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

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Submitted 18 June 2002; accepted in final form 26 December 2002

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 (12, 28). The potential stimulatory effect of growth factor administration on vessel formation has created a possible new treatment option for this patient group (7, 17). It is important to distinguish two different forms of compensatory vessel growth, angiogenesis and arteriogenesis, as has recently been recognized by several other groups (4, 10, 11, 19, 33). Angiogenesis refers to the formation of new small capillaries in response to ischemia. Arteriogenesis refers to the remodeling of preexisting arterioles to mature collateral arteries. In this process, not ischemia, but increased shear stress due to redistribution of blood flow over these arterioles, is the driving force for the remodeling of these vessels into true collateral arteries (24, 32). Most likely, the therapeutic stimulation of arteriogenesis is to be preferred over angiogenesis, because arteriogenesis is more efficient to compensate flow reduction due to the larger diameter and better functionality of the formed vessels compared with capillary networks in angiogenesis (28). A number of experimental peripheral ligation models in mainly small animals have been used to study the stimulation of these processes with growth factors (13, 15). 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 hindlimb ligation model that may be more suitable for the extrapolation of the observed effects to patients with peripheral arterial obstructive disease.

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 with a combination of azaperone (5 ml, 40 mg/ml), midazolam (3 ml, 5 mg/ml), and ketamine hydrochloride (2 ml, 100 mg/ml) and were subsequently intubated and ventilated with a respirator (Engström 300, Engström Medical; Solna, Sweden) with...
N\textsubscript{2}O-O\textsubscript{2} in a ratio of 2:1. General anesthesia was maintained with isoflurane (0.8 to 2.0 vol\% in O\textsubscript{2}). The left arteria femoralis was exposed using a sterile surgical technique and ligated immediately distal to 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 and externalized and connected to a portable elastomeric infusion system (2.0 ml/h, Multiday Infusor; Baxter Healthcare). The animals were examined acutely after ligation (n = 4), after 2 wk infusion with vehicle (PBS; n = 11), or after treatment with 2.0 µg/h monocye chemoattractant protein 1 (recombinant MCP-1, Boehringer Ingelheim) for 48 h, followed by 12 days of vehicle (n = 13) or 2 wk continuous infusion of MCP-1 (n = 12).

**Experimental design.** For the terminal study after 2 wk of ligation, the animals were anesthetized again with the previously described doses of azaperone, ketamine, and midazolam. Anesthesia was subsequently maintained by the administration of pentobarbital sodium (60 mg/animal bolus, followed by a continuous intravenous infusion in a dose of 10 mg·kg\textsuperscript{-1}·h\textsuperscript{-1}). 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 by using ECG, and heart rate and arterial oxygenation were measured by using a 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 was retrogradely cannulated with a polyethylene catheter. These tubings were connected to a portable elastomeric infusion system (2.0 ml/h, Multiday Infusor; Baxter Healthcare). The animals were examined acutely after ligation (n = 4), after 2 wk infusion with vehicle (PBS; n = 11), or after treatment with 2.0 µg/h monocye chemoattractant protein 1 (recombinant MCP-1, Boehringer Ingelheim) for 48 h, followed by 12 days of vehicle (n = 13) or 2 wk continuous infusion of MCP-1 (n = 12).

**Resting blood flow and pressures.** The resting blood flow and peripheral pressures of all treatment groups were subsequently fitted in a linear regression. All conductance indexes were calculated from the equation of the pressure-flow relation as the slope of the distal vascular bed at a pressure (P) gradient (P\textsubscript{perfusion} - P\textsubscript{distal}) of 100 mmHg. Animals were excluded if the linear fit of the conductance calculation did not result in a regression coefficient (r\textsuperscript{2}) > 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).

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

**Resting blood flow and pressures.** The resting blood flow and peripheral pressures of all treatment groups are depicted in Table 2. 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 2 wk of vehicle infusion. Whereas distal pressure did not show a significant increase (55 ± 12, 57 ± 11, and 57 ± 11 mmHg after 2 wk of PBS and 2 days and 2 wk of MCP-1, respectively; \( P \) = not significant), blood flow increased after treatment with MCP-1 (from 54 ± 30 to 105 ± 60 ml/min and 88 ± 38 ml/min after 2 wk of PBS and 2 days and 2 wk of MCP-1, respectively; \( P < 0.05 \)). Figure 2 shows that resting blood flow to the leg increased from 27% of the contralateral leg after acute ligation to 53% after 2 wk of treatment with vehicle (\( P < 0.05 \)). This is in contrast with a marked increase of flow after 2 days of treatment with MCP-1 (81%; \( P < 0.05 \)), although this flow did not further increase if MCP-1 administration was extended to 2 wk (81%). Distal pressures increased from 45% of the normal directly after ligation to 73%, 75%, and 76% after 2 wk of the vehicle infusion and 2 days and 2 wk of treatment with MCP-1, respectively (\( P = \) not significant). 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.

