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EFFECTS OF LOCAL MCP-1 PROTEIN THERAPY ON THE DEVELOPMENT OF THE COLLATERAL CIRCULATION AND ATHEROSCLEROSIS IN WATANABE HYPERLIPIDEMIC RABBITS

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Abstract

**Background:** The objective of our study was to quantify the arteriogenic potency of Monocyte Chemoattractant Protein-1 (MCP-1) under hyperlipidemic conditions. Additionally, we aimed to determine the effects of locally applied MCP-1 on systemic serum lipid levels as well as on atherosclerosis.

**Methods and results:** A total of sixty-four Watanabe rabbits was treated with either low dose MCP-1 (1 μg/kg/week), high dose MCP-1 (3.3 μg/kg/week) or PBS as a control substance. Substances were applied directly into the collateral circulation via an osmotic minipump with the catheter placed in the proximal stump of the ligated femoral artery. Either one week or six months after initiation of the treatment, X-ray angiography was performed as well as measurements of collateral conductance using fluorescent microspheres. The extent of atherosclerosis was quantified in whole aortas using Sudan IV staining. One week after ligation of the femoral artery a significant increase in collateral conductance was observed in animals treated with high dose MCP-1 (control: 2.2 ± 0.8 ml/min/100 mmHg vs. MCP-1 high dose: 8.9 ± 2.0 ml/min/100 mmHg, p<0.05). Six months after femoral artery ligation no differences were found between the treated and the control group (PBS: 44.9 ± 11.6 ml/min/100 mmHg, MCP-1: 47.8 ± 11.5 ml/min/100 mmHg, p=NS). No influence was found on serum lipids or on the development of atherosclerosis in the present model.

**Conclusions:** MCP-1 accelerates arteriogenesis upon femoral artery ligation under hyperlipidemic conditions. Six months after treatment these pro-arteriogenic effects of MCP-1 can no longer be observed. The present data do not show an effect of local MCP-1 treatment on serum lipids or on atherosclerosis. It should be noted however that a high standard deviation was observed for the data on atherosclerotic surface area, necessitating additional experiments in a different model of atherosclerosis.
Introduction

Monocyte Chemoattractant Protein 1 (MCP-1) significantly increases the process of arteriogenesis when locally applied in a rabbit hindlimb model of femoral artery occlusion. The arteriogenic action of MCP-1 is thought to be based on an increased number of attracted monocytes to the place of interest and this factor holds promise as a new therapeutic modality to treat peripheral and coronary obstructive arterial disease.

However, it is not known whether the arteriogenic properties of MCP-1 are preserved in a hyperlipidemic environment. Moreover, arteriogenesis and atherogenesis display several similarities like monocyte infiltration, increased expression of growth factors and cytokines and smooth muscle cell mitosis.

Therefore, a possible limitation to every arteriogenic therapy is the acceleration or initiation of atherosclerotic lesion development. This is an even more realistic potential side-effect when using MCP-1 as arteriogenic substance, since this cytokine is believed to be directly involved in the initiation of atherogenesis.

The Watanabe Heritable Hyper-Lipidemic (WHHL) rabbit possesses an inheritable deficiency of LDL receptors, similar to familial hypercholesterolemia in humans, leading to high LDL plasma levels and early development of atherosclerosis. We used this animal model to study the short- and long-term effects of locally applied MCP-1 on both atherogenesis and arteriogenesis under hyperlipidemic conditions. Additionally, we determined the effects of MCP-1 treatment on serum lipid levels.

Methods

WHHL rabbit model

A total of sixty-four WHHL rabbits were used for the current study after securing the appropriate institutional approval conforming with 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). Animals were anaesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazin (8 mg/kg). A skin incision of approximately 3 cm was performed to visualize and ligate the right femoral artery. A catheter with the tip being placed upstream in the proximal stump of the occluded artery was connected to an osmotic minipump (Alzet, 2ML1, Alza Corp., Palo Alto, USA). Thereby the substance was directly delivered into the collateral circulation for a defined time period of one week. The absence of any residual volume in the minipumps (<3%) after the experiment verified delivery of the contents. 12 animals were treated with a total MCP-1 dose of 1 μg/kg (low dose), 24 animals were treated with a total MCP-1 dose of 3.3 μg/kg (high dose) and 24 animals received Phosphate Buffered Saline (PBS) as a control substance. Animals were then divided in six groups. Group I consisted of 12 animals treated with low dose MCP-1 and analyzed after one week, group II consisted of 12 animals treated with high dose MCP-1 and analyzed after one week,
group III consisted of 12 animals treated with PBS and analyzed after one week, group IV consisted of 12 animals treated with high dose MCP-1 and analyzed after six months and group V consisted of 12 animals treated with PBS and analyzed after six months. The sixth group consisted of 4 non-operated animals that were used to obtain normal values of collateral conductance in WHHL rabbits. Age of the animals at the time of the operation was either 6 months or 1 year, divided over the groups in such a way that for the final experiment each animal was around 1 year. Animals weighed between 2.4 and 3.3 kg.

