Experimental and clinical studies on collateral and epicardial flow in obstructive arterial disease

Voskuil, M.

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Chapter 6

Modulation of collateral artery growth in a porcine hind limb ligation model using MCP-1

Michiel Voskuil, Niels van Royen, Imo E Hoefer, Randolph Seidler, Brian D Guth, Christoph Bode, Wolfgang Schaper, Jan J Piek, Ivo R Buschmann

Abstract
For an appropriate extrapolation to patients with peripheral arterial obstructive disease we tested the efficacy of monocyte chemoattractant protein 1 (MCP-1) treatment in a porcine hind limb ligation model. In 40 minipigs, a femoral artery ligation was performed. Control animals was examined immediately after ligation (n = 4), or after two weeks of intra-arterial infusion of phosphate buffered saline (PBS; n = 11). A second group of animals was evaluated after intra-arterial infusion of 2.0 μg/h of MCP-1 for 48 hours (followed by 12 days of PBS; n = 13) or two weeks continuously (n = 12). In the terminal experiment after two weeks, resting flow to the leg and peripheral arterial pressures were assessed, without vasodilatation. Subsequently, vascular conductance was determined using a pump driven extra corporal circulation, during maximal vasodilatation. The results showed that resting blood flow to the hind limb was 53% of normal after two weeks of infusion of PBS, compared to 81% in both MCP-1 treatment groups (p < 0.05). Collateral conductance was 645 ± 346 ml/min/mmHg after two weeks of infusion with PBS, compared to 1070 ± 530 and 1158 ± 535 ml/min/mmHg after 48 hours and two weeks treatment with MCP-1, respectively (p < 0.05).
Modulation of the process of arteriogenesis is feasible in this large animal model via intra-arterial infusion of the c-c-chemokine MCP-1.
MODULATION OF ARTERIOGENESIS IN THE PIG HIND LIMB

Introduction

Patients with obstructive peripheral or coronary disease may benefit from the progress made during the last decades in both medical and invasive treatment modalities focusing on the restoration of blood flow. Nevertheless, the group of patients that remains symptomatic, despite these currently available treatment options, is still growing and therefore constitutes a major clinical problem in the western world. The potential stimulatory effect of growth factor administration on vessel formation has created a possible new treatment option for this patient group. It is important to distinguish two different forms of compensatory vessel growth, angiogenesis and arteriogenesis, as has recently been recognized by several other groups. Angiogenesis refers to the formation of new small capillaries in response to ischemia. Arteriogenesis refers to the remodeling of pre-existing arterioles to mature collateral arteries. In this process, not ischemia, but increased shear stress due to redistribution of blood over these arterioles is the driving force for the remodeling of these vessels into true collateral arteries. Most likely, the therapeutic stimulation of arteriogenesis is to be preferred over angiogenesis, since arteriogenesis is more efficient to compensate flow reduction due to the larger diameter and better functionality of the formed vessels, compared to capillary networks in angiogenesis.

A number of experimental peripheral ligation models in mainly small animals have been used to study the stimulation of these processes with growth factors. In these studies, monocyte chemoattractant protein 1 (MCP-1) has been shown to be one of the strongest stimulators of the arteriogenesis process. The purpose of the present study was to evaluate the potency of MCP-1 for the stimulation of collateral artery growth in a porcine hind limb ligation model that may be more suitable for extrapolation of the observed effects to patients with peripheral arterial obstructive disease (PAOD).

Methods

Surgical preparation

For this study 40 Göttinger Minipigs of either sex and weighing 28 ± 6 kg (Ellegaard, Dalmose, Denmark) were used. The animals were handled in accordance with the American Physiological Society guidelines for animal welfare. Animals were housed in standard cages and fed water and chow ad libitum. The pigs were sedated using a combination of azaperone (5ml; 40 mg/ml), midazolam (3 ml, 5 mg/ml) and ketamine hydrochloride (2 ml; 100mg/ml), and were subsequently intubated and ventilated with a respirator (Engström 300, Engström Medical AB, Solna, Sweden) with N2O : O2 in a ratio of 2 : 1. General anesthesia was maintained using isoflurane (0.8 to 2.0 vol% in O2). The left artery femorals was exposed using a sterile surgical technique and ligated immediately distal from the bifurcation with the arteria profunda femoris. A double ligation was performed with a 4-cm distance in-between the two ligation sites. Also, the arteria circumflexa femoris lateralis
was ligated to prevent 'bridging' collateral artery formation.

**Intra-arterial infusion**

A 1.6 mm silicon infusion catheter was retrogradely inserted with the tip placed just distal to the bifurcation to ensure a first-pass effect of the compound over the collateral vascular bed. The catheter was subcutaneously tunneled to the animal's back, externalized and connected to a portable elastomeric infusion system (Multiday Infusor 2.0 ml/hour; Baxter Healthcare Corporation, Deerfield). The animals were examined acutely after ligation (n = 4), after two weeks infusion with vehicle (phosphate buffered saline, PBS; n = 11) or after treatment with 2.0 μg/h monocyte chemoattractant protein 1 (recombinant MCP-1, Boehringer Ingelheim, Austria) for 48 hours, followed by 12 days of vehicle (n = 13) or two weeks continuous infusion of MCP-1 (n = 12).

**Experimental design**

For the terminal study after two weeks of ligation the animals were anesthetized again using to the above-described doses of azaperone, ketamine and midazolam. Anesthesia was subsequently maintained using administration of sodium pentobarbital (60 mg/animal bolus, followed by a continuous intravenous infusion in a dose of 10 mg/kg/h). The jugular vein was cannulated for the maintenance of the anesthesia. Heparin was injected in a dose of 20.000 IU/animal. The animals were monitored during the experiment using ECG and measurement of heart rate and arterial oxygenation using pulse-oximetry. A solid-state pressure gauge manometer was placed in the left carotid artery for the continuous measurement of systemic arterial pressure. The saphenous arteries were exposed at the level of the metatarsus and cannulated with fluid-filled polyethylene catheters. These tubings were connected to pressure transducers for the measurement of distal arterial pressure. With the use of a laparotomy, the abdominal aorta and both artery iliac externae were isolated. For the measurement of volume flow to the region of interest, flow probes (Transonic Systems Inc., Ithaca, NY) were placed around each of the artery iliac externae just proximal of the bifurcation of the arteria femoralis and the arteria profunda femoris. The mesenteric artery was cannulated with a polyethylene-heparinized catheter for the measurement of the perfusion pressure after installation of an extracorporeal circulatory system. For this extracorporeal system, the aorta was dissected and specially designed glass cannulas were inserted proximally and distally into the aorta, immediately before the aortic bifurcation. The glass cannulas were connected at both ends to a silicone tube (aortic bypass). The silicone tube was inserted into an electronic roller pump (ISM 726, Ismatec GmbH, Wertheim, Germany) for controlled perfusion of the hind limbs. After a steady state was reached papaverine-HCl (Sigma Chemicals Co.; St. Louis, MO) was continuously infused in a dose of 20 mg/min into the perfusion line to achieve a stable maximal local vasodilatation. The
pump speed was then stepwise increased until the systemic blood supply was exhausted. Each step was maintained until a stable flow was achieved. Continuous hemodynamic recordings were made using the data acquisition software Notocord-Hem 3.3 (Notocord systems SA, Croissy, France).

**In vivo angiography**

In a total of 4 minipigs (2 PBS treated and 2 two weeks MCP-1 treated animals), a sheath was inserted directly into the right carotid artery and a 7F diagnostic catheter was positioned in either the distal iliac artery or the arteria profunda femoris for the selective injection of a single 20-50 ml bolus of nonionic contrast agent (Solutrast 300, Byk Gulden, Konstanz, Germany). Images were digitally recorded on a desktop personal computer.

**Hemodynamic measurements**

Before the insertion of the extra corporal circulatory system, values of mean left and right resting blood flow through the arteria iliaca externa and mean left and right peripheral and central blood pressures were assessed, without the use of vasodilatation. Subsequently, the pump driven extra corporal circulatory system was applied to control perfusion pressures to the legs. Using this technique, perfusion pressure was enhanced in several steps under continuous maximal vasodilatation, using papaverine. Both femoral artery volume flow and pressure gradient over the ligated and unligated arteria Femoralis were assessed for the calculation of arterial conductance.

