Differential effects of MCP-1 and leptin on collateral flow and arteriogenesis

Stephan H. Schirmera,*,1, Ivo R. Buschmannb,a, Marco M. Josta, Imo E. Hoefera, Sebastian Grundmannb,a, Jan-Philip Anderta, Susann Ulusansa, Christoph Bodea, Jan J. Piekb, Niels van Royenb

Research Group for Experimental and Clinical Arteriogenesis, Department of Internal Medicine III-Cardiology and Angiology, University Hospital Freiburg, Breisacher Strasse 66 (ZKF), D–79106 Freiburg, Germany

Department of Cardiology, Academic Medical Center, Amsterdam, Netherlands

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Abstract

Objective: Strategies to therapeutically stimulate collateral artery growth in experimental models have been studied intensively in the last decades. However, the experimental methods to detect collateral artery growth are discussed controversially and vary significantly. We compared different methods in a model of arteriogenesis in the rabbit hind limb and determined the effects on collateral flow of a known pro-arteriogenic factor, monocyte chemoattractant protein-1 (MCP-1), and a cytokine not previously evaluated for its arteriogenic efficacy, the adipocytokine leptin.

Methods and results: Forty-two New Zealand White rabbits received either MCP-1, leptin or PBS after ligation of the right femoral artery. The pro-arteriogenic effect of MCP-1 was confirmed by flow measurements during reactive hyperemia, as demonstrated by increased flow ratio (PBS 0.56±0.07 vs. MCP-1 0.77±0.06, no unit, p<0.0001), ankle-brachial index and microsphere-based conductance measurements (PBS 50.8±2.1 vs. MCP-1 225.8±8.8 ml/min/100 mm Hg, p<0.001). Biological activity of leptin on rabbit monocytes was shown by a dose dependent increase in Mac-1 expression. In-vivo administration of leptin also led to an increase in hyperemic flow and flow ratio (leptin 0.69±0.03, p<0.05 vs. PBS), but not to an increase in collateral conductance (leptin 54.7±4.1 ml/min/100 mm Hg, p=ns vs. PBS) or proliferation of vascular smooth muscle cells (Ki-67 staining: PBS 24.7±3.9%, leptin 22.7%±0.8% (p=ns), MCP-1 32.0±1.9% (p<0.01)). Ki-67 mRNA measured by real-time polymerase chain reaction increased (8.8±3.1-fold, p<0.01) during natural arteriogenesis, and was further enhanced (25.5±8.1-fold, p<0.005) after stimulation with MCP-1.

Conclusion: MCP-1 and leptin increase collateral flow in the rabbit hind limb model. In contrast to MCP-1, leptin does not enhance direct markers of vascular proliferation such as collateral conductance under maximal vasodilation and proliferation indices. The observed increase in hyperemic collateral flow thus most probably can be attributed to the well-documented vasodilatory effects of leptin. These data stress the necessity of the use of proliferation markers and microsphere-based conductance measurements under maximal vasodilