Therapeutic arteriogenesis: from experimental observations towards clinical application [cum laude]
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LOCAL MONOCYTE CHEMOATTRACTANT PROTEIN-1 THERAPY INCREASES COLLATERAL ARTERY FORMATION IN APOLIPOPROTEIN-E DEFICIENT MICE BUT INDUCES SYSTEMIC MONOCYTIC CD11B EXPRESSION, NEOINTIMA FORMATION AND PLAQUE PROGRESSION

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Abstract

Background: Monocyte Chemoattractant Protein-1 (MCP-1) stimulates the formation of a collateral circulation upon arterial occlusion. The present study served to determine whether these pro-arteriogenic properties of MCP-1 are preserved in hyperlipidemic ApoE -/- mice and whether it affects systemic development of atherosclerosis.

Methods and Results: A total of 78 ApoE -/- mice was treated with local infusion of low dose MCP-1 (1 μg/kg/week), high dose MCP-1 (10 μg/kg/week) or Phosphate Buffered Saline (PBS) as a control after unilateral ligation of the femoral artery. Collateral hindlimb flow, measured with fluorescent microspheres, significantly increased upon a one-week high dose MCP-1 treatment (PBS: 22.6%±7.2%, MCP-1: 31.3%±10.3%, p<0.05). These effects were still present two months after the treatment (PBS: 44.3%±4.6%, MCP-1: 56.5%±10.4%, p<0.001). The increase in collateral flow was accompanied by an increase in the number of perivascular monocytes/macrophages upon MCP-1 treatment. However, systemic CD11b expression by monocytes also increased, as well as monocyte adhesion at the aortic endothelium and neointima formation (PBS: 0.097±0.011 vs. MCP-1: 0.257±0.022, intima/media ratio, p<0.0001). Moreover, Sudan IV staining revealed an increase in aortic atherosclerotic plaque surface (PBS: 24.3%±5.2% vs. MCP-1: 38.2%±9.5%, p<0.01). Finally, a significant decrease in the percentage of smooth muscle cells was found in plaques (control: 15.0%±5.2% vs. 5.8%±2.3%, p<0.0001).

Conclusions: Local infusion of MCP-1 significantly increases collateral flow upon femoral artery ligation in ApoE -/- mice up to two months after the treatment. The local treatment however did not preclude systemic effects on atherogenesis, leading to increased atherosclerotic plaque formation and changes in cellular content of plaques.
The present paper was accompanied by a cover picture on the February 7, 2003 issue of Circulation Research. Monocytes are shown, accumulating around a collateral artery.
Arteriogenesis refers to the outgrowth of preexisting collateral arteriolar connections into large conductance arteries. Due to the high capacity of these vessels as compared to the capillary networks that are formed during angiogenesis, arteriogenesis is believed to be the most efficient form of vessel growth to restore tissue perfusion upon arterial occlusion. Thus, pro-arteriogenic substances potentially can be utilized to increase the capacity of collateral arteries in patients suffering from arterial obstructive disease, thereby alleviating symptoms of claudication intermittens or angina pectoris.

The large majority of arterial obstructions are caused by atherosclerotic disease and thus substances that are envisioned to be of use for therapeutic purposes in this population should be tested for their 'neglected, potential serious side-effects' on atherosclerosis. For substances that stimulate angiogenesis this is of particular interest since the process of angiogenesis is directly involved in atherosclerotic plaque progression. Although arteriogenesis as a process is not directly involved in atherogenesis, several pathophysiological entities are displayed by both arteriogenesis as well as atherogenesis like monocyte infiltration and increased expression of certain growth factors and cytokines. MCP-1 is a known pro-arteriogenic factor, accelerating significantly the formation of collateral arteries upon arterial occlusion in rabbits. The pro-atherogenic properties of MCP-1 have been subject of many studies and it has been shown that overexpression of MCP-1 directly at the vessel wall leads to an increased macrophage infiltration and neointima formation.

Local and intravascular delivery of angiogenic/arteriogenic proteins most efficiently restores perfusion upon arterial obstruction. Moreover, such local application limits systemic side-effects and maximizes selectivity. However, even local application might lead to systemic side-effects and therefore the objective of the current study was to determine whether a local protein infusion of MCP-1 directly into the hindlimb collateral circulation, in a dosage that significantly stimulates arteriogenesis, promotes atherosclerosis systemically.

