large and

gracilis muscles and the fluorescence was detected by confocal microscopy as described above.

3.4.4. ELISA

The concentration of MCP-1 protein in supernatants from mSMCs and HUVECs was determined by ELISA kits from R&D Systems® according to the manufacturer's instructions. The human MCP-1 immunoassay was carried out with 1:10 diluted media samples and the mouse MCP-1 immunoassay with the media samples diluted 1:5. For both assays, the values obtained under control conditions were set to 100%.

3.5. Statistical analysis

All results are expressed as means±SEM. Differences between experimental groups were analyzed by unpaired Student's *t*-test using the Instat™ version 3.06 statistics software package (Graph Pad Software, San Diego, CA, USA). *P*-values < 0.05 were considered statistically significant.

4. Results

4.1. Hind limb ischemia model

4.1.1. Arteriogenic remodelling is characterized by an increase in diameter of collateral arterioles after femoral artery ligation

In order to analyze the arteriogenic remodelling process of collateral arterioles in mouse hindlimbs, the femoral artery was ligated just distal to the origin of the deep femoral artery. The 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 (Scholz 2002; Deindl 2003). On day 7 after femoral artery ligation the remodelled collaterals were easily identified by their corkscrew-like morphology (Figures 12A-B, arrows) and increase in diameter (Figure 12C).

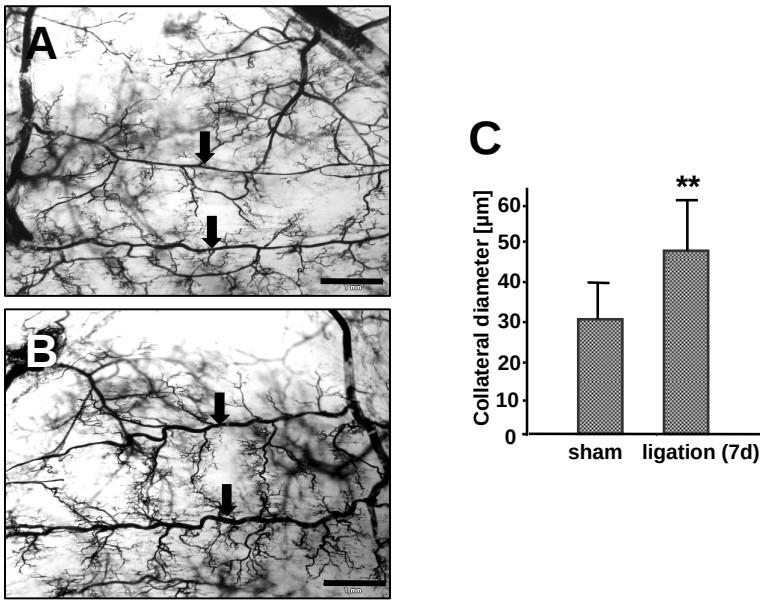

Figure 12. Analysis of collateral arteriolar remodelling in the mouse hindlimb. Images of particle-perfused transparent mouse hindlimbs (A, sham operated; B, ligated, scale bar=1 mm). Arteriogenic remodelling of collateral arterioles is indicated by their tortuous morphology (arrows) and increased diameter (C, **P<0.01 vs. sham, n=5).

4.1.2. MCP-1 is one of the first molecules up-regulated during the onset of arteriogenesis

The remodelling process of collateral arterioles after femoral artery ligation in mouse hindlimbs was accompanied by an increase in the expression of MCP-1 which was observed by using real-time PCR analysis already at 24 hours as well as 7 days post ligation (Figure 13A). Due to the defined and thus predictable anatomy of collateral arterioles in the mouse hindlimb, expression of MCP-1 in these vessels could also be analyzed 24 hours after ligation of the femoral artery even without establishing their tortuous morphology. Similarly, the expression of ICAM-1, another well known gene product the up-regulation of which is associated with arteriogenesis (Hoefer 2004; Hur 2007), was also up-regulated 7 days post occlusion in the remodelling collateral arterioles (Figure 13B). ICAM-1 is expressed mainly in endothelial cells, therefore analysis of its mRNA abundance was normalized by using the endothelial cell marker CD31.

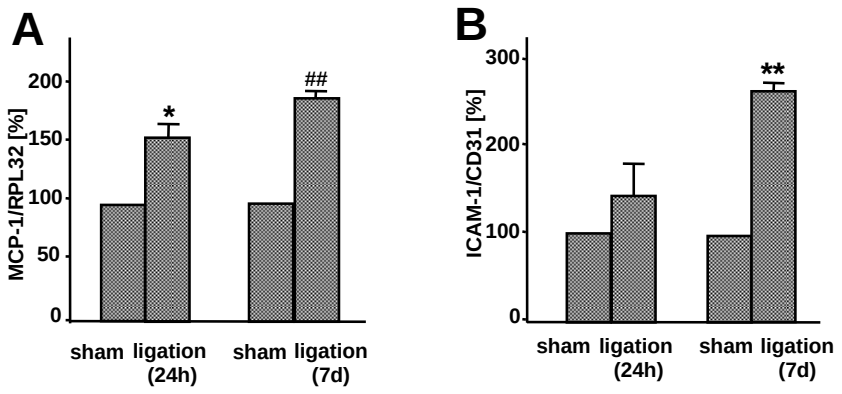

Figure 13. Expression of MCP-1 and ICAM-1 in collateral arterioles undergoing arteriogenesis in mouse hindlimbs after femoral artery ligation. Quantitative real-time PCR analyses of MCP-1 (A, *P<0.05 vs. sham, $^{\#\#}$P<0.01 vs. sham, n=5) and ICAM-1 (B, **P<0.01 vs. sham, n=5) expression in individual arteriogenic arterioles after femoral artery occlusion in the hindlimb ischemia model in mice (MCP-1/RPL and ICAM-1/CD31 ratio under control conditions was set to 100%).

4.2. Ear artery ligation model

4.2.1. Arteriogenic remodelling in the mouse ear is characterized by an increase in number and diameter of corkscrew-like arterioles after artery occlusion

In order to analyse the arteriogenic remodelling process more closely, a mouse ear artery ligation model was employed. Similar to the hindlimb ischemia model, ligation of one first order mouse ear artery resulted in an increase in diameter (Figure 14C) as well as the number (Figure 14D) of corkscrew-like collateral arterioles (Figures 14B, arrows).

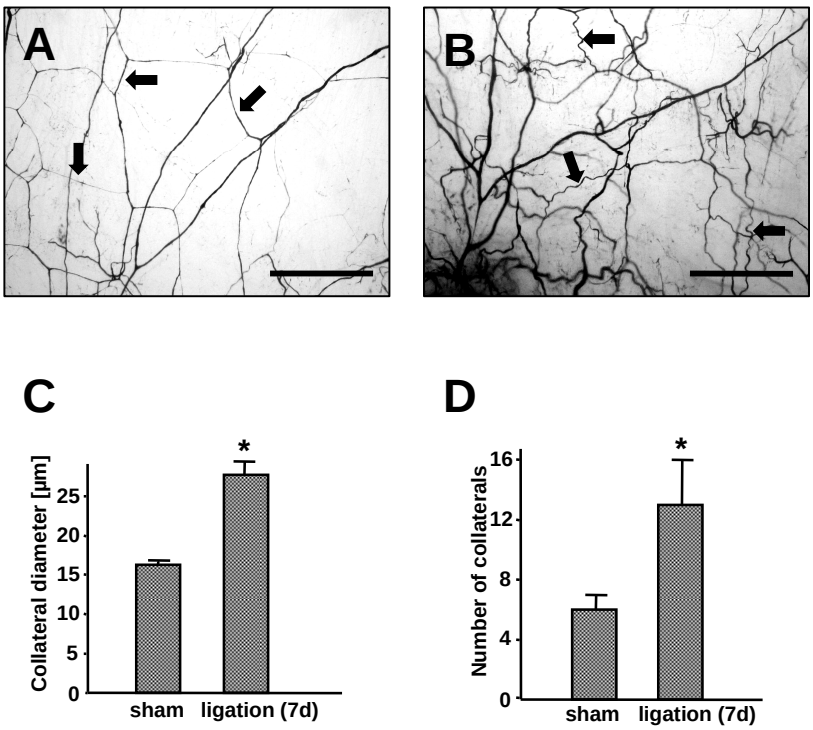

Figure 14. Analysis of collateral arteriolar remodelling in the mouse ear. Images of particle-perfused transparent mouse ears (A, sham operated; B, ligated, scale bar: 0.5 mm) 7 days after arterial occlusion. Arteriogenic remodelling of collateral arterioles is indicated by their tortuous morphology (arrows), increased diameter (C, *P<0.05 vs. sham, n=5) and number (D, *P<0.05 vs. sham, n=5).

4.2.2. Adaptive remodelling of collateral arterioles after ear artery ligation is accompanied by an increased expression of MCP-1 and ICAM-1

The expression of MCP-1 and ICAM-1 in single isolated collateral arterioles from the mouse ear was analyzed as well by using real-time PCR analysis. Likewise, expression of MCP-1 (Figure 15A) and ICAM-1 (Figure 15B) in corkscrew-like collateral arterioles from the mouse ear 7 days post artery occlusion was significantly increased compared to collaterals isolated from sham-operated ears.

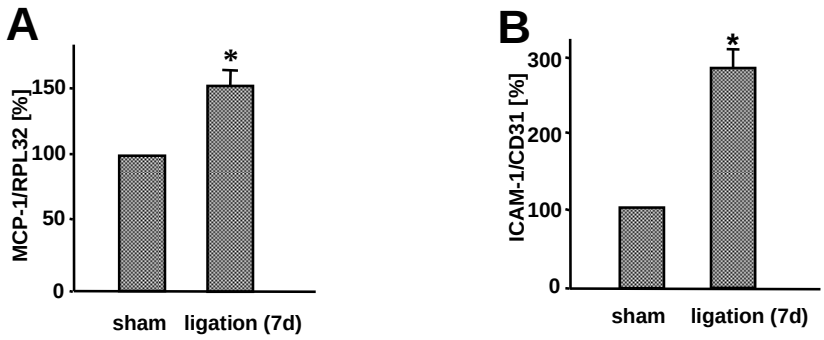

Figure 15. Expression of MCP-1 and ICAM-1 in arteriogenic collateral arterioles isolated from the mouse ear. MCP-1 (A, *P<0.05 vs. sham, n=5) and ICAM-1 (B, *P<0.05 vs. sham, n=5) expression in isolated control and arteriogenic arterioles was determined by real-time PCR analyses in the mouse ears (MCP-1/RPL32 and ICAM-1/CD31 ratios under control conditions were set to 100%).

4.3. Perfusion model of isolated branches of the mouse mesenteric artery

4.3.1. Pro-arteriogenic perfusion conditions up-regulate MCP-1 expression in vascular smooth muscle cells

To mimic the changes in arteriolar perfusion which occur after occlusion of the femoral artery, an *in situ* perfusion model was established. 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 (Helisch 2003; Heil 2004). As a result of the experimentally set pressure gradient (50 mm Hg vs. 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. Immunohistochemical analyses revealed an increased abundance of MCP-1 in the media of these segments upon perfusion under pro-arteriogenic conditions for 6 hours (compare Figures 16A and B).

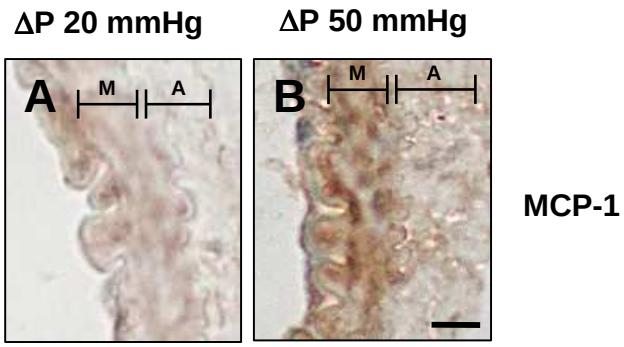

Figure 16. Analysis of the expression of MCP-1 in isolated perfused small mouse arteries. Immunohistochemical staining of MCP-1 (brown colour) revealed an increased abundance of the cytokine in the media upon exposure to an increased pressure gradient (B; M – media; A – adventitia; scale bar=10 µm).

To localize the source of MCP-1 expression in the perfused branches of the mesenteric artery, the endothelial cell layer was mechanically removed by denuding the vessels just after termination of perfusion. Immunohistochemical analyses confirmed the increase in MCP-1 abundance in the media upon perfusion under pro-arteriogenic conditions (compare Figures 17A and B).

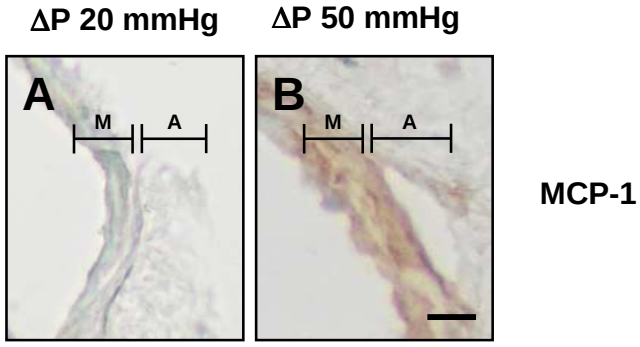

Figure 17. Analysis of the expression of MCP-1 in isolated perfused small mouse arteries after removal of the EC monolayer. Immunohistochemical staining of MCP-1 (brown colour) revealed an increased abundance of the cytokine in the media after perfusion under pro-arteriogenic conditions (B; M – media; A – adventitia; scale bar=10

Moreover, real-time PCR analyses of the perfused arteries confirmed that mechanical removal of the endothelial cell monolayer did not affect the relative change in MCP-1 mRNA abundance under pro-arteriogenic flow conditions (Figure 18).

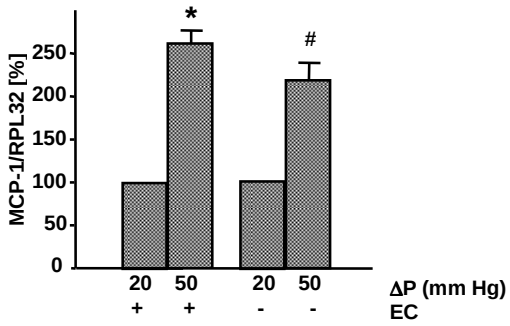

Figure 18. Analysis of MCP-1 mRNA abundance in isolated perfused branches of the mouse mesenteric artery. Compared to the perfused arteries with the intact EC monolayer, its mechanical removal after perfusion did not alter the increase in the MCP-1 mRNA after exposure to pro-arteriogenic perfusion conditions as evidenced by real-time PCR. (*$P<0.05$ vs. control, i.e. $\Delta P=20$ mm Hg, $^{\#}P<0.05$ vs. control, n=6; MCP-1/RPL32 ratio under control conditions was set to 100%).

4.4. Effects of shear stress and cyclic stretch application *in vitro*

As an increase in flow with the resulting distension of the vessel wall affects both shear stress and circumferential wall strain, the impact of these two biomechanical forces on MCP-1 expression in cultured cells was analyzed individually.

4.4.1. Cyclic stretch rather than shear stress up-regulates MCP-1 expression in cultured vascular cells

Cultured HUVECs were exposed to fluid shear stress (30 dyn/cm^2) for 6 hours and analyzed by real-time PCR and ELISA for MCP-1 expression. An increase in shear stress had no effect on MCP-1 mRNA (Figure 19A) and protein (Figure 19B) in the cultured HUVECs.

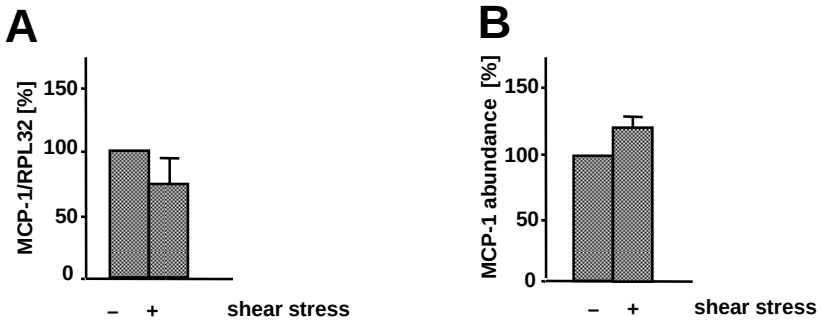

Figure 19. Analysis of MCP-1 expression in cultured endothelial cells in response to shear stress. MCP-1 mRNA levels (A, real-time PCR analysis) and MCP-1 protein levels in supernatants of HUVECs (B, ELISA) were not significantly altered following exposure to shear stress (30 dyn/cm^2) for 6 hours (n=3; MCP-1 expression/release under static conditions was set to 100%).

This effect was also confirmed in endothelial cells isolated from the mouse lung microvasculature (Figure 20A) and in the mouse EC cell line bEnd.3 isolated from the cerebral cortex (Figure 20B and 21).

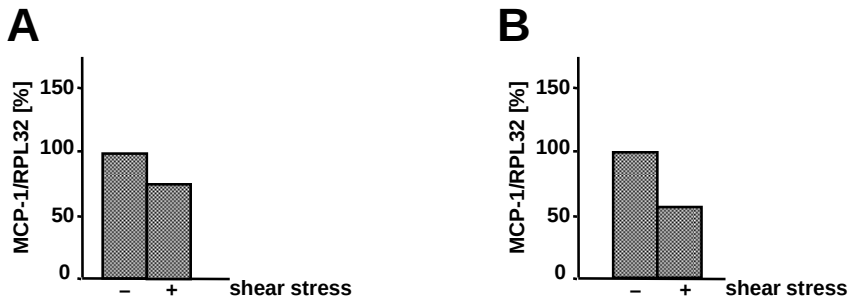

Figure 20. Exemplary analysis of MCP-1 expression in cultured mouse endothelial cells in response to shear stress. MCP-1 mRNA levels (real-time PCR) in endothelial cells isolated from mouse lung microvasculature (A) and in the mouse EC cell line bEnd.3 (B) were not significantly changed after exposure to shear stress (30 dyn/cm^2) for 6 hours (n=1, the level of MCP-1/RPL32 under control conditions was set to 100%).

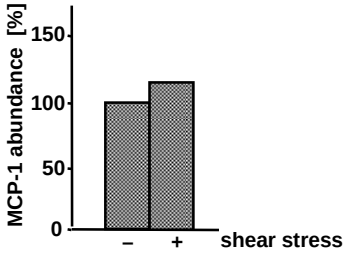

Figure 21. Exemplary analysis of MCP-1 expression in cultured endothelial cells in response to shear stress. MCP-1 protein abundance (ELISA) in supernatants of the in mouse EC cell line bEnd.3 was not significantly changed after exposure to shear stress (30 dyn/cm^2) for 6 hours (n=1, the level of MCP-1 release under control conditions was set to 100%).

As a surrogate parameter for an increase in circumferential wall strain, cyclic stretch was applied to the cultured HUVECs and mSMCs for 6 hours (15% elongation at 0.5 Hz). In contrast to the shear stress experiments, quantitative real-time PCR analyses revealed an increased expression of MCP-1 in both cell types on the mRNA level (Figure 22A). MCP-1 protein abundance determined by ELISA was also significantly increased in both types of cells upon exposure to cyclic stretch as compared to the static controls (Figure 22B).

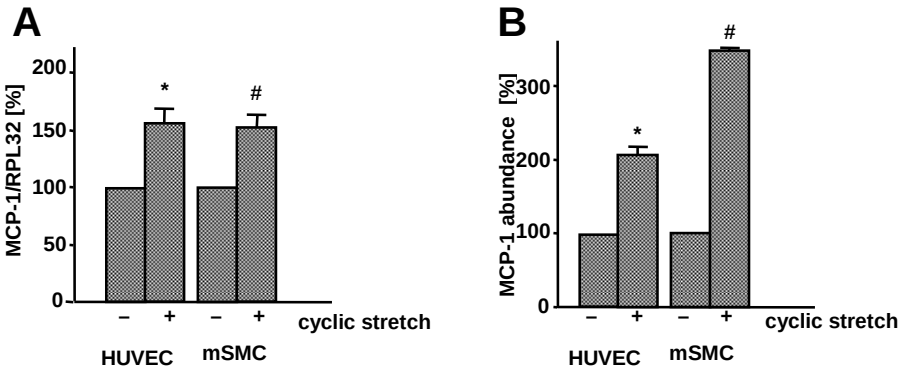

Figure 22. Analysis of MCP-1 expression in cultured endothelial and smooth muscle cells in response to cyclic stretch. Exposure to cyclic stretch (15% elongation at 0.5 Hz) of both HUVEC and mSMC for 6 hours similarly increased the abundance of MCP-1 mRNA (A, real-time PCR; *P<0.05, #P<0.05 vs. static control, n=3) and protein (B, ELISA; *P<0.05, #P<0.05 vs. static control, n=3). The level of MCP-1 expression/release under control conditions was set to 100%.