**Histology**

A contrast medium, based on barium sulfate, was infused into the donor artery (arteria profunda femoris) for macroscopical detection of the collateral arteries (n = 4; 2 PBS treated and 2 two weeks MCP-1 treated animals). Tissue samples were taken after identification of the formed collateral arteries, based on recognition of the typical stem, midzone and reentry region and corkscrew appearance. Histological sections (5μm) were prepared from paraffin-embedded tissue samples and were evaluated for morphological appearance with hematoxylin-eosin (HE) staining. For detection of proliferating vascular wall cells, frozen sections (5 mm thick) were placed on gelatine-coated slides and fixed for 10 min in aceton. Tissue sections were then exposed for 10 min in 0.1% carboxylated bovine serum albumin in PBS, followed by incubation overnight at 4°C with a primary monoclonal antibody against Ki-67 (clone MIB-1). After repeated washes in PBS, the sections were then incubated for 1 hour at RT with goat anti-mouse IgG conjugated with FITC. Specificity of the labeling was confirmed by omission of the primary antibody. Nuclei were stained with Hoechst 33342.
Data analysis

Values of volume flow and pressure were obtained during the plateau of each perfusion level and were averaged. The assessed flows and pressure gradients were subsequently fitted in a linear regression. All conductance indices were calculated from the equation of the pressure-flow relation as the flow level of the distal vascular bed at a pressure gradient (P perfusion - P distal) of 100 mmHg. Animals were excluded if the linear fit of the conductance calculation did not result in a regression coefficient (r²) > 0.94 in one of the legs. Results are expressed as means ± SD. Differences between sample means were determined with an ANOVA with a Dunnett’s (post) test and were considered statistically significant when the p-value was < 0.05.

Results

No differences were present regarding age and body weight between the different treatment groups (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Age, month ± SD</th>
<th>Vehicle Acute</th>
<th>2 weeks</th>
<th>MCP-1 2 days</th>
<th>2 weeks</th>
</tr>
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<tbody>
<tr>
<td>16.1 ± 6</td>
<td>16.9 ± 7</td>
<td>19.5 ± 5</td>
<td>18.1 ± 6</td>
<td></td>
</tr>
<tr>
<td>28.5 ± 8</td>
<td>22.8 ± 3</td>
<td>30.8 ± 7</td>
<td>27.4 ± 8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>13</td>
<td>10</td>
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</tbody>
</table>

Angiography

Examples of in vivo angiographies of animals that received PBS for 2 weeks or that were treated for two weeks with MCP-1 are shown in Figure 1. Collateral arteries, connecting the arteria profunda femoris (stem-zone) and the distal zone of the femoral artery (reentry-zone), could be observed.
Figure 1

In vivo angiography. **A:** No arterial ligation. **B:** 2 weeks of PBS infusion after ligation of the femoral artery. **C:** 2 weeks of continuous MCP-1 infusion. The arrows depict several visible collateral connections.
Resting blood flow and pressures

The resting blood flow and peripheral pressures of all treatment groups are depicted in Table 2.

Table 2

Hemodynamic data of the four treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>MCP-1</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>2 weeks</td>
</tr>
<tr>
<td><strong>Pressure (mmHg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic</td>
<td>108 ± 24</td>
<td>78 ± 12</td>
</tr>
<tr>
<td>Ligated leg</td>
<td>43 ± 8</td>
<td>55 ± 12*</td>
</tr>
<tr>
<td>Unligated leg</td>
<td>94 ± 16</td>
<td>72 ± 13</td>
</tr>
<tr>
<td><strong>Volume flow (ml/min)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ligated leg</td>
<td>28 ± 17</td>
<td>54 ± 30*</td>
</tr>
<tr>
<td>Unligated leg</td>
<td>110 ± 58</td>
<td>98 ± 37</td>
</tr>
<tr>
<td><strong>Heart rate (bpm)</strong></td>
<td>113 ± 31</td>
<td>84 ± 21</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