Apolipoprotein E-deficient mice (ApoE -/-) show high levels of serum lipids and formation of atherosclerotic plaques, similar to human atherosclerotic plaques. In a newly developed mouse model we delivered the MCP-1 protein directly into the hindlimb collateral circulation of ApoE knockout mice. This enabled us to test in one model simultaneously the pro-arteriogenic effects of local MCP-1 protein therapy under hyperlipidemic conditions as well as the systemic negative side-effects on atherosclerosis.

Methods

Animals
A total of 50 ApoE -/- mice of 8 weeks (N10, backcrossed onto C57/B16, Jackson Laboratory, Bar Harbor, Maine, USA) as well as 28 ApoE -/- mice of 6 months in age were used after securing the appropriate institutional approval conforming with the Guide for the Care and Use of Laboratory Animals published by the US
Figure 1: Flow ratios after femoral artery ligation in control and MCP-1 high-dose treated animals. Collateral flow increases upon MCP-1 treatment both at day 7 (PBS: 22.6 ± 7.2%, MCP-1 high: 31.3 ± 10.3%, p<0.05) as well as 2 months after ligation (PBS: 44.3 ± 4.6%, MCP-1 high: 56.5 ± 10.4%, p<0.001).

Figure 3: Monocytes adhere to the aortic endothelium, already 3 days after initiation of MCP-1 treatment (PBS: 12.4 ± 4.0 vs. MCP-1: 20.1 ± 9.9 monocytes/mm endoluminal wall, p<0.001). Two months after the MCP-1 treatment this effect is still present (PBS: 16.5 ± 5.6 vs. MCP-1: 41.7 ± 9.8 monocytes/mm endoluminal wall, p<0.0001).
Figure 2: Accumulation of monocytes/macrophages around collateral arteries of different sizes of animals treated with either PBS (A,C,E) or MCP-1 (B,D,F). MOMA-2 is used to detect monocytes/macrophages (red), alpha-smooth muscle actin is labelled green and nuclei are labelled blue with Hoechst 33342. Upon MCP-1 treatment a strong increase is observed in perivascular monocytes/macrophages (white arrows) (PBS: 26.9 ± 19.3, MCP-1 high: 64.2 ± 37.1, MOMA-2-positive cells per square, p<0.01).
Figure 4: Increased neointima formation in MCP-1 treated animals (control: 0.097 ± 0.011 vs. MCP-1: 0.257 ± 0.022, intima/media ratio, p<0.0001). Black arrows indicate regions of neointima formation.

Figure 5. Monocytic infiltration in aortic atherosclerotic plaques. CD11b is used to detect monocytes/macrophages (red-yellow), and nuclei are labelled blue with Hoechst 33342. Strong mononuclear cell invasion of pre-existing atherosclerotic plaques and aortic wall is observed, directly following the one-week MCP-1 treatment (panels B and D).
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National Institute of Health (NIH Publication No. 85-23, revised 1996). The 8-week old mice were treated with either low dose MCP-1 (1 μg/kg/week, n=8), high dose MCP-1 (10 μg/kg/week, n=21) or PBS as a control substance (n=21). Measurements were performed either at day 3 during treatment (MCP-1 high: n=5, PBS: n=5), directly after the 1 week treatment (MCP-1 low: n=8, MCP-1 high: n=8, PBS: n=8) or 2 months after treatment (MCP-1 high: n=8, PBS: n=8). The 6 month old mice were divided into 2 groups (MCP-1 high dose, n=14; PBS, n=14) and were analyzed directly following treatment (MCP-1 high: n=3, PBS: n=3) or after a 2 month period (MCP-1 high: n=11, PBS: n=11). The treatment period with either PBS or MCP-1 was one week for all animals in all groups. The one-week treatment period was ensured by using osmotic minipumps (Alzet, 1007D, Alza Corp., Palo Alto, USA), especially designed for delivery of content over 7 days.