These results were confirmed with cultured human smooth muscle cells where application of cyclic stretch rapidly induced MCP-1 expression on the protein level (Figure 23).

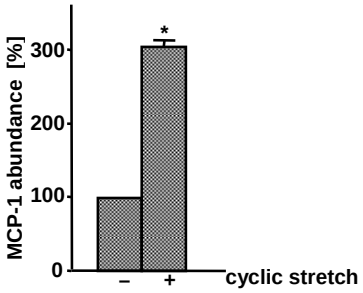

Figure 23. Analysis of MCP-1 expression in cultured human smooth muscle cells in response to cyclic stretch. Exposure to cyclic stretch (15% elongation at 0.5 Hz) of SMC isolated from human thymus veins for 6 hours increased MCP-1 protein expression (ELISA; *P<0.05 vs. static control, n=6). The level of MCP-1 release under control conditions was set to 100%.

4.5. The impact of AP-1 activation on the regulation of MCP-1 expression

Among several 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 pro-inflammatory gene products (Wung 1997; Park 1999; Kumar 2003). Therefore, the role of AP-1 in stretch-induced MCP-1 expression was analyzed.

4.5.1. Expression of MCP-1 induced by pro-arteriogenic perfusion conditions is dependent on the activation of AP-1

In order to analyze whether activation AP-1 is necessary for MCP-1 expression, the decoy oligodeoxynucleotides technique was employed first in the isolated branches of the mouse mesenteric artery. The gene expression studies showed that pre-treatment with an appropriate AP-1 dODN completely abrogated the increase in MCP-1 expression in arteries exposed to pro-arteriogenic perfusion conditions (Figure 24).

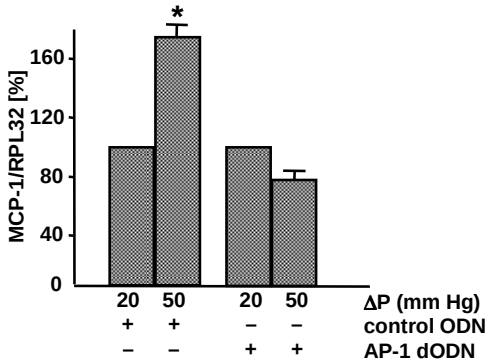

Figure 24. Analysis of the role of AP-1 in the mechanosensitive expression of MCP-1. Pre-treatment of arterial segments with the AP-1 dODN (10 µmol/L) for 4 hours abolished the increase in MCP-1 expression under pro-arteriogenic perfusion conditions (ΔP = 50 mmHg) whereas treatment with mutant control ODN had no effect (real-time PCR analysis; *P<0.05 vs. control, i.e. ΔP=20 mm Hg, n=6; MCP-1 mRNA under control conditions was set to 100%).

To verify the specificity of the AP-1 dODN, a mutant control ODN was administered in parallel to the isolated branches of the mouse mesenteric artery, but did not affect MCP-1 expression induced by pro-arteriogenic perfusion conditions (Figure 24).

4.5.2. Activation of AP-1 is critical for stretch-induced MCP-1 expression *in vitro*

Cultured mouse smooth muscle cells were pre-treated with the AP-1 dODN and exposed to cyclic stretch for 6 hours (15% elongation at 0.5 Hz). Gene expression analysis by real-time PCR showed that inhibition of AP-1 activity abolished stretch-induced MCP-1 expression on the mRNA level (Figure 25A). ELISA further confirmed that stretch-induced expression of MCP-1 protein was completely abrogated as well (Figure 25B). Treatment with the control ODN, on the other hand, had no effect on the expression of MCP-1 mRNA or protein in the cultured mSMCs (Figure 25).

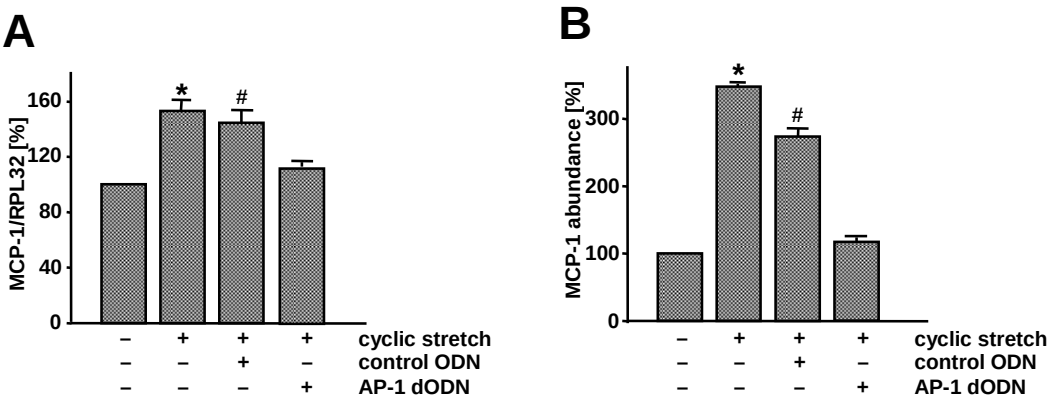

Figure 25. Analysis of MCP-1 expression in stretch-stimulated cultured mouse smooth muscle cells treated with the AP-1 dODN. Treatment with the AP-1 dODN but not the mutant control ODN inhibited stretch-induced expression of both MCP-1 mRNA (A, real-time PCR; *P<0.05, $^{\#}$P<0.05 vs. static control, n=3) and protein (B, ELISA; *P<0.05, $^{\#}$P<0.05 vs. static control, n=3). The level of MCP-1 expression/abundance under control conditions was set to 100%.

4.6. Analysis of ROS formation in cells exposed to cyclic stretch and in the perfused arteries

4.6.1. Cyclic stretch increases production of ROS in cultured smooth muscle cells

The transcription factor AP-1 has been shown to be activated by increased formation of reactive oxygen species (ROS). Therefore, formation of ROS in the cultured smooth muscle cells upon exposure to cyclic stretch was analyzed. Incubation of the cultured cells with dihydroethidium revealed a marked increase in ROS formation 6 hours after exposure to cyclic stretch which was abolished in the presence of the ROS scavenger Idebenone (Figure 26) verifying the specificity of the dehydroethidium detection method.

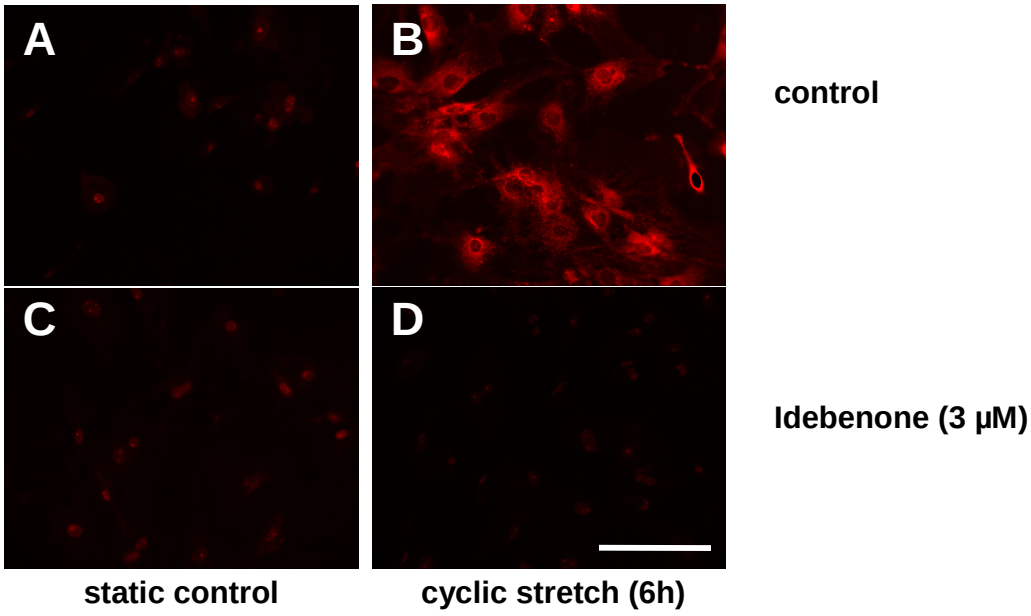

Figure 26. Formation of reactive oxygen species (ROS) in stretched mSMCs. Detection of ROS by dihydroethidium in stretch-stimulated mSMCs in the absence (A, static control; B, cyclic stretch: 15% elongation at 0.5 Hz for 6 hours) and in the presence of the ROS scavenger Idebenone (3 µM; C, control; D, cyclic stretch; scale bar=50 µm).

In order to delineate a role for ROS in the regulation of MCP-1 expression, cultured mouse smooth muscle cells were exposed to cyclic stretch in the presence of Idebenone (3 µM) for 6 hours. Quantitative real-time PCR analyses revealed that in contrast to the previous series of experiments there was no stretch-induced increase in MCP-1 expression both on the mRNA (Figure 27A) and protein level (Figure 27B) in the presence of Idebenone.

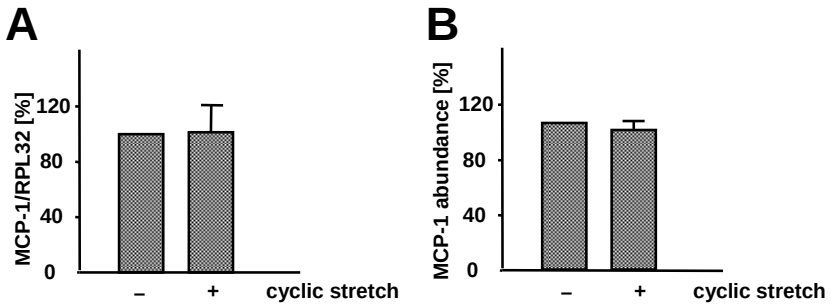

Figure 27. Analysis of MCP-1 expression in cultured mouse smooth muscle cells in response to cyclic stretch in the presence of Idebenone. Exposure to cyclic stretch (15% elongation at 0.5 Hz) for 6 hours in the presence of Idebenone (3 µM) failed to raise MCP-1 mRNA (A, real-time PCR; n=3) or protein (B, ELISA; n=3) levels. The level of MCP-1 expression/release under control conditions was set to 100%.

4.6.2. Formation of ROS is necessary for MCP-1 expression induced by pro-arteriogenic flow conditions *in situ*

Similarly, an enhanced ROS formation was detected in the isolated perfused branches of the mouse mesenteric artery. Immediately after perfusion the vessel segments were frozen in liquid nitrogen and 5 µm sections were incubated with dihydroethidium. Analysis of the fluorescence intensity revealed that perfusion under pro-arteriogenic flow conditions for 6 hours induced an increase in ROS formation mainly in the media of the vessels (Figure 28).