Conductance measurements. Figure 3A shows that acutely after ligation, conductance over the distal vascular bed decreased to a value of 158 ± 112 ml·min\(^{-1}\)·100 mmHg\(^{-1}\). After 2 wk of infusion with PBS, conductance increased to 645 ± 346 ml·min\(^{-1}\)·100 mmHg\(^{-1}\) compared with 1,070 ± 530 and 1,158 ± 535 ml·min\(^{-1}\)·100 mmHg\(^{-1}\) after 48 h and 2 wk treatment with MCP-1, respectively (PBS compared with both MCP-1 groups; \( P < 0.05 \)). Similar differences were observed when the conductance was corrected for the conductance in the unligated leg (Fig. 3B).

Histology. An increased number of inflammatory cells (monocytes/neutrophils) were present in the perivascular space around developing collateral arteries (Fig. 4). Furthermore, Ki67 staining for proliferating cells revealed dividing smooth muscle cells in the tunica media of the developing collateral arteries (Fig. 5). Ki67 is a nuclear antigen expressed by proliferating cells but downregulated in cells reentering the G0 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.


**DISCUSSION**

The present study demonstrates the efficacy of stimulation of collateral artery growth in a porcine hindlimb ligation model using exogenous administration of the C-C-chemokine MCP-1. Blood flow was increased twofold after 2 days of treatment, whereas extension of treatment to 2 wk did not further increase this positive effect on hindlimb perfusion.

**Collateral artery growth in the peripheral circulation in pigs.** It has been shown previously that the pig has limited potential for the development of (endocardial) collateral arteries in the coronary circulation compared with the extensive (epicardial) coronary collateral vascular bed in the dog (8, 21, 30). For the hindlimb 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 hindlimb model (2, 13, 15, 31). 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 because it enables the assessment of dose-effect relationships on arterial remodeling in a large animal.

MCP-1 in arteriogenesis. After obstruction of a main feeding artery, a redistribution of blood flow occurs over the preexisting arterioles. The subsequent presence of increased intravascular shear stress due to the redistribution of blood flow occurs over the preexisting arterioles. The subsequent presence of increased intravascular shear stress due to the

**Table 2. Hemodynamic data of the four treatment groups**

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<th>Vehicle</th>
<th>MCP-1</th>
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<tr>
<td></td>
<td>Acute</td>
<td>2 Wk</td>
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<tr>
<td>Pressure, mmHg</td>
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<tr>
<td>Systemic</td>
<td>108 ± 24</td>
<td>78 ± 12</td>
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<tr>
<td>Ligated leg</td>
<td>43 ± 8</td>
<td>55 ± 12</td>
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<tr>
<td>Unligated leg</td>
<td>94 ± 16</td>
<td>72 ± 13</td>
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<tr>
<td>Volume flow, ml/min</td>
<td></td>
<td></td>
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<tr>
<td>Ligated leg</td>
<td>28 ± 17</td>
<td>54 ± 30</td>
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<tr>
<td>Unligated leg</td>
<td>110 ± 58</td>
<td>98 ± 37</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>115 ± 31</td>
<td>84 ± 21</td>
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<td>n</td>
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<td>9</td>
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Values are means ± SD; n, number of animals. *P < 0.05 compared with value acutely after ligation. †P < 0.05 compared with value after 2 wk of vehicle infusion.

**Fig. 2.** Resting volume flow, peripheral pressure, and the ankle-brachial index (all expressed as a percentage of the unligated hindlimb) of the different treatment groups. *P < 0.05 compared with value acutely after ligation. †P < 0.05 compared with value after 2 wk of vehicle infusion.