Animal microsurgery

Animals were anesthetized and the femoral artery was dissected free under a stereoscopic microscope (Leica MZ6, Leica, Bensheim, FRG). A small incision was made in the femoral artery, distal from the arteria profunda femoris, and a catheter (inner diameter 0.28 mm; outer diameter 0.61 mm) was inserted into the proximal stump of the femoral artery with the tip of the catheter pointing upstream. The catheter was then secured with two ligations around the femoral artery, thereby also obstructing completely femoral artery flow. Before introducing the catheter it was connected to the osmotic micropump for local delivery of either MCP-1 or PBS over a one-week period. Micropumps were then secured under the skin.

Collateral flow measurements

Collateral flow measurements were performed as previously described. In summary: a catheter was inserted in the abdominal aorta with the tip just proximal to the aortic bifurcation. Both hindlimbs were then perfused at 4 different pressure levels with differently labeled microspheres. Adequate mixing of the microspheres was ensured by vortexing for 30 seconds, shortly before injection. After performance of all 4 infusions, animals were sacrificed and distal hindlimb muscles (i.e. gastrocnemius and peroneus muscles) were dissected. Tissue was digested and homogenized and the number of accumulated microspheres in both hindlimbs was counted using flow cytometry (Beckman Coulter, Epics XL-MCL, Germany). Restoration of flow was then expressed as a percentage, derived from the ratio between the flows in the occluded versus the non-occluded hindlimb.

Serum measurements

A total of 1 ml blood was withdrawn from each animal, shortly before sacrifice. Triglycerides, total cholesterol, VLDL, LDL, HDL and CRP were measured enzymatically in serum using standard protocols.
FACS-analysis of CD11b expression on monocytes

CD11b expression on circulating monocytes was determined using FACS analysis (Epics XL-MCL, Coulter, Miami, FL, USA). Therefore 0.3 ml blood was withdrawn from the left ventricle and stained with an RPE-labeled F4/80 marker (Caltag, Burlingame, CA, USA) as well as a FITC-labeled CD11b antibody (Serotec, Oxford, UK) using standard protocols for whole blood staining. Monocytes were identified based on scatter properties and positive staining for F4/80. CD11b expression by monocytes was expressed as fluorescence intensity (arbitrary units).

Immunohistochemistry and atherosclerotic lesion size quantification

Aortas of 8 week old mice were harvested, stored at -80°C and 5 μm sections of the ascending aorta were placed on cationic coated slides (Superfrost Plus, MJ Research, Waltham, USA). For all histological examinations a total of five slides per animal was analyzed, with 50 μm distance between the samples. All quantitative analyses were performed by two blinded observers. A mouse-specific marker for CD11b (Serotec, Oxford, UK) with FITC as a secondary antibody (Southern Biotechnologies, Birmingham, AL, USA) was used in order to detect monocytes. The number of adhering monocytes was quantified at a magnification of 400X at either day 3, day 7 or after 2 months. Therefore, the total number of monocytes per aortic ring was counted visually and endoluminal wall length was measured using Qfluoro software (Leica, Wetzlar, FRG). Data were then expressed as monocytes/mm endoluminal wall in order to correct for different aortic diameters and cutting angles. To quantify neointima formation, photographs were taken of the complete aortic ring at the level of the ascending aorta (7-9 photographs for each aortic ring, 5 rings per animal) at a magnification of 400X with a Leica DC 300F digital camera (Leica, Wetzlar, FRG). Neointima was then quantified planimetrically using ImageJ software (available on the internet as shareware, http://rsb.info.nih.gov/ij/). Measurements of adhering monocytes and neointima formation were performed on tissues derived from the 8 week old ApoE -/- mice. Aortas from 6 month old animals, sacrificed 2 months after femoral artery ligation were dissected and immersed in formalin 4% and stained with Sudan IV for detection of atherosclerotic plaques (PBS-treated n=6, MCP-treated n=6). Stained aortas were then photographed with a digital camera (Coolpix 900, Nikon, Tokyo, Japan) and the percentage of atherosclerotic surface compared to total aortic surface was calculated planimetrically.