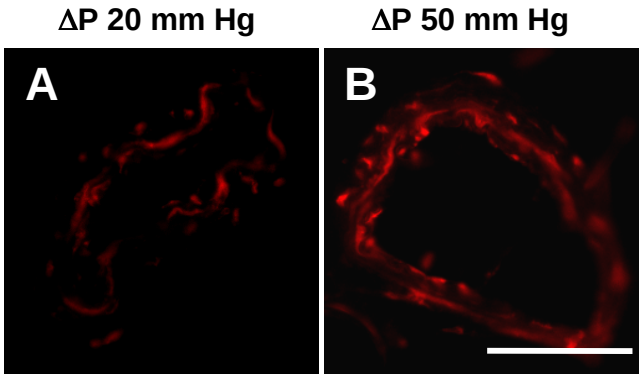

Figure 28. Analysis of ROS formation in isolated perfused mouse arteries. Detection of ROS by dihydroethidium (A, perfusion under control conditions; B, perfusion under pro-arteriogenic conditions; scale bar=100 µm) revealed that pro-arteriogenic flow conditions enhance ROS formation in the isolated arterial segments.

Similarly, the effect of Idebenone was investigated in the perfused branches of the mouse mesenteric artery where it inhibited MCP-1 mRNA expression induced by pro-arteriogenic flow conditions (Figure 29).

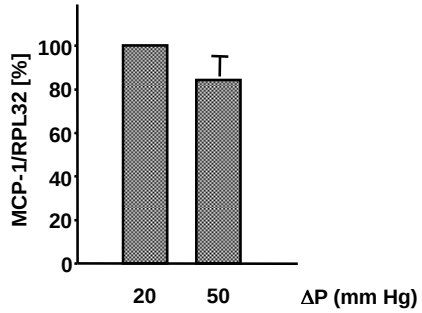

Figure 29. Analysis of MCP-1 expression in isolated perfused branches of the mouse mesenteric artery in the presence of Idebenone. In the presence of Idebenone (3 µM) there was no increase in MCP-1 mRNA abundance, as analyzed by real-time PCR, upon perfusion under pro-arteriogenic conditions (n=6, MCP-1/RPL32 ratio under control conditions was set to 100%).

4.6.3. An increased ROS formation is associated with collateral arterioles undergoing arteriogenesis

Furthermore, ROS formation in the collateral arterioles of the mouse hindlimbs was analyzed after femoral artery occlusion. Shortly before animals were sacrificed DHE solution in PBS and BS I Lectin labeled with FITC (to identify the vessels in the muscle of the hindlimb) were injected into the mouse tail vein. By using confocal microscopy an increased ROS formation was detected associated with the collateral arterioles in the mouse hindlimbs 3 days after occlusion of the femoral artery compared to arterioles from sham-operated animals (Figure 30).

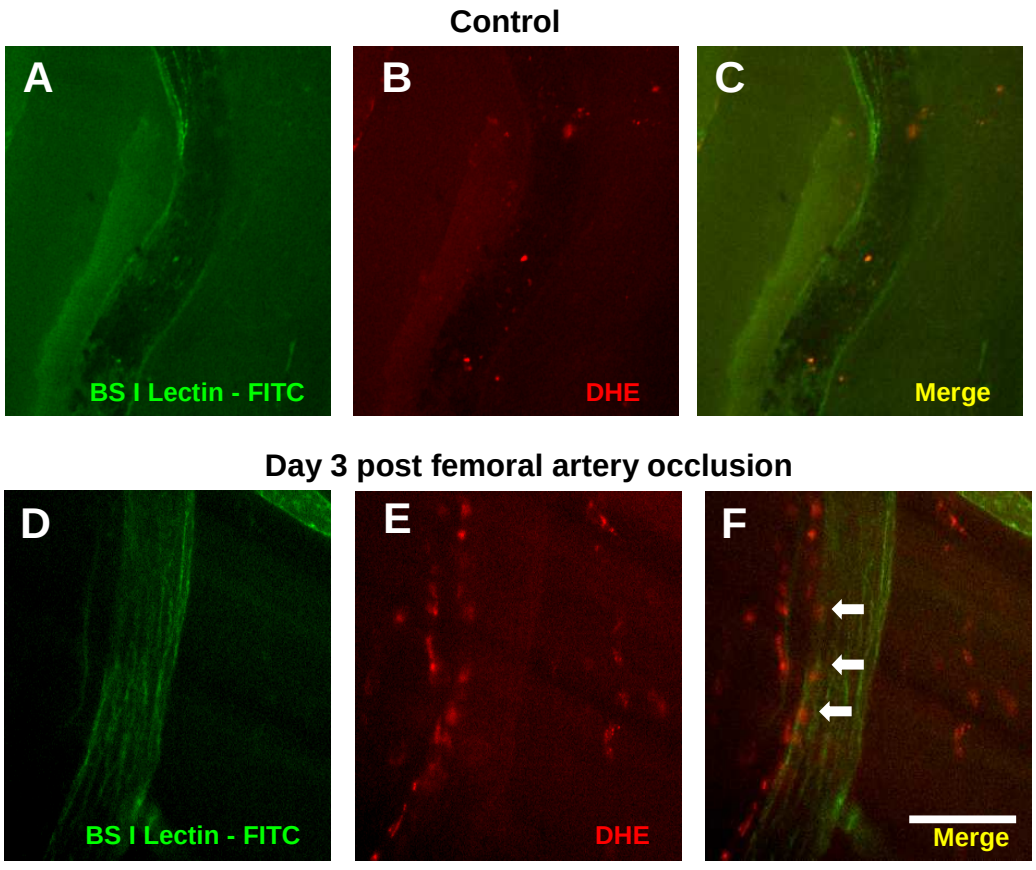

Figure 30. Formation of ROS in collateral arterioles after ligation of the femoral artery (mouse hindlimb ischemia model). Detection of ROS by dehydroethidium (red fluorescence), combined with BS I lectin-FITC labeling of the vessel wall (green fluorescence) in collateral arterioles of the mouse hindlimb 3 days after ligation of the femoral artery (A-C, sham; D-F, ligation; scale bar=50 µm).

4.6.4. Expression of MCP-1 can be induced by an increase in exogenous ROS formation *in vitro*

Cultured smooth muscle cells were stimulated with glucose oxidase to induce exogenous formation of hydrogen peroxidase and analyze its effect on MCP-1 expression in these cells. Quantitative real-time PCR analysis demonstrated an increased expression of MCP-1 in the mSMCs after exposure to glucose oxidase (Figure 31A). Unexpectedly, MCP-1 protein abundance did not change significantly in the presence of glucose oxidase (Figure 31B).

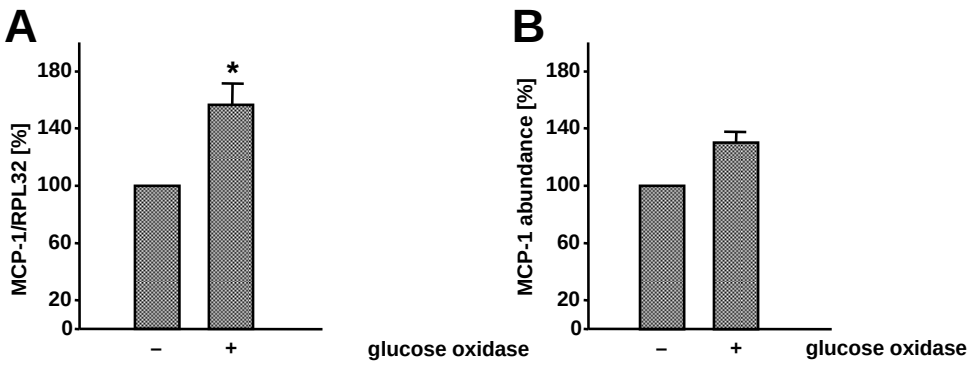

Figure 31. Expression of MCP-1 in cultured mouse smooth muscle cells stimulated with glucose oxidase. Exposure to glucose oxidase (12 mU/ml) for 6 hours at 37°C resulted in increased MCP-1 expression on the mRNA level (A, real-time PCR analysis, n=3, *P<0.05) while that on the protein level did not reach statistical significance (B, ELISA, n=3). The level of MCP-1 expression/release under control conditions was set to 100%.

4.7. Activation of AP-1 is crucial for MCP-1 expression and monocyte recruitment during arteriogenesis

Since the aforementioned data had identified AP-1 as a pivotal factor in stretch-induced MCP-1 expression in vascular SMCs *in vitro* and *in situ*, the role of AP-1 during arteriogenesis *in vivo* was further analyzed. To this end, the mouse ears were treated with a topical formulation of the AP-1 dODN or the corresponding control ODN during the induction and early manifestation phase of the remodelling process. Topical administration of the fluorescent dye-labelled dODN as an ointment resulted in an excellent penetration of the nucleic acid through the skin into the wall of the ear blood vessels within 24 hours after the first application where it was still detectable after 48 hours (Figure 32). Thus, the ointments containing the AP-1 dODN and the corresponding control ODN were topically administered to the mouse ear the day before the ligation was made, on the day of the ligation and every 48 hours following the ligation of the ear artery.

Seven days after occlusion of the main supplying artery in one ear the animals were sacrificed and the ears harvested for further analyses. Morphological analysis by using coloured particle-perfusion showed that topical application of the AP-1 dODN completely abrogates the arteriogenic remodelling process when compared to treatment with the mutant control ODN (Figures 33A-C).