**Fig. 3.** Conductance measurements of ligated hindlimb under maximal vasodilatation. A: absolute values of conductance of the ligated hindlimb (in ml·min⁻¹·100 mmHg⁻¹). B: percent conductance of the ligated hindlimb corrected for the conductance of the unligated hindlimb. *P < 0.05 compared with value acutely after ligation. †P < 0.05 compared with value after 2 wk of vehicle infusion.

This effect may be markedly different in larger sized animals, considering 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 2 wk ("natural course") was demonstrated. Angiography showed that the porcine hindlimb collateral circulation has a similar anatomy compared with the human situation according to Longland’s classification (20). The pig hindlimb thus provides an excellent large animal model for the evaluation of collateral artery growth in the peripheral circulation, which provides a broad spectrum of functional hemodynamic parameters and allows the assessment of vascular conductance under conditions of maximal vasodilatation.
enhanced blood flow causes a local activation of the endothelium (14, 18, 23). This activated endothelium causes an upregulation of monocyte adhesion receptors such as intercellular and vascular cell adhesion molecule and endogenously produces factors such as transforming growth factor-β and MCP-1 (6, 25, 27). MCP-1 is a potent agonist for the β-chemokine receptors CCR-2 and CCR-4 that are expressed on monocytes (22). 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,

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**Fig. 4.** Paraffin sections of collateral arteries with hematoxylin-eosin staining. A: section of preexisting arteriolar connection. B: remodeled collateral artery after 2 wk of treatment with MCP-1. C: section of preexisting arteriolar connection. D: remodeled collateral artery after 2 wk of PBS infusion. Arrows allocate perivascular cell infiltration. Black bars depict 50 μm.

**Fig. 5.** Frozen sections of remodeled collateral arteries with Ki67 staining of a PBS (A) and a MCP-1-treated animal (B and C). Arrows allocate dividing cells, as shown by green fluorescence of (blue) cell nucleus. D: magnification of selected inset of C. White bars depict 50 μm.
their local production of a cocktail of factors is generally believed to be the primary stimulatory mechanism for collateral vessel growth (1, 16). The cocktail of factors that is produced by the monocyte [i.e., matrix metalloproteinases (MMPs), tumor necrosis factor-α (TNF-α), basic fibroblast growth factor (b-FGF), and platelet-derived growth factor (PDGF)] facilitates a locally active process of mitosis of endothelial and smooth muscle cells (5, 29). TNF-α and MMPs induce an inflammatory environment and the degradation of existing structures, whereas 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 is in contrast to the development of small capillaries during angiogenesis, consisting exclusively of endothelial cells (3). This is important with respect to the functionality and capacity of these vessels, because 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 hindlimb could be positively modulated using an intraarterial administration of MCP-1. This effect (leading to approximately a doubling of the spontaneous increase of conductance) seems to be less pronounced compared with the strong effects that were observed in the rabbit model (MCP-1-treated three- to eightfold increase of conductance compared with PBS) (13, 15).

The total dose that was used in the 2 wk-treated pigs is about five- to sixfold the dose (corrected for weight and treatment period) as used in the rabbit studies. However, in the animals that were treated for only 48 h, the total amount of MCP-1 per kilogram body weight that was administered was similar to the amount used in the rabbit model. No further improvement of hindlimb perfusion was observed after a prolonged duration (2 wk) 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 occur within the first days after acute arterial occlusion (1, 16). 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.

End points. In the current study, assessment of hindlimb perfusion was performed after 2 wk of femoral artery ligation, irrespective of the treatment period. Although angiography and histology were performed, hemodynamic parameters were used as the primary end point to evaluate the effects of MCP-1 on hindlimb perfusion, because the correlation between the number of visible arteries and the grade of perfusion is generally believed to be doubtful (9). Relative small positive effects were observed on resting blood flow. However, no (statistical significant) effects were seen on resting peripheral pressures and on the calculated ankle-brachial index (which is an end point in many clinical studies), which may be due to the high level of “spontaneous” recovery of the resting distal pressure to ~75% of normal. This reduces the therapeutic window for growth factor therapy for these end points. 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 using 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 hindlimb and that an improvement of perfusion can be achieved using intrarterial administration of MCP-1. Moreover, our data show that 2 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 proarteriogenic effect.

Boehringer Ingelheim Pharma is acknowledged for financial and technical assistance in this project. T. Dietze, A. Sterner, and S. Germeyer contributed to this project. This work was supported by the German Volkswagenfoundation.

REFERENCES