In order to determine cellular content, plaques from the descending aortas of the remaining animals (PBS-treated n=5, MCP-treated n=5) were stained for monocytes/macrophages (CD11b, see above), lymphocytes (FITC-labeled mouse-specific CD3 marker, Serotec, Oxford, UK) and smooth muscle cells (FITC-labeled anti-human alpha smooth muscle marker with cross-reactivity for mouse tissue, Sigma, St. Louis, Missouri, USA). Nuclear staining was performed with Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA). Negative controls were performed for all immunological stainings by omission of the primary antibody.
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Figure 6: Sudan IV staining of aortas of 6 month old ApoE mice (A: PBS, B: MCP-1 high dose). The treatment with high dose MCP-1 leads to an increased percentage of atherosclerotic plaque surface in total aortas, 2 months after initiation of the treatment as shown in panel C (PBS 24.3% ± 5.2% vs. MCP-1 38.2% ± 9.5%, p<0.01).

Figure 7: Representative pictures of immunohistological staining for alpha smooth muscle actin (green). Panels A and C are derived from a PBS treated control animal. It is shown clearly that in the MCP-1 treated animals, as shown in panels B and D the percentage of smooth muscle cells in the plaque region has decreased. As shown in panel E, this difference was found to be statistically significant (PBS: 15.0% ± 5.2% vs. MCP-1: 5.8% ± 2.3%, p<0.0001).
Six additional mice of 6 months old were treated with either high-dose MCP-1 or PBS and tissue was harvested directly following the one-week treatment period, in order to detect the influence of MCP-1 on monocyte infiltration into aortic plaques and hindlimb tissue directly following treatment. Monocyte infiltration into plaques was performed as described above. Monocytes/macrophages around collateral vessels in hindlimb tissue (quadriceps and adductor muscles) were detected with the use of a mouse monocyte/macrophage specific monoclonal antibody against MOMA-2 (BMA Biomedicals, Augst, Switzerland) and Cy3 (DPC Bierrmann, Bad Nauheim, Germany) as a secondary antibody. In addition, tissue was stained with the above mentioned antibody against smooth muscle cells in order to ensure the arterial aspect of selected vessels. Photomicrographs were taken with a 400X magnification, and the number of monocytes/macrophages was counted in predefined squares of 273 μm X 345 μm around muscular collateral arteries. Moreover, monocytes/macrophages were expressed as a percentage of total cell population in the predefined squares.

**Results**

**Collateral flow measurements**

In the 8-week old ApoE mice, collateral flow did not increase in the low dose MCP-1 treated animals as compared to control animals, seven days after femoral artery ligation (PBS: 22.6%±7.2%, MCP-1 low: 20.3%±4.3%, p=ns).

When comparing the high dose MCP-1 group with the control animals no statistical significant difference was observed at day 3 (PBS: 10.2%±2.7%, MCP-1 high: 11.8%±3.3%). However at day 7 after femoral artery ligation a significant difference could be observed between treated and control animals (PBS: 22.6%±7.2%, MCP-1 high: 31.3%±10.3%, p<0.05). Two months after the ligation and the one-week treatment the difference in collateral conductance between the MCP-1 treated and the control animals was maintained (PBS: 44.3%±4.6%, MCP-1 high: 56.5%±10.4%, p<0.001). This increase in collateral flow was accompanied by an increased number of monocytes/macrophages around muscular arteries in the quadriceps and adductor muscles of the ligated leg (PBS: 26.9%±19.3, MCP-1 high: 64.2±37.1, p<0.01, figure 1). Also when expressed as a percentage of total cells around collateral vessels, an increase of monocytes/macrophages was detected in MCP-1 treated animals as compared to control animals (PBS: 19.0%±6.5%, MCP-1 high: 34.9%±9.8%, p<0.001).

**Serum measurements**

No increase in serum levels of CRP was found in any of the groups. High values for triglycerides, total cholesterol, VLDL and LDL as well as low levels of HDL were found in all groups. However, the treatment with MCP-1 had no influence on any of these values, either 7 days or 2 months after initiation of the one-week treatment (table 1).
**FACS-analysis of CD11b expression on monocytes**

The local infusion of high dose MCP-1, directly in the peripheral collateral circulation led to an increased expression of CD11b by circulating monocytes that were withdrawn from the left ventricle. Fluorescence intensity was 214.5±8.7 in the control mice and 256.7±11.4 (arbitrary units, p<0.001) in the high dose MCP-1 group. No increase in CD11b expression by circulating monocytes was detected in the low dose MCP-1 group (data not shown).