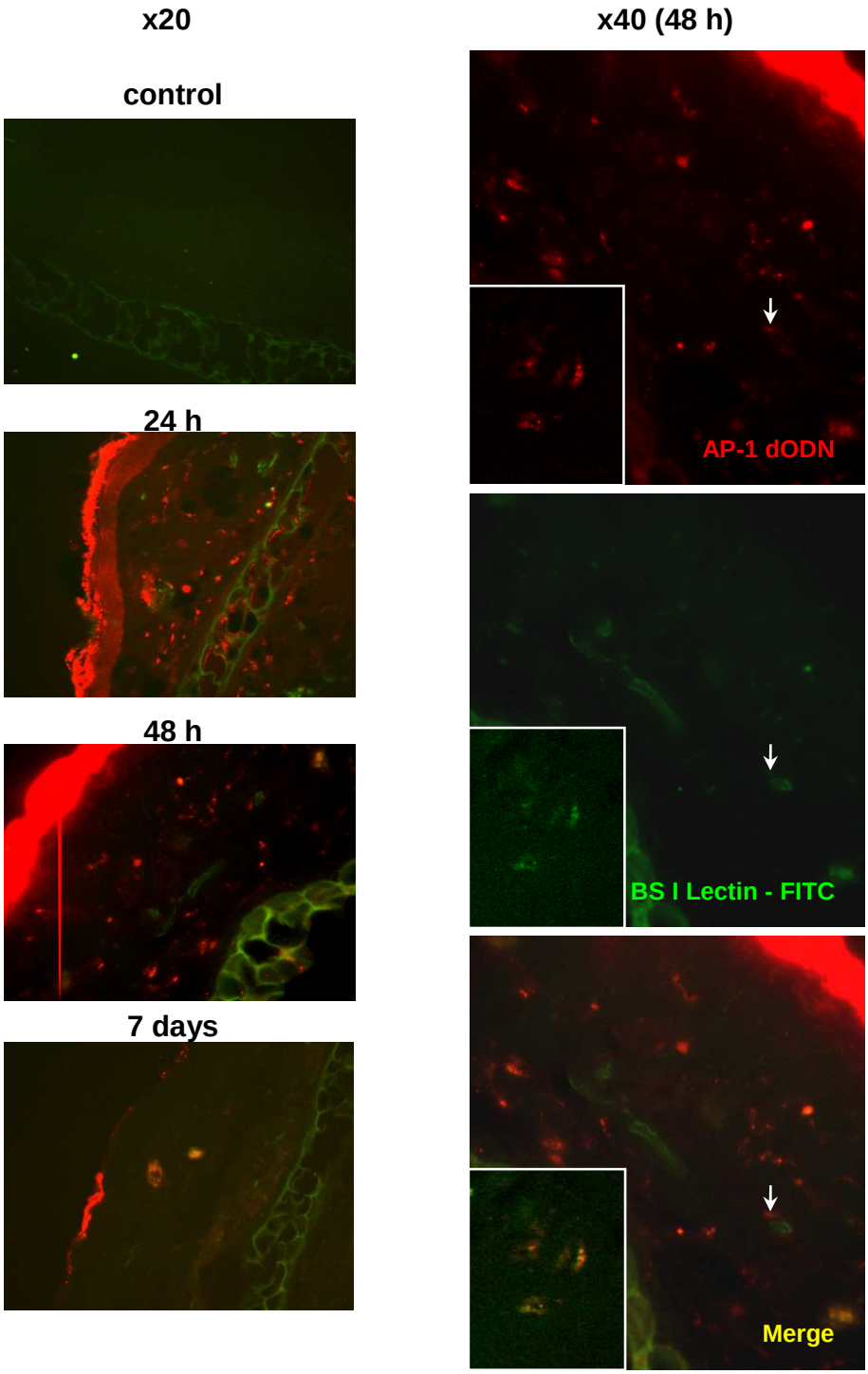

Figure 32. Monitoring of the penetration of the AP-1 dODN through the skin of the mouse ear. Fluorescence dye ATTO*565-labelled AP-1 dODN (red fluorescence) was homogeneously administered once to the surface of the ear and monitored 1, 2 and 7 days later by way of confocal fluorescence microscopy (left panel, endothelial cells in the ear blood vessels were labelled by perfusion with BS I lectin-FITC, green fluorescence). Labelled AP-1 dODN was detected adjacent to ear blood vessels (right panel, arrow) and in endothelial cells (right panel, inserts).

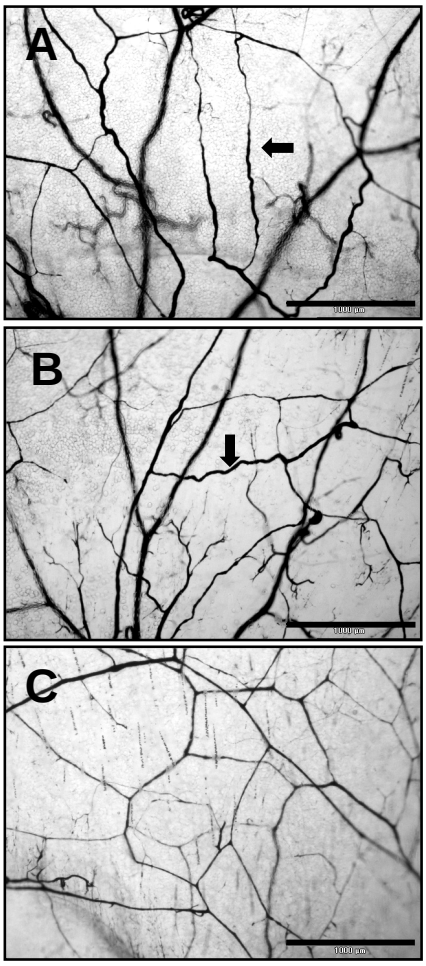

Figure 33. Visualization of the effects of AP-1 dODN blocking AP-1 activity on the arteriogenic remodelling process in the mouse ear 7 days after occlusion of the main artery (A, arrows). Topical treatment with the AP-1 dODN (C) but not the mutant control ODN (B, for details of the treatment regimen refer to the Methods section) inhibited collateral arteriolar remodeling (B, C, arrows; scale bar: 1 mm) as indicated by the reduced number of corkscrew-like arterioles.

Closer analysis revealed that both the enlargement (Figure 34A) and increase in the number of collateral arterioles with corkscrew-like morphology (Figure 34B) was virtually blunted upon treatment with the AP-1 dODN, while treatment with the mutant control ODN had no such effect. In addition, real-time PCR analyses confirmed that there was no arteriogenesis-associated increase in MCP-1 expression in the collateral arterioles of AP-1 dODN-treated animals (Figure 34C). Treatment with the mutant control ODN, on the other hand, did not affect the increase in MCP-1 mRNA level in the remodelling collateral arterioles.

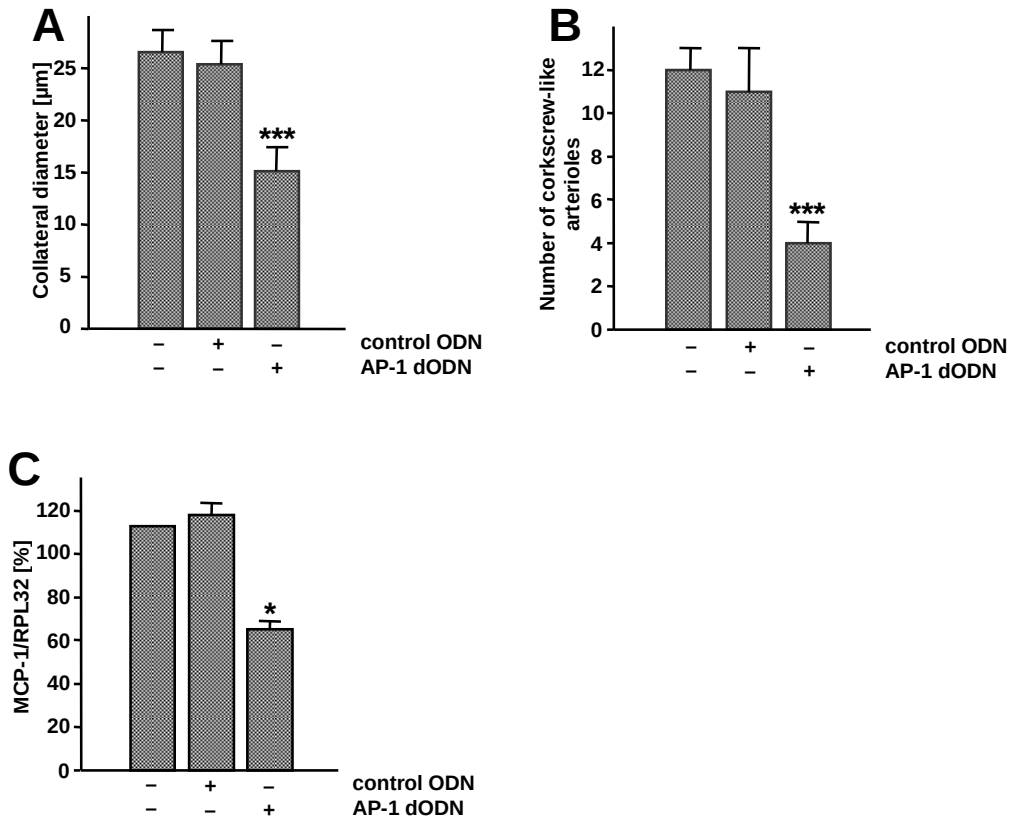

Figure 34. Analysis of the effects of AP-1 inhibition in the mouse ear artery ligation model. Diameter and number of corkscrew-like blood vessels is decreased by treatment with the dODN 7 days post ear artery occlusion and accompanied by a decrease in MCP-1 expression as evidenced by real-time PCR analyses (A-C, *P<0.05, ***P<0.001 vs. non-treated control mouse ears, n=5).

Since MCP-1 is a powerful chemoattractant for circulating monocytes (Ernst 1994), the effect of the AP-1 dODN treatment on the recruitment of monocytes to the arteriogenic vessel wall was analyzed. Following treatment with the AP-1 dODN but not the mutant control ODN there was a marked decline in the number of F4/80-positive macrophages surrounding the arteriolar vessel wall (Figure 35).