 Serum levels of lipids
A total of 2 ml blood was withdrawn from the ear vein shortly before sacrifice. Triglycerides, total cholesterol, VLDL, LDL and HDL were then measured enzymatically in serum.

 X-ray angiography
From group 1 to V a total of 6 animals per group was randomly selected for X-ray angiography. After sacrifice of the animals a canula was inserted into the abdominal aorta and a bismuth/gelatine contrast was slowly infused under constant pressure of 80 mmHg. Animals were then put on ice for 2 hours. The angiogram was performed with a Balteau chamber (30 kV, 1.45 minutes). Of each animal, 2 angiograms were performed at different angles. The number of collateral arteries was then counted using stereoscopic imaging. Only collateral arteries with a clearly identifiable stem, mid-zone and re-entry region were counted, according to the Longland criteria. The visualization threshold of these angiograms is approximately 50 μm.

 Collateral conductance measurements
One week (MCP-1 low dose: n=6, MCP-1 high dose: n=6, control: n=6) or 6 months (MCP-1 high dose: n=6, control: n=6) after femoral artery ligation, measurements of collateral conductance were performed as previously described. In short: the left and right arteria saphena magna were canulated with sterile polyethylene heparinized catheters for peripheral pressure measurements. The left femoral artery was canulated for microsphere reference sampling. A pump-driven shunt, installed in the abdominal aorta, ensured oxygenated blood flow from the carotid artery in the abdominal aorta into the right and left hindlimbs. Additionally, donor blood was used from animals that were selected for X-ray angiography. A three-way stopcock was attached to the aortic canula in order to measure pressure at the level of the abdominal aorta, distal from the shunt. An in-line flow probe (Transonics, Ithaca, NY, USA) was installed in the shunt system for measurements of total flow to both hindlimbs. Maximal vasodilatation was achieved by injecting adenosine to the shunt at a constant rate of 1 mg/kg/min. After stabilization of peripheral and central pressures,
both legs were perfused via the aortic canula at 6 different pressures. The 6
perfusion pressure levels were generated in vivo with a roller pump, installed in line
with the shunt between carotid artery and abdominal aorta. For each pressure level,
15 μm microspheres (Molecular Probes, Eugene, Oregon, USA) with each a
different fluorescent color were injected into the mixing chamber, that was installed
in the carotid-abdominal aortic shunt system. For each colour a total of 2 million
microspheres was injected, ensuring a large enough amount of microspheres in the
individual tissue samples. The amount of microspheres in hindlimb tissue was then
quantified using FACS-analysis (Becton Dickinson & Co, Lincoln Park, NJ, USA).
Flows for each hindlimb sample were then calculated from the number of
microspheres in the tissue, the microsphere count in the reference sample, the
internal standard in the sample, the internal standard in the reference sample, the
weight of the reference sample and the time-interval in which the reference sample
is withdrawn. For each pressure step the difference between peripheral and central
pressure was calculated. Collateral conductance was then retrieved from the slope of
the curve of flow and pressure difference.

Quantification of atherosclerotic plaque formation
Complete aortas were isolated, embedded in formalin 4% and stained with Sudan
IV. After the staining procedure, a planimetric quantification of the percentage of
atherosclerotic plaque was performed from digitized photographs using NIH Image

Statistical analysis
Results are presented as mean ± standard deviation. Significant differences between
sample means were determined with an independent-samples T-test. Differences
with a p-value < 0.05 were classified as significant. However, when more than two
groups were compared, a Bonferroni correction was performed and the level of
significance was lowered to 0.05 divided by the number of groups.