**Immunohistochemistry and atherosclerotic lesion size quantification**

Upon MCP-1 treatment of the 8 week old animals, an increase in endoluminal monocytes/macrophages in the ascending aorta was observed already at day 3 (PBS: 12.4±4.0, MCP-1: 20.1±9.9 monocytes/mm endoluminal vessel wall, p<0.001) as well as at day 7 (PBS: 13.7±3.1, MCP-1: 21.2±9.6 monocytes/mm, p<0.001). Two months after femoral artery ligation, the difference between the treated and the control group had further increased (PBS: 16.5±5.6, MCP-1: 41.7±9.8 monocytes/mm, p<0.0001), (figure 3). This was accompanied by an increased neointima formation in the MCP-1 treated animals (control: 0.09±0.011, MCP-1: 0.25±0.022, intima/media ratio, p<0.0001), (figure 4). In the 6 month old ApoE mice an increase in monocyte content of atherosclerotic plaques could be appreciated directly following MCP-1 treatment. Several plaques of MCP-1 treated animals consisted almost exclusively of monocytes/macrophages accompanied by aortic wall invasion of monocytes/macrophages. This was encountered solely in MCP-1 treated animals, whereas control animals showed normal cellular content of plaques, directly following the one-week PBS infusion (figure 5). In the 6 month old ApoE mice, treatment with high dose MCP-1 led to an increased percentage of atherosclerotic plaque surface in total aortas, 2 months after initiation of the treatment (PBS: 24.3±5.2%, MCP-1: 38.2±9.5%, p<0.01) (figure 6). This increased total plaque surface could be attributed almost completely to increased plaque formation in the thoracical and abdominal aorta (PBS: 14.8±5.1%, MCP-1: 30.9±7.8%, p<0.05), whereas plaque percentage in the aortic arch remained almost unchanged (PBS: 54.4±3.9%, MCP-1: 60.1±7.8%, p=NS). However, cellular content of plaques from the aortic arch was changed upon MCP-1 treatment. Two months after MCP-1 treatment a significant decrease in the percentage of smooth muscle cells was observed in MCP-1 treated animals (PBS: 15.0±5.2%, MCP-1: 5.8±2.3%, p<0.001). No significant change was found in either monocyte/macrophage or lymphocyte content of atherosclerotic plaques in the 6 month old animals, two months after treatment (figure 7).

**Discussion**

In a first step we have determined the dosage of MCP-1 required to induce arteriogenesis in ApoE mice. Our data show that the arteriogenic potency of MCP-1 is preserved under hyperlipidemic conditions in the ApoE-/- mice up to two months after ligation, at a dosage of 10 μg/kg/week. At the same time, using this dosage, a systemic increase in monocyteic CD11b expression was observed upon the local MCP-1 treatment. This was accompanied by an increased monocyte adhesion to the
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aortic endothelium and an increased neointima formation. Finally, in 6 month old animals the treatment with MCP-1 increased total plaque surface in the aorta as well as modulated the cellular composition of plaques towards a morphology that is potentially more prone to rupture.

The potential to form a collateral circulation upon arterial obstruction is distributed very heterogeneously among the population. Several factors influencing this potential have been identified in recent years like age or the presence of diabetes. Hyperlipidemia also negatively influences the formation of a collateral circulation and therefore we first determined the natural time-course of arteriogenesis in ApoE-/- mice and the additive pro-arteriogenic effects of MCP-1. The natural arteriogenic response in the PBS-treated control group restored flow to about 45% of normal. This seems to be in contradiction with the observed 60% restoration of flow as observed 5 weeks after femoral artery ligation as published previously. This difference most probably relates to the different methodological approaches. In contrast to that study we performed measurements of tissue perfusion using fluorescent microspheres instead of Laser Doppler fluxmetry. In a previous study we showed that Laser Doppler fluxmetry in the mice hindlimb model leads to overestimation of tissue perfusion as compared to microsphere-based measurements. This is in agreement with the observed differences between the present study and that of Couffinhal et al.