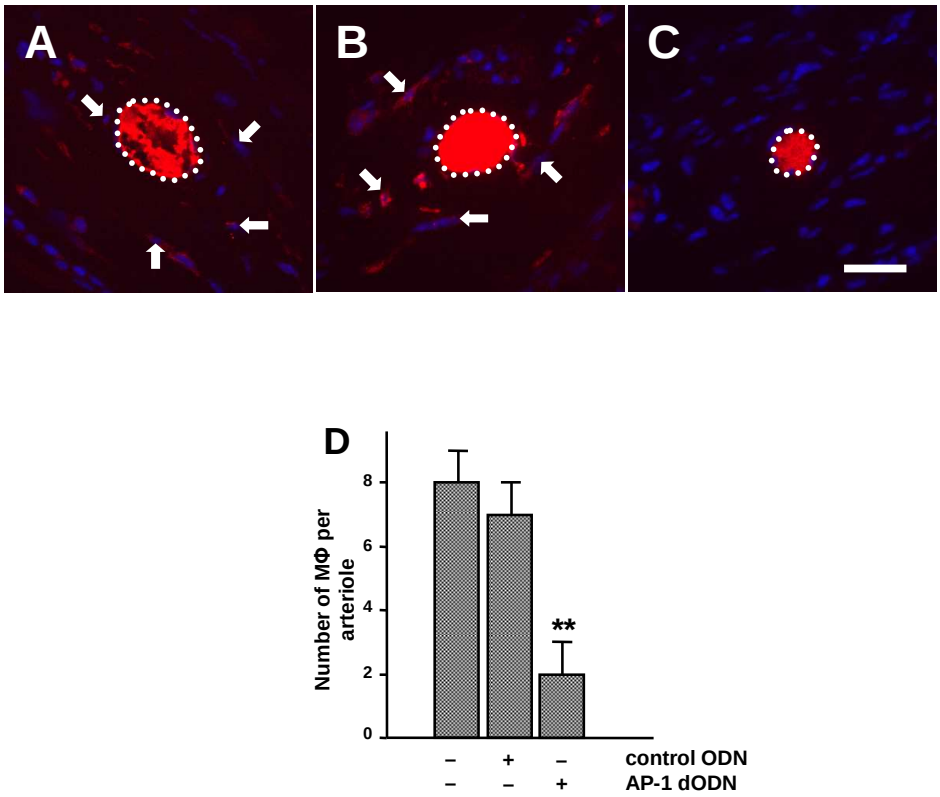

Figure 35. Effect of the topical AP-1 dODN treatment on arteriogenesis-associated monocyte recruitment. Representative immunofluorescence images showing F4/80-positive macrophages (red fluorescence, arrows) surrounding collateral arterioles (nuclei, blue fluorescence; dotted circle: arteriole filled with particles showing intense red autofluorescence; scale bar: 25 μm) 7 days post occlusion of the main artery in control (A), mutant control ODN-treated (B) and AP-1 dODN-treated (C) mouse ears. The number of F4/80-positive macrophages surrounding arteriogenic collateral arterioles is significantly reduced by treatment with the AP-1 dODN (D, **P<0.01 vs. untreated arteriogenesis control, n=5).

4.8. Inhibition of STAT-1 activity does not affect arteriogenic remodelling of collateral arterioles

In order to delineate effects associated with the activity of AP-1 from that of other pro-inflammatory transcription factors on the arteriogenic remodelling process, decoy oligonucleotides specific for neutralizing signal transducer and activator of transcription-1 (STAT-1) were administration in the same manner to the skin of

mouse ear. On day 7 after ligation of an ear artery the mice were sacrificed and ears dissected as described above.

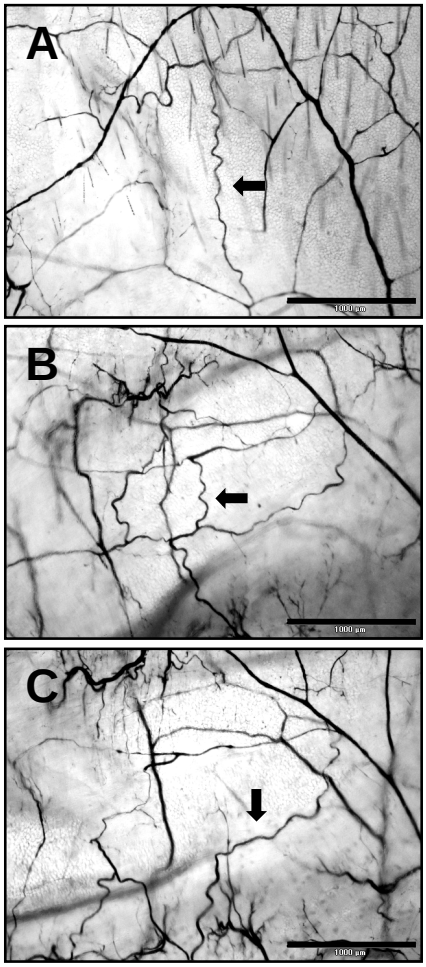

Figure 36. Visualization of the effects of STAT-1 inhibition on arteriogenic remodelling of the arterioles in the mouse ear 7 days after occlusion of the main artery (A, arrows). Topical treatment with the STAT-1 dODN (C) as well as the mutant control ODN (B, for details of the treatment regimen refer to the Methods section) did not affect collateral arteriolar remodelling (arrows; scale bar: 1 mm).

Morphological analysis with coloured particle-perfusion showed that blockade of STAT-1 activity did not affect the arteriogenic remodelling process in the mouse ear (Figure 36). This lack of effect was further detailed by quantification of the number and diameter of the corkscrew-like collateral arterioles (Figure 37).

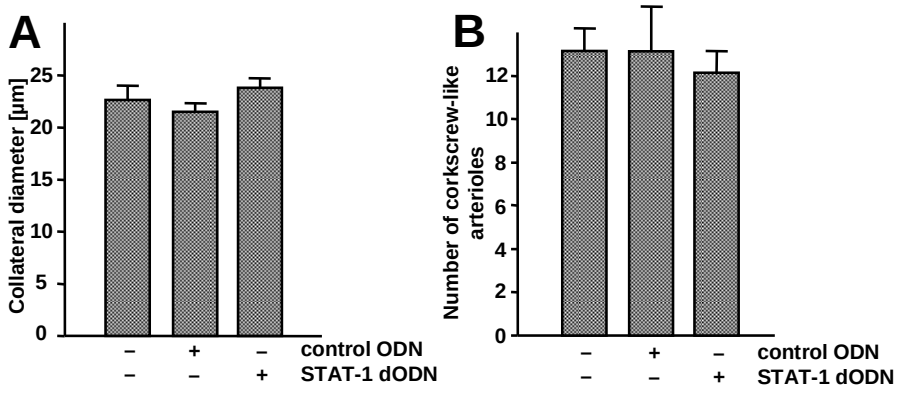

Figure 37. Analysis of the effects of STAT-1 inhibition in the mouse ear artery ligation model. Diameter (A, n=3) and number (B, n=3) of corkscrew-like collateral arterioles is not affected by STAT-1 dODN treatment 7 days post ear artery occlusion compared to ears treated with the mutant control ODN.

5. Discussion

Occlusive vascular diseases continue to be the most common cause of death in the Western world and hence constitute a high unmet medical need. In the Cardiovascular Health Study, peripheral artery disease was found in 13.9% of 2214 men aged ≥ 65 years and in 11.4% of 2870 women aged ≥ 65 years without known cardiovascular disease (Newman 1993). In Germany, prevalence was 9.2% of 6880 males over 65 years of age in primary care (Diehm 2004). With the progress in the field of percutaneous intervention and vascular surgery, development of atherosclerotic vascular disease has become treatable in a growing number of patients. However, in a significant amount of cases the disease can not be treated effectively. Patients suffering from peripheral artery disease have a large number of co-morbidities including coronary heart disease (angina, myocardial infarction) and cerebrovascular disease (stroke) increasing the risk particularly of surgical therapies (Nikol 2007).

Alternative approaches to restore blood flow to the ischemic tissue are needed for those patients with untreatable lesions or contraindications to surgical interventions. As a consequence, several strategies are currently tested to stimulate collateral artery growth and the formation of natural bypasses (arteriogenesis). Therapeutic stimulation of this protective mechanism, which is far more efficient than angiogenesis, has the potential to become a significant treatment option for vascular occlusive diseases.

Despite major advances during the past years, a large discrepancy exists between the successful stimulation of arteriogenesis in experimental animal models and the disappointing outcome of recent clinical trials (van Royen 2005). For example, in the past decade, cardiovascular research particularly profited from knowledge gained from studying the possible inhibition of tumor angiogenesis (*de novo* formation of capillaries). Tumors with high rates of angiogenesis grow faster and usually become larger than those with low capillary density. The first clinical trials, therefore, were focused on influencing angiogenesis in cancer patients to inhibit further tumor growth or metastases by preventing angiogenesis and thus microcirculation in the tumor tissue. In ischemic vessel disease, the opposite of anti-angiogenesis is required. The hypoxia-driven sprouting of capillaries observed

in angiogenesis is fundamentally different from mechanical forces-mediated beneficial arteriogenesis in cardiovascular diseases (Grundmann 2007). The incorrect assumption of an equal efficacy of angiogenesis and arteriogenesis is a likely explanation for the negative outcome of the first clinical trials with the objective to stimulate collateral artery formation in cardiovascular disease (Heil 2006). Blood flow to the regions distal to the site of occlusion is limited and oxygen supply is decreased. Therefore, the insights from anti-angiogenesis strategies were first used to enhance microcirculatory blood flow in the ischemic regions and improve clinical outcome of patients with stenotic vessel disease by promoting angiogenesis. Although not all turned out positive, various placebo-controlled trials have been initiated in recent years, indicating the potential of VEGF and FGFs to stimulate therapeutic angiogenesis (e.g. TRAFFIC, AGENT, VIVA), (Grines 2002; Lederman 2002; Simons 2002; Henry 2003). However, the focus on the capillary sprouting instead of stimulating growth of functional collateral arteries resulted in the negative outcome of therapeutic treatment of occlusive vascular disease.

Intensive research on the basic mechanism of arteriogenesis is progressing towards first clinical trials with pro-arteriogenic compounds (Seiler 2001; Simons 2002; van Royen 2005) which by and large might facilitate arteriogenesis but would not induce it. Moreover, the processes regulating arterial enlargement are far from being understood and, especially, the translation from basic research to clinical practice remains challenging.

The purpose of the present work was to delineate mechanisms responsible for the onset of arteriogenesis, particularly the mechanism of mechanosensitive expression of MCP-1 and its regulation of expression during this process. Moreover, the role of AP-1 activation was evaluated for the first time in the context of arteriogenesis.

5.1. *In vivo* models of arteriogenesis - development of experimental tools

Several animal models have been developed to outline factors that promote arteriogenesis (Scholz 2001). The first detailed studies of growing collateral vessels were performed in dog hearts, where the initial level of collateral development

seemed to predict the final development of collateral vessels after repetitive coronary occlusion (Yamamoto 1984). The events of collateral vessel growth observed in pig hearts are somewhat less clear than those in dogs. Due to their smaller size pre-existing porcine coronary collateral vessels are more difficult to define and to study serially (White 1992). In rabbit hindlimbs, pre-existing collateral arterioles have been identified in the thigh, and their transformation into collateral arteries upon artery occlusion is very similar to the growth process of canine coronary collateral vessels including neointima formation (Scholz 2000). In another model of arteriogenesis in rats, a pronounced remodelling of pre-existing collateral thigh arterioles was observed within 7 days of femoral artery occlusion with subsequent neointima formation during the first three weeks (Hoefer 2001). And finally, in the mouse hindlimb, the growth of collateral arterial anastamoses observed after femoral artery ligation seems to be based on pre-existing arteriolar connections that remodel into larger vessels with one to two layers of vascular smooth muscle cells forming the *tunica media* (Scholz 2002).
Although differing in the spatiotemporal progression of the remodelling process, the common feature of these models is that the acute occlusion of a main feeding artery is employed to increase the blood flow in the collateral arterioles (Schaper 2003). And despite the anatomical differences in these models, the primary importance of pre-existing collateral vessels for the natural revascularization process does not seem to be species-specific. The abrupt occlusion of a larger feeding artery, on the other hand, is one of the biggest hurdles of arteriogenesis research today – especially in terms of translational research – since in humans a relevant stenosis in a larger artery leading to compensation arteriogenesis, develops rather slowly over time.