Heart rate, systemic pressure and distal pressure and blood flow in the unligated leg remained similar in all animal groups. Blood flow and distal pressure in the ligated leg increased after two weeks of vehicle infusion. While distal pressure did not show a significant increase (55 ± 12, 57 ± 11 and 57 ± 11 mmHg after 2 weeks of PBS, 2 days and 2 weeks of MCP-1, respectively; p = NS), blood flow increased after treatment with MCP-1 (from 54 ± 30 to 105 ± 60 and 88 ± 38 ml/min after 2 weeks of PBS, 2 days and 2 weeks of MCP-1, respectively; p < 0.05). Figure 2 shows that resting blood flow to the leg increased from 27% of the contra lateral leg after acute ligation to 53% after two weeks of treatment with vehicle (p < 0.05). This is in contrast with a marked increase of flow after two days of treatment with MCP-1 (81%; p < 0.05), although this flow did not further increase if MCP-1 administration was extended to two weeks (81%). Distal pressures increased from 45% of normal directly after ligation to 73%, 75% and 76% after two weeks of vehicle infusion, two days and two weeks of treatment with MCP-1, respectively (p = NS). Likewise, no statistically significant differences were present regarding the calculated ratio of the systemic and peripheral pressure ("ankle-brachial index") between the groups of animals that were treated with either vehicle or MCP-1.
MODULATION OF ARTERIOGENESIS IN THE PIG HIND LIMB

Resting volume flow, peripheral pressure and the ankle-brachial index (all expressed as a percentage of the unligated hind limb) of the different treatment groups. *p < 0.05 compared to value acutely after ligation. †p < 0.05 compared to value after 2 weeks of vehicle infusion.

Conductance measurements

Figure 3A shows that acutely after ligation, conductance over the distal vascular bed decreased to a value of 158 ± 112 ml/min/100mmHg. After two weeks of infusion with PBS, conductance increased to 645 ± 346 ml/min/mmHg, compared to 1070 ± 530 and 1158 ± 535 ml/min/mmHg after 48 hours and two weeks treatment with MCP-1, respectively (PBS compared to both MCP-1 groups; p < 0.05). Similar differences were observed when the conductance was corrected for the conductance in the unligated leg (Figure 3B).

Histology

An increased number of inflammatory cells (monocytes/neutrophils) were present in the perivascular space around developing collateral arteries (Figure 4). Furthermore, Ki67 staining for proliferating cells revealed dividing smooth muscle cells in the tunica media of the developing collateral arteries (Figure 5). Ki67 is a nuclear antigen expressed by proliferating cells but down-regulated in cells re-entering the GO phase.26 However, no quantitative differences in the number of infiltrating cells or dividing smooth muscle cells could be observed between the MCP-1 treated and control animals.
Conductance measurements of ligated hind limb under maximal vasodilatation. Absolute values of conductance of the ligated hind limb in ml/min/100mmHg.

Figure 3 A

Conductance measurements of ligated hind limb under maximal vasodilatation. Percent conductance of the ligated hind limb, corrected for the conductance of the unligated hind limb. * p < 0.05 compared to value acutely after ligation. † p < 0.05 compared to value after 2 weeks of vehicle infusion.

Figure 3 B
Figure 4

Discussion

The present study demonstrates the efficacy of stimulation of collateral artery growth in a porcine hind limb ligation model using exogenous administration of the c-c-chemokine MCP-1. Blood flow was increased two-fold after two days of treatment, whereas extension of treatment to two weeks did not further increase this positive effect on hind limb perfusion.

Collateral artery growth in the peripheral circulation in pigs

It is has been shown previously that the pig has limited potential for the development of (endocardial) collateral arteries in the coronary circulation, compared to the extensive (epicardial) coronary collateral vascular bed in the dog. For the hind limb circulation, the development of collateral arteries in pigs has not been studied until now. The efficacy of different growth factors has been shown in the rabbit hind limb model. However, for the extrapolation of the effects of growth factors on arteriogenesis to the clinical situation of peripheral arterial obstructive disease the present animal model is valuable, since it enables the assessment of dose-effect relationships on arterial remodeling in a large animal. This effect may be markedly different in larger sized animals, consi-
dering the number of cell divisions required for maturation of the collateral vessels. As shown in the present study, no overt ischemic damage to the femoralis-perfused tissue was observed. Moreover, a spontaneous increase of blood flow after two weeks ('natural course') was demonstrated. Angiography showed that the porcine hind limb collateral circulation has a similar anatomy compared to the human situation according to Longland's classification. The pig hind limb thus provides an excellent large animal model for the evaluation of collateral artery growth in the peripheral circulation, that provides a broad spectrum of functional hemodynamic parameters and allows the assessment of vascular conductance under conditions of maximal vasodilatation.