At a dosage of 1 µg/kg/week no increase in collateral flow was observed as compared to the control group. The dosage of 10 µg/kg/week significantly increased flow ratio by approximately 30%, 7 days after femoral artery ligation. Interestingly, this positive effect of the one-week treatment on collateral flow was still observed 2 months after initiation of the therapy showing an ongoing benefit of the treatment. The issue remains whether the beneficial effects of MCP-1 in the current model can be attributed to enlargement of pre-existing collateral vessels or the de novo formation of new arteries. The mouse model of femoral artery ligation as performed in the current study is designed to study arteriogenesis specifically. Therefore, the A. Profunda is left intact and the ligation is at a relative distal site of the femoral artery. Using this model, about 6 pre-existing collateral arterioles are readily recruitable and macroscopically visible. Immediately upon ligation these pre-existing vessels partially restore flow to jeopardized tissues as a natural escape mechanism from massive ischemic tissue damage upon acute femoral artery ligation. Over time, the lumen of these pre-existing vessels widens via active proliferation of vascular wall cells, thereby restoring flow to values of up to 45% of normal in the present model. Finally, when analyzed histologically, these arteries are always in close anatomic relation with veins and nerves. It can be postulated that arteries and nerves develop in a coordinated fashion as shown recently in a different experimental setting in embryonic mouse limb skin, however the simultaneous development of veins in the present model seems redundant since the femoral vein is left intact. Moreover, proliferation markers like KI-67 were only found to be positive in the arterial wall and not in accompanying veins or nerves (own observations). Taken together, in the current model the proliferation of pre-existing

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collateral vessels seems to be the dominating form of vessel growth, rather than de novo formation of collateral arteries. Having determined the pro-arteriogenic properties of both the low and the high MCP-1 dosage we then used the 10 μg/kg/week MCP-1 dosage for all further experiments, focusing on the pro-atherogenic properties of MCP-1. In recent years numerous pre-clinical as well as clinical studies were conducted in order to identify pro-angiogenic or pro-arteriogenic strategies, but only few addressed the possible negative side-effects of such therapies as was recently stressed by Epstein. Barger first proposed that angiogenesis is an integral part of atherosclerotic plaque formation. Subsequently, Folkman showed that the inhibition of angiogenesis via TNP-470 or endostatin diminishes plaque formation. Furthermore, basic Fibroblast Growth Factor (bFGF), either as gene or as protein therapy, induces neointimal hyperplasia as well as increased neovascularization of the intima in porcine arteries. More recently it was shown that the exogenous application of a low dose of the angiogenic factor VEGF strongly stimulates plaque formation in both cholesterol-fed rabbits and knock-out mice, doubly deficient in apolipoprotein E/apolipoprotein B100.

Arteriogenesis plays no direct role during atherogenesis. However, arteriogenesis and atherogenesis share numerous common features. Shear stress upregulates the expression of endothelial cell adhesion receptors like intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and this occurs during both atherogenesis and arteriogenesis. The subsequent monocyte/macrophage invasion plays a pivotal role in both arteriogenesis and atherogenesis. Other features shared by arteriogenesis and atherogenesis include smooth muscle cell mitosis and elastolysis. A direct role for MCP-1 in atherogenesis was suggested by two studies showing that a deficiency in either MCP-1 or its receptor CCR2 leads to diminished plaque formation in mice. In addition, in irradiated apoE-deficient mice that were repopulated with bone marrow cells from MCP-1 transgenic mice, the localized overexpression of MCP-1 by macrophages resulted in amplification of atherosclerosis. Finally, local overexpression of MCP-1 in the vessel wall of rabbits on a high cholesterol diet leads to a local increase in monocyte/macrophage infiltration as well as lesion formation.