Nonetheless, in the present study a mouse ear artery ligation model (North 1958) was adapted to analyze the arteriogenic remodelling of collateral arterioles, thereby exploiting the easy accessibility and simplicity of the nearly two-dimensional architecture of the ear vasculature. Occlusion of a distinct first order ear artery up-regulated the expression of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) in the remodelling arterioles which could be identified by their corkscrew-like morphology after four to seven

days. Moreover, the classical mouse hindlimb ischemia model was used for comparison, in which an increased expression of ICAM-1 and MCP-1 was also observed 7 days after femoral artery occlusion. These findings correspond to earlier results obtained by *Lee et al.* (Lee 2004), where transcriptional profiling of the nonischemic adductor muscle in the thigh after artery excision had revealed an increased expression of MCP-1. They also resemble previously published observations made already in 1958 by *North et al.* that division of a blood vessel in the mouse ear is followed by a progressive enlargement of its collateral vessels (North 1958).

5.2. MCP-1 orchestrates early steps in arteriogenesis

Due to the predictability of collateral arteriolar enlargement in the mouse hindlimb, MCP-1 expression was analyzed already 24 hours after femoral artery occlusion. The observed significant up-regulation of MCP-1 expression during this period suggests that this chemokine may orchestrate rather early steps in the pro-arteriogenic cascade. The release of MCP-1 is a prerequisite for the recruitment of monocytes to the arteriolar vessel wall which in turn initiate its transformation to a small conductance artery (Scholz 2000). After occlusion of an artery activated vascular cells of the collateral arterioles express MCP-1 and transfer it to their luminal cell surface where it is immobilized by proteoglycans (Heil 2007). Changes in the expression and conformation of adhesion molecules transform the collateral endothelium from a quiescent vessel layer into a “sticky” surface, now supporting attraction, adhesion and invasion of leukocytes. Consequently, expression of selectins, ICAM-1 and -2, and vascular cell adhesion molecules (VCAM-1) is increased (Scholz 2000). In addition, adhesion molecules undergo a fast conformational change and are clustered in so-called focal adhesion complexes.
Attracted by MCP-1 and probably other chemoattractants, the monocyte binding to the collateral surface is mediated by intergrin receptors like Mac-1 and LFA-1 – counterparts of endothelial ICAM-1, -2 and VCAM-1. Monocytes, which mature to macrophages after entering the collateral vessel wall, have a central function in the induction of proliferation of vascular cells as well as in vascular cell remodelling. They function as micro-bioreactors and potently express proteases like matrix-metalloproteinases and urokinase-type plasminogen activator (uPA)

(Kusch 2002; Menshikov 2002) that weaken the cellular matrix. It was also observed that monocytes invade deeper layers of the collateral arteriolar wall and accumulate in perivascular clusters.

Correspondingly, it has been shown earlier that local infusion of MCP-1 with the aid of an osmotic minipump into the proximal stump of an occluded femoral artery in rabbits markedly enhanced the recruitment of monocytes and the speed of arteriogenesis. In comparison to other growth factors, MCP-1 is the strongest pro-arteriogenic peptide known (Ito 1997). In line with this, collateral artery growth and reperfusion in the ischemic tissue was diminished in MCP-1 and CC chemokine receptor 2 (CCR2) deficient mice (Heil 2004; Voskuil 2004).

Interestingly, CCR2 - the primary receptor for MCP-1 – is not only expressed on monocytes but also on other leukocytes such as activated T-cells. This could mean that other leukocytes also recruited by this pathway which has been shown for lymphocytes are - similar to monocytes – leave the blood stream and localize in the vicinity of the growing arteriolar collaterals. Likewise, *Stabile et al.* (Stabile 2003) concluded that T-cells contribute to arteriogenesis by releasing chemoattractive factors, hence supporting monocyte recruitment and supporting their paracrine activity during arteriogenesis.

5.3. Expression of MCP-1 is restricted mainly to smooth muscle cells

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, a new experimental *in situ* model was established, where small second order branches of the mouse mesenteric artery were subjected to control (low flow) and pro-arteriogenic (high flow) conditions. Despite the common notion of MCP-1 being expressed in activated endothelial cells in the collateral arteriolar vessel wall during the onset of arteriogenesis (Scholz 2001), here the smooth muscle cells of the media were identified as the main source of MCP-1.

Earlier studies were already pointing towards a localization of this chemokine to the smooth muscle cell layer of the remodelling collateral arterioles in the mouse hindlimb *in vivo*. *Jost et al.* observed that in mice treated with GM-CSF after ligation of the femoral artery expression of MCP-1 in the hindlimbs was restricted to cells constituting the vessel wall (media) after 3 days (Jost 2003).

Next, the question was addressed as to what triggers the expression of MCP-1 in the smooth muscle cells of the collateral arterioles undergoing remodelling. In this context, hemodynamic forces were characterized to be important for triggering arteriogenesis. Particularly, fluid shear stress has been intensively discussed as a molding force that probably initiates the cascade of events (Pipp 2004). It acts mainly on the endothelium which cushions the effect on the underlying smooth muscle cells that are further shielded by the internal elastic lamina. The related primarily but not exclusively force acting on the smooth muscle cell layer is circumferential wall strain (Schaper 1967; Scheel 1979). Its magnitude is much higher than that of fluid shear stress, and its increase in arteriogenesis is due to the initial volume-mediated distention (by Laplace's law: dP = T/R, dP - transmural pressure difference, T- wall tension, R - and radius) and later, with the onset of active remodelling, by the thinning of the vessel wall (Scholz 2001). Proliferation of the smooth muscle cells normalizes circumferential wall strain again and probably contributes to the premature halt of the arteriogenic process (Scheel 1979).

In this context, the observations made in this study suggest that of the two putative biomechanical determinants of arteriogenesis, FSS and CWS (Schaper 2003; Heil 2004), the latter most likely is responsible for the increase in MCP-1 expression, since changes in fluid shear stress can only be directly sensed by endothelial cells and an increase in fluid shear stress inhibited rather than augmented MCP-1 expression in both mouse and human endothelial cells.

5.4. MCP-1 expression *in vitro* is induced only by cyclic stretch

Further, the individual effects of laminar FSS and CWS on the expression of MCP-1 were investigated in cultured cells. Exposing cultured endothelial cells to shear stress resulted in a decrease rather than an increase in MCP-1 expression both on the mRNA and protein level. This finding is in agreement with previous studies demonstrating that endothelial cell MCP-1 expression is in fact down-regulated in response to shear stress over a similar period (Shyy 1994). Likewise, up-regulation of expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) critical for arteriogenesis has been shown to be inhibited in endothelial cells in response to laminar shear stress (Chiu 2003).

In contrast, exposure of cultured cells to cyclic stretch – a substitute of circumferential wall strain - resulted in a robust increase in MCP-1 expression both in cultured endothelial and smooth muscle cells. Moreover, the increase in MCP-1 abundance in the supernatant of vascular smooth muscle cells was particularly striking. These results are in line with reports showing that cyclic stretch induces MCP-1 expression in cultured human endothelial cells (Okada 1998), and extend this finding also to vascular smooth muscle cells. On the other hand, smooth muscle cells are also shown to be capable of up-regulating MCP-1 in response to certain pro-inflammatory cytokines (Cattaruzza 2002).

Furthermore, the data support the hypothesis that cyclic or static stretch, as the result of an increase in circumferential wall strain, plays a decisive role in the expression of MCP-1 in the collateral arteriolar wall during the early phase of arteriogenesis. Conversely, the observations of this study question the view of arteriogenesis as a predominantly shear stress-mediated remodelling process.

5.5. Up-regulation of MCP-1 expression depends on AP-1 activation

As mentioned above, the mechanism by which MCP-1 expression is regulated during the onset of arteriogenesis it is not yet understood. Chemokine expression is regulated primarily at the level of gene transcription, although contributions by posttranscriptional mechanisms have also been reported (Villarete 1996; Martin 1997; Olszewska-Pazdrak 1998). Previously it has been shown that the promoters of many chemokine genes, including interleukin-8 (IL-8), chemokine ligand 5 (CCL5 or RANTES), and MCP-1 contain binding sites for the redox-sensitive transcription factors activator protein-1 (AP-1) and nuclear factor-kB (NF-kB), both of which are thought to be involved in the transcriptional control of the aforementioned genes (Roebuck 1999).

AP-1 and NF-kB are inducible transcription factors critical for the expression of many genes involved in the inflammatory response, including cytokines and adhesion molecules (Baeuerle 1994). Activator protein-1 is one of the main transcription factors activated by either ERK or JNK and plays a central role in a variety of cellular responses (Shaulian 2001). For example, in a series of experiments in rats, it has been shown that MAP kinases and AP-1 are significantly

activated in the hypertrophied heart, the balloon-injured artery and hypertensive vascular or renal tissue (Kim 2003).

In the present study, the decoy oligodeoxynucleotide (dODN) technique (Mann 2005) was employed to inhibit AP-1 activity in the cultured vascular cells and in the perfused branches of the mouse mesenteric artery. These short double-stranded DNA sequences contain at least one consensus binding motif for AP-1 and even in the absence of surrounding DNA can bind the transcription factor in a highly specific manner (Dzau 2002). Taken together, the results revealed that stretch-induced MCP-1 expression in cultured smooth muscle cells is abolished in the presence of the AP-1 dODN. The same effect was observed in the branches of the mouse mesenteric artery perfused under pro-arteriogenic flow conditions. Moreover, these results are in line with previous reports where AP-1 has been shown to regulate the expression of many stress response genes, like connexin 43 and MCP-1, including those associated with a pro-inflammatory phenotype of endothelial or smooth muscle cells (Wung 1997; Wu 2007).