Results
Serum levels of lipids
High levels of serum lipids were observed in both the control groups as well as in
the MCP-1 treated groups. However, no significant differences were observed
between any of the groups for values of either cholesterol, triglycerides, HDL or
LDL neither at 1 week after the treatment (see table 1) nor at 6 months after
treatment (data not shown).
Table 1: Serum lipids in the different treatment groups.

<table>
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<th>PBS control</th>
<th>MCP-1 low dose</th>
<th>MCP-1 high dose</th>
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<tbody>
<tr>
<td>Triglycerides</td>
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<td>453.8 ± 55.2 mg/dl</td>
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<td>Cholesterol</td>
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<td>467.3 ± 62.5 mg/dl</td>
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<tr>
<td>HDL</td>
<td>35 ± 2.4 mg/dl</td>
<td>32.3 ± 6.2 mg/dl</td>
<td>33.7 ± 3.4 mg/dl</td>
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<tr>
<td>LDL</td>
<td>362.8 ± 90 mg/dl</td>
<td>295.3 ± 36.1 mg/dl</td>
<td>360.8 ± 95.6 mg/dl</td>
</tr>
</tbody>
</table>

Figure 1: Upon femoral artery ligation several collateral arteries develop in hindlimbs of WHHL rabbits. The number of visible collateral arteries is increased upon high dose MCP-1 (1C). Figure 1D illustrates the process of pruning, referring to the decrease over time of the number of visible collateral arteries after their initial recruitment upon femoral artery ligation. X-ray angiography.
One week after ligation several collateral arteries could be observed, spanning mainly from the arteria profunda to the arteria genualis and the arteria saphena parva. The number of visible collateral vessels was 12.6 ± 2.3 in the WHHL rabbit control group. The low dose MCP-1 group did not differ significantly from the control group (14.3 ± 3.4). The 1-week treatment with high dose MCP-1 caused a significant increase in the number of visible collateral vessels (19.4 ± 2.4, p<0.05/3 as compared to the control group). Six months after femoral artery ligation the number of visible arteries had regressed in both groups (a process referred to as “pruning”) showing no longer a difference in the number of visible collateral arteries between difference in the number of visible collateral arteries between the high-dose MCP-1 treated group and the control group (MCP-1: 8.2 ± 0.9, PBS: 7.5 ± 0.8, p=NS, figure 1 and 2).

Collateral conductance measurements
The normal value for collateral conductance in the WHHL rabbit was 76.0 ± 6.6 ml/min/100 mmHg. One week after femoral artery ligation, collateral conductance was 2.2 ± 0.8 ml/min/100 mmHg. In the low dose MCP-1 group no significant increase in collateral conductance could be observed (3.4 ± 1.6 ml/min/100 mmHg, p=NS as compared to the control group). However, treatment with high dose MCP-1 significantly increased collateral conductance, 1 week after femoral artery ligation (8.9 ± 2.0 ml/min/100 mmHg, p<0.05/3 as compared to the control group). Six months after femoral artery ligation a restoration of flow to approximately 60% of normal was found in both the treated as well as the control group. At this time-point the difference in collateral conductance between the MCP-1 treated animals and the control animals was no longer significant (PBS: 44.9 ± 11.6 ml/min/100 mmHg, MCP-1: 47.8 ± 11.5 ml/min/100 mmHg, p=NS, figure 3).

Quantification of atherosclerotic plaque formation
Aortas of both treated and control WHHL rabbits showed massive plaque formation. Plaque formation was especially strong in the aortic arch and was found to a lesser extent in the thoracical and abdominal aorta (figure 4). However, no significant differences were found between the control and the MCP-1 treated group in percentage of atherosclerotic plaque/total endoluminal wall, 6 months after femoral artery ligation (control: 26.1% ± 18.1%, vs. MCP-1: 33.5% ± 14.4% p=NS, figure 5).

Discussion
The main finding of our study is that the arteriogenic potency of MCP-1 is preserved under hyperlipidemic conditions in the Watanabe rabbit. However, the effects are somewhat diminished as compared to the effects in normal New
Figure 2: One week after femoral artery ligation the number of visible collateral arteries was significantly greater in the high dose MCP-1 group as compared to the control group. In the low dose MCP-1 group no significant increase could be observed (Control: 12.6 ± 2.3, low dose MCP-1: 14.3 ± 3.4, high dose MCP-1: 19.4 ± 2.4). Six months after ligation the number of visible collateral arteries had regressed again (MCP-1: 8.2 ± 0.9, PBS: 7.5 ± 0.8, the high-dose MCP-1 treated group and the control group (MCP-1: 8.2 ± 0.9, PBS: 7.5 ± 0.8, p=NS, figure 1 and 2), p=NS).