In the present study we have shown for the first time that a local treatment with MCP-1, administered as a protein, affects clearly the several steps in systemic atherogenesis. First of all, we could show that the local treatment induced activation of circulating monocytes as measured by CD11b expression, even in the absence of detectable systemic MCP-1 levels as measured by ELISA (data not shown). Most probably, monocytes are activated by high local MCP-1 levels. Some of these activated monocytes will adhere to the endothelium of collateral arteries, increasing the arteriogenic response and explaining in part the beneficial effects of MCP-1 on the development of the collateral circulation. However, another fraction of the activated monocytes will recirculate and adhere to the endothelium at distant sites like the atherosclerotic-prone regions in the aortic arch. Indeed the increased expression of CD11b on circulating monocytes was accompanied by a strongly
increased amount of adhering monocytes in the aortic arch, both directly as well as 2 months after treatment. Moreover, monocyte infiltration in existing advanced lesions in 6 month old ApoE was observed, directly following the one-week treatment period. Two months after MCP-1 treatment an increase in intima/media ratio was observed as compared to the control animals, showing that local MCP-1 treatment did not result in a transient effect on monocyte adhesion but rather induced atherogenesis.

The positive correlation between increased CD11b expression by circulating monocytes, increased monocyte infiltration and the progression of atherosclerotic disease after MCP-1 treatment confirms data that were published recently, showing the reversed phenomenon after Leukotriene B4 receptor antagonism, leading to decreased CD11b expression of circulating monocytes, decreased monocyte infiltration and the reduction of lesion progression. It should be noted that CD11b expression was used in our study as a marker of monocyte activation. A direct role of CD11b in MCP-1 induced atherogenesis remains to be elucidated but seems less probable since it was shown recently that atherosclerosis develops normally in LDL-R −/− mice also when CD11b expression on leukocytes is absent, as was achieved in a chimera model using CD11b −/− bone marrow. It could still be postulated however that the induction of CD11b overexpression, as was the case in our study, does influence monocyte trafficking to atherosclerotic lesions directly and we hope to further unravel the exact mechanistic background of MCP-1 induced atherogenesis in future studies.

Interestingly, 2 months after MCP-1 treatment we also observed an increased expression of ICAM-1 on the aortic endothelium (data not shown). ICAM-1 is essential for monocyte adhesion to atherosclerosis prone regions and ICAM-1 upregulation is one of the earliest events occurring in atherogenesis. A correlation exists between ICAM-1 expression on the aortic endothelium and atherosclerotic disease progression in ApoE −/− mice. Since in our model increased ICAM expression on the aortic endothelium was only detected 2 months after MCP-1 treatment we postulate that this was also merely an indicator of more progressed atherosclerotic disease in treated animals rather than a direct effect of the MCP-1 treatment. This can also be concluded from the earlier mentioned ELISA data, showing no increase in circulating MCP-1 after treatment and excluding direct effects of MCP-1 on ICAM expression on aortic endothelium.

To test whether MCP-1 treatment led to increased lesion progression via induction of plaque neovascularization we performed a CD31 staining of plaques. Although neovascularization was present, especially in large-sized advanced lesions, no obvious difference between treated and non-treated animals could be detected when comparing size-matched plaques (data not shown).

The pro-atherogenic effects of MCP-1 treatment were further confirmed by the data from the 6 month old ApoE mice showing an increase in total plaque surface upon treatment. This increase in plaque surface of the whole aorta could be attributed mainly to increased plaque formation in the abdominal and the thoracic aorta whereas the plaque surface in the aortic arch remained unchanged. Cellular content
of plaques in the aortic arch did change though, leading to a 3-fold decrease in relative smooth muscle cell content of plaques upon MCP-1 treatment. It can be postulated that this decrease in smooth muscle cell content drives plaques towards a more rupture-prone form of atherosclerotic lesions.

Taken together, MCP-1 exerts a strong arteriogenic effect, even under hyperlipidemic conditions. The beneficial effects of MCP-1 were ongoing, still present 2 months after the treatment. The local treatment with MCP-1 however did not preclude negative systemic effects on atherogenesis. These pro-atherogenic properties of MCP-1 confirm earlier observations on the proatherogenic properties of the pro-angiogenic substances b-FGF and VEGF. Strategies need to be identified, focusing at either different dosage regimens in order to minimize the pro-atherogenic effects or the combination with other substances that might act in an anti-atherogenic fashion. Of interest in this regard is the recent identification of two other pro-arteriogenic substances, GM-CSF \cite{55,34} and TGF-β1 \cite{35}, that have also been reported to exert anti-atherogenic properties \cite{36-38}.

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