5.6. ROS formation is increased during arteriogenic remodelling

A class of highly diffusible and ubiquitous molecules, termed reactive oxygen species (ROS), has previously been shown to function as signalling molecules leading to changes in gene expression during inflammatory episodes (Suzuki 1997) moreover, they can act as second messengers in cells exposed to various stimuli such as cytokines (Lo 1996). ROS encompass species such as the superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radicals (Halliwell 1990). These highly reactive molecules are known to regulate many important cellular events, including gene expression (Lo 1995), transcription factor activation (Schreck 1991), DNA synthesis (Crawford 1988), and cell proliferation (Murrell 1990).

Moreover, ROS have been shown to regulate chemokine gene expression. Particularly *Satriano et al*. have demonstrated that ROS act as second messengers for cytokine-induced MCP-1 expression (Satriano 1993). Although this redox regulation of MCP-1 expression appears to be at the transcriptional level and involves redox-sensitive transcription factors such as AP-1 and NF-kB, the exact mechanisms are unknown. On the other hand, in most cases, the activation of AP-1 is triggered by an increase in the formation of ROS - another hallmark of vascular

stress responses (Shono 1996; Wung 1997), and inhibited by antioxidants such as the thiol compound *N*-acetyl cysteine (Pinkus 1996).

In the present study, a marked increase in ROS formation was observed in smooth muscle cells upon exposure to cyclic stretch as well as in the isolated small arteries perfused under pro-arteriogenic conditions. Moreover, an increased formation of ROS associated with collateral arterioles undergoing remodelling was detected in the mouse hindlimb after femoral artery ligation. These findings support the hypothesis that the arteriogenesis-associated up-regulation of MCP-1 expression in the arteriolar vessel wall most probably is triggered through a ROS – AP-1 pathway. This notion was verified by the finding that exposure of cultured smooth muscle cells to glucose oxidase lead to a rapid increase in MCP-1 mRNA confirming a role for the H_2O_2-generation in this context.

5.7. AP-1 dependent regulation of arteriogenesis

Collectively, the facts discussed so far suggest that in the early phase of arteriogenesis an increase in circumferential wall strain, hence stretch, triggers an increase in ROS formation in 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 to be highly valuable due to the fact that it allowed administration of the AP-1 dODN in a dermal formulation. Topical application of this ointment resulted in an excellent penetration of the dODN through the skin into the wall of the ear blood vessels within 24 hours after the application. The resulting neutralization of AP-1 not only abrogated the arteriogenic response in the mouse ear 7 days post artery ligation, but also prevented the increase in MCP-1 expression. Furthermore, the infiltration of monocytes into the wall of the remodelling collateral arterioles was significantly decreased. The specificity of the AP-1 dODN approach was confirmed by the lack of effect of a mutant control ODN. Moreover, a dODN directed against the potent pro-inflammatory transcription factor signal transducer and activator of transcription-1 (STAT-1) also had no effect on the remodelling of the collateral

arterioles after ligation of the main artery. Thus, the AP-1 dependent adaptive response occurring in the remodelling collateral arterioles must be different from the more potent and potentially chronic inflammatory reaction which transcription factors such as STAT-1 are typically involved with.

In contrast to many other forms of genetic manipulation both in cells *in vitro* and in intact tissues, transcription factor decoys have become a versatile tool for researches attempting both to define the role of various genes in normal and pathologic cell biology and to consider novel interventions. In just the past few years, for example, decoys have been studied in models of inflammation ranging from arthritis, sepsis, cerebral ischemia/reperfusion and transplant vasculopathy (NF-kappaB) to glomerulonephritis (E2F and AP-1), allergic airway reactivity and asthma (STAT-1, NF-kappaB) (Mann 2005). The most advanced clinical program involving the use of transcription factor decoys in human patients has been modification of the biology of venous bypass graft vasculopathy through intra-operative administration of an E2F dODN (Ehsan 2001).

In this study performed in the mouse ear artery ligation model, the application of dODNs in the dermal formulation not only showed a promising therapeutic approach, but also accentuated the pivotal role of AP-1 activation in the arteriogenic remodelling process.

5.8. An outlook

Even though MCP-1 is only one among many AP-1 target genes, the findings of this study corroborate that MCP-1 dependent monocyte recruitment is a rate-limiting step in arteriogenesis (Heil 2002). Moreover, they establish AP-1 as a critical modulator of MCP-1 expression in an as yet unrecognized context and point towards stretch-induced AP-1 dependent changes in gene expression in general as a decisive step in the initiation of arteriogenesis.

These results are in line with previous reports where such AP-1 dependent changes in gene expression have also been shown to play a role in hypertension-induced arterial remodelling, where the activation of mitogen-activated protein (MAP) kinases was followed by c-*fos* and *c-jun* gene expression, hence activation of AP-1 *in vivo* (Xu 1996). Moreover, accumulating evidence suggest that activation of AP-1

is a critical step in restenosis following angioplasty and stent placement (Buchwald 2002).

Therefore, a systematical comparison of the initial steps of these adaptive remodelling processes with that of arteriogenesis may ultimately lead to the identification of the main trigger of arteriogenesis and thus spur the development of a pro-arteriogenic therapy.

Increasing the concentration of circulating monocytes is one potential target for therapeutic arteriogenesis nowadays, as there is a direct correlation between the number of circulating monocytes and arteriogenic remodelling (Heil 2002). Another approach would be to locally raise the number of monocytes, and one promising factor for this would be MCP-1 (Hoefer 2006). However, even though administered downstream of the abdominal aorta (i.e. into the collateral arteries of a murine hindlimb), MCP-1 significantly increased atherosclerotic plaque formation in the aortas of apolipoprotein E deficient mice (van Royen 2003), diminishing the chance that this potent pro-arteriogenic compound will make its way into clinical application.

Other compounds, such as GM-CSF, have been shown to present anti-atherogenic properties as well as strong pro-arteriogenic actions. However, in the START trial *van Royen et al*. demonstrated that GM-CSF treatment does not have a beneficial effect in the patients with peripheral artery disease (van Royen 2005). For TGF, the effect of a pro-arteriogenic therapy on the underlying pathology, atherosclerosis, is not yet known and seems to be dose-dependent (Grundmann 2007). Previous studies indicate that TGF has at least a neutral, if not inhibiting effect on atherosclerotic plaque formation (Lutgens 2002).

The ideal candidate molecule should have a pro-arteriogenic and anti-atherosclerotic effect at the same time, stimulate collateral artery growth at any rate independent of the history of the patient and the affected vascular bed, and it should be easy to administer. Thus, by enhancing the current mechanistic knowledge in this field, the observations of this study may contribute to the understanding of a well-orchestrated and self-limiting arterial remodelling process that may eventually be exogenously induced for therapeutic purposes.

6. Summary

Occlusive vascular diseases represent the most frequent cause of death in industrialized nations. Treatment of the end-stage of these diseases such as myocardial infarction, stroke, peripheral artery disease and others is usually limited to interventions such as angioplasty, bypass surgery or limb amputation. An alternative approach to restore blood flow to the ischemic tissue can be the therapeutic induction and/or enhancement of the endogenous collateral circulation (arteriogenesis), bypassing the site of stenosis and protecting the downstream tissue from ischemic injury. Since biomechanical forces have been implicated in the initiation of arteriogenesis, their impact on the remodelling process during the onset of arteriogenesis and on the mechanosensitive expression of a pivotal pro-arteriogenic molecule, monocyte chemoattractant protein-1 (MCP-1), was investigated in the present study. MCP-1 governs the recruitment of circulating monocytes to the wall of the remodeling collateral arterioles – an essential step in the initiation of collateral artery formation.

Employing two different *in vivo* mouse models – the hindlimb ischemia model and a novel ear artery ligation model – revealed that MCP-1 expression is significantly increased in collateral arterioles undergoing arteriogenesis already 24 hours after its onset. To define the mechanism triggering MCP-1 expression, an *in situ* perfusion model of small mouse arteries was established, mimicking the pro-arteriogenic perfusion conditions. Subsequent gene expression analyses showed that MCP-1 expression is predominantly up-regulated in the smooth muscle cells of these arterial segments which solely sense changes in circumferential wall strain or stretch. Further analyses of cultured endothelial and smooth muscle cells confirmed that elevated levels of shear stress do not up-regulate MCP-1 expression in these cells while application of cyclic stretch to both cell types resulted in a robust increase in MCP-1 expression both on the mRNA and especially on the protein level.

Inhibition of the mechanosensitive transcription factor activator protein-1 (AP-1) by employing the decoy oligodeoxynucleotide (dODN) technique abolished the stretch-induced expression of MCP-1 in the cultured cells as well as in the isolated perfused segments of the mouse mesenteric artery.

Further analyses revealed an increased ROS formation in the cultured smooth muscle cells upon exposure to cyclic stretch as well as in the second order branches of mouse mesenteric artery perfused under pro-arteriogenic flow conditions *in situ*. Inhibition of ROS production by the ROS scavenger Idebenone abolished stretch-induced MCP-1 expression.

Finally, topical application of the AP-1 dODN to the mouse ear after arterial occlusion completely abrogated remodelling of the collateral arterioles through down-regulating MCP-1 expression and monocyte recruitment. Administration of a dODN against transcriptional factor signal transducer and activator of transcription-1 (STAT-1) in the same model did not affect the arteriogenic remodelling process pointing towards a more specific role of AP-1 signaling to the nucleus therein.

In summary, the present study establishes the ear artery ligation model of the mouse as a new experimental tool to investigate adaptive arteriolar remodelling processes *in vivo*. Furthermore, the data point towards a stretch-induced AP-1 mediated rise in MCP-1 expression in vascular smooth muscle cells as a critical determinant for the initiation of arteriogenesis. Conversely, the observations of this study question the view of arteriogenesis as a predominantly shear stress-mediated remodelling process and contribute to the deeper understanding of a well-orchestrated arterial remodelling process that may eventually be exogenously induced for therapeutic purposes.

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Curriculum Vitae

Name:	**Elena Demicheva**
Date of birth:	April, 13th, 1983
Place of birth:	Moscow, Russia
Nationality/ Citizenship:	Russia

EDUCATION

2004 – current	Ph.D. program in Biology at the University of Heidelberg. Presumably graduating in April, 2008. Institute of Physiology and Pathophysiology, Medical Faculty, University of Heidelberg, Heidelberg, Germany.
1999 - 2004	Diploma (equivalent to Master's degree) in Biology, specialization Biochemistry (with honours). Department of Biochemistry, Faculty of Biology, Moscow State University (MSU), Moscow, Russian Federation.
1989 - 1999	Karamzin Classic Gymnasium Certificate with extensive knowledge in humanities. Moscow, Russian Federation.