**MCP-1 in arteriogenesis**

After obstruction of a main feeding artery a redistribution of blood flow occurs over pre-existing arterioles. The subsequent presence of increased intravascular shear stress, due to the enhanced blood flow, causes a local activation of the endothelium. This activated endothelium causes an up regulation of monocyte adhesion receptors such as intercellular and vascular cell adhesion molecule and endogenously produces factors such as transforming growth factor Beta and MCP-1. MCP-1 is a potent agonist for the β-chemokine receptors CCR-2 and CCR-4 that are expressed on monocytes. The presence of a gradient of MCP-1 induces chemotaxis of monocytes via this pathway. The attraction of monocytes, their diapedesis through the vessel wall, transformation into macrophages and finally, their local production of a cocktail of factors is generally believed to be the primary stimulatory mechanism for collateral vessel growth.

The cocktail of factors that is produced by the monocyte (i.e. matrix metalloproteinases; MMP's, tumor necrosis factor alfa; TNF-alfa, basic fibroblast growth factor; b-FGF, platelet derived growth factor; PDGF) facilitates a locally active process of mitosis of endothelial and smooth muscle cells. TNF-alfa and MMP's induce an inflammatory environment and the degradation of existing structures, while b-FGF and PDGF stimulate mitogenesis of endothelial and smooth muscle cells. This remodeling process leads to the development of functional arteries with multiple smooth muscle layers that are capable to carry substantial volumes of blood due to their relatively low resistance and responsiveness to vasoactive substances (i.e. during exercise). This in contrast to the development of small capillaries during angiogenesis, consisting exclusively of endothelial cells. This is important with respect to the functionality and capacity of these vessels, since these vessels have to compensate for a substantial amount of loss of blood flow after obstruction of a large feeding artery, as also depicted in the current study (flow decrease of 75%). In the present study, the accumulation of monocytes around the formed collateral arteries was confirmed histologically and it was shown that the process of arteriogenesis in the porcine hind limb could be positively modulated using an intra-arterial administration of MCP-1. This effect (lea-
ding to approximately a doubling of the spontaneous increase of conductance) seems to be less pronounced, compared to the strong effects that were observed in the rabbit model (MCP-1 treated 3-8 fold increase of conductance compared to PBS). The total dose that was used in the 2 weeks treated pigs is about 5-6 fold the dose (corrected for weight and treatment period) as used in the rabbit studies. However, in the animals that were treated for only 48 hours, the total amount of MCP-1 per kg body weight that was administered was similar to the amount used in the rabbit model. No further improvement of hind limb perfusion was observed after a prolonged duration (two weeks) of treatment with MCP-1. This finding may be explained by the fact that local attraction and extravasation of monocytes around a developing collateral artery merely occurs within the first days after acute arterial occlusion. Hence, a short duration of MCP-1 infusion may be sufficient for the attraction and activation of the monocytes, which are required for collateral artery growth.

Endpoints
In the current study assessment of hind limb perfusion was performed after two weeks of femoral artery ligation, irrespective of the treatment period. Although angiography and histology were performed, hemodynamic parameters were used as primary endpoint to evaluate the effects of MCP-1 on hind limb perfusion, since the correlation between the number of visible arteries and the grade of perfusion is generally believed to be doubtful. Relative small positive effects were observed on resting blood flow. However, no (statistical significant) effects were seen on resting peripheral pressures and the calculated ankle-brachial index (that is an endpoint in many clinical studies), which may be due to the high level of ‘spontaneous’ recovery of the resting distal pressure to approximately 75% of normal. This reduces the therapeutic window for growth factor therapy for these endpoints. After induction of increasing perfusion pressures using the pump driven system under maximal vasodilatation, the positive effects of MCP-1 treatment were detected more clearly. This result reflects the importance of the use of vasodilators and the testing of the maximal capacity of the vascular system, rather than only measuring at resting conditions.

In summary, our results have shown that collateral arteries develop in the pig hind limb and that an improvement of perfusion can be achieved using intra-arterial administration of MCP-1. Moreover, our data show that a two days of infusion of MCP-1 is sufficient to induce a significant arteriogenic response, whereas a longer duration of therapy did not further increase this pro-arteriogenic effect.
Acknowledgments

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MODULATION OF ARTERIOGENESIS IN THE PIG HIND LIMB