Figure 3: Both absolute values of collateral conductance as well as percentages of normal conductance are shown. Treatment with high dose MCP-1 significantly increased collateral conductance, 1 week after femoral artery ligation (control: 3.4 ± 1.6 ml/min/100 mmHg MCP-1 high dose: 8.9 ± 2.0 ml/min/100 mmHg, p<0.05/3 as compared to the control group).
Figure 4: Representative Sudan IV stainings of aortas from a control animal (4A) and from a MCP-1 treated animal (4B), six months after treatment.

Figure 5: Planimetry of atherosclerotic surface in aortas from control animals and MCP-1 treated animals did not reveal a statistical significant difference between the two groups six months after treatment. However, a large standard deviation was observed in both groups (control: 26.1% ± 18.1%, MCP-1: 33.5% ± 14.4%, p=NS).

Figure 6: Comparison between Watanabe and NZW rabbits for either absolute conductance values (6A) or percentages of normal conductance (6B). As shown clearly, the arteriogenic effects of MCP-1 are significantly reduced in Watanabe rabbits as compared to normal NZW rabbits (* indicates a p-value lower than 0.05).
Zealand White Rabbits\textsuperscript{6} and higher dosages of MCP-1 are required to elicit an arteriogenic response. There was no longer an effect seen on the conductance of the collateral circulation six months after the treatment. The treatment with either low or high dose MCP-1 had no effect on serum lipids. Moreover, using Sudan IV staining we could not detect a statistical significant difference in the development of atherosclerotic plaques between MCP-1 treated animals and control animals, six months after initiation of the treatment. It should be noted however that the standard deviation of the data derived with the Sudan IV staining were of such magnitude that a potential pro-atherogenic effect of MCP-1 cannot be excluded based on these data.

Hyperlipidemia negatively influences the arteriogenic properties of MCP-1 in WHHL rabbits. The WHHL rabbits were treated with an MCP-1 dose that was shown in a previous study to significantly increase arteriogenesis in normal New Zealand White (NZW) rabbits\textsuperscript{6}. Additionally, a group was treated with a higher dose of MCP-1 since it has been described previously that collateral formation is diminished under hyperlipidemic conditions\textsuperscript{7,8}. Indeed we could show that the low dose MCP-1 treatment, that is sufficient to induce a strong arteriogenic response in NZW rabbits, has no significant arteriogenic effect in WHHL rabbits. In the high dose MCP-1 group an accelerated arteriogenic response was found, increasing collateral conductance approximately 4-fold, one week after femoral artery ligation. The salutary effect of high dose MCP-1 treatment on collateral conductance was no longer detectable six months after treatment. This indicates that the MCP-1 dose that was used in the present study induces an acceleration of the natural arteriogenic response upon femoral artery ligation but diminishes over time. It is currently not known whether final effects on collateral conductance can be altered by either higher dosages of MCP-1 or the combination of MCP-1 with other arteriogenic growth factors like TGF-\textbeta{} or b-FGF\textsuperscript{9-11}. This is of interest in view of earlier findings showing that the combination of MCP-1 and GM-CSF in a chronic model of more matured collateral arteries could still induce an arteriogenic response, in contrast to the lack of arteriogenic efficiency of both MCP-1 and GM-CSF when applied as monotherapy in this chronic model\textsuperscript{12}.

In figure 6 we have made a direct comparison between Watanabe and NZW rabbits for both absolute values of conductance as well as for the percentages of normal conductance. The latter correction is important since in WHHL rabbits normal conductance without femoral artery ligation was about 45\% of the conductance that we observed in normal rabbits of equal weight\textsuperscript{6}. It has been shown previously that the responsiveness of the rabbit hindlimb resistance vasculature to adenosine is not impaired by hypercholesterolemia\textsuperscript{13}. Thus, the increased peripheral resistance in WHHL rabbits (and thus the decreased conductance) most probably is due to the atherosclerotic disease itself\textsuperscript{14}. The NZW rabbit data presented in figure 6 are previously published historical data, except for the high dose MCP-1 treatment\textsuperscript{6}.
The measurements of collateral conductance were performed under exactly the same conditions as described for the current manuscript. As shown, low-dose MCP-1 treatment significantly increases collateral conductance in NZW rabbits, in contrast to the lack of arteriogenic efficacy in Watanabe rabbits. In the high dose MCP-1 group an accelerated arteriogenic response was found in both Watanabe as well as NZW rabbits. However, the 4-fold increase in percentage of normal conductance in Watanabe rabbits is still a diminished response as compared to the 9-fold increase as observed upon high dose MCP-1 treatment in New Zealand White rabbits. In the PBS-treated control groups a significant difference in arteriogenic response was found between Watanabe and NZW rabbits only for absolute conductance values (Figure 6A).

The potential of angiogenic or arteriogenic substances to stimulate atherogenesis is a topic that requires careful attention. Barger first proposed that angiogenesis, i.e. the formation of new capillary networks via endothelial sprouting, is an integral part of atherosclerotic plaque formation. Subsequently, Folkman showed that the inhibition of angiogenesis via TNP-470 or endostatin diminishes plaque formation. More recently it was shown by Celletti et al that the exogenous application of a low dose of the angiogenic factor Vascular Endothelial Growth Factor (VEGF) stimulates plaque formation in both cholesterol-fed rabbits and knock-out mice, doubly deficient in apolipoprotein E/apolipoprotein B100.

Arteriogenesis, i.e. the transformation of small pre-existing vascular collateral pathways into large-sized collateral conductance arteries, plays no direct role during atherogenesis. However, arteriogenesis and atherogenesis share numerous common features. Shear stress upregulates the expression of endothelial cell adhesion receptors and this occurs during both atherogenesis and arteriogenesis. Also 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.

During flow-restoration upon arterial obstruction, monocytes act in a pro-arteriogenic fashion. The endothelial expression of ICAM-1, an adhesion molecule for monocytes, is increased early during arteriogenesis and, using electron-microscopy and immuno-histochemistry, subsequently large numbers of adhering monocytes and perivascular macrophages are observed. These perivascular macrophages produce several growth factors, like basic Fibroblast Growth Factor and Tumour Necrosis Factor alpha, required for the transformation of pre-existent collateral arterioles into large conductance arteries.

However, in contrast to their beneficial pro-arteriogenic capacity it is believed that monocytes play a detrimental role during atherogenesis, leading for example to weakening of the fibrous cap and plaque instability. Thus it is obvious, that stimulation of atherogenesis is a potential hazardous side-effect of therapeutic arteriogenesis. This is even more true for MCP-1 since a direct
role for this cytokine 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. Therefore, additional insight into the pro-atherogenic properties
of MCP-1 when applied as a pro-arteriogenic substance are mandatory prior to the
initiation of a clinical trial. The route of administration of MCP-1 in such a clinical
study should preferably be local and intra-arterial since this form of application
most efficiently restores perfusion upon arterial obstruction and may limit
systemic side-effects.

In the WHHL rabbits, the atherosclerotic plaque surface in the aorta was not
significantly increased upon high dose MCP-1 treatment as compared to the control
animals six months after the one-week treatment. However, these data are somewhat
difficult to interpret. It should be noted that a trend could be recognized towards
more atherosclerotic plaque surface in the MCP-1 treated animals (26.1% vs.
33.5%). This difference did by no means reach statistical significance (p-value of
0.32) but this might also be due to the relatively high standard deviation that was
observed in both groups. It could still be postulated that this large variety within the
groups masks the pro-atherogenic effects of high dose MCP-1.

In conclusion, it was found that MCP-1 elicits an arteriogenic response, even under
severe hyperlipidemic conditions as observed in WHHL rabbits. The dosage
required for the arteriogenic response is however 3–fold higher as compared to the
dosage needed under normolipidemic conditions. Treatment with MCP-1 did not
influence serum lipids and in the present model we did not detect a pro-atherogenic
effect of MCP-1. However, in order to be able to definitely exclude pro-atherogenic
side-effects of MCP-1 when used as a pro-arteriogenic substance, more studies are
warranted in different models of atherosclerosis.

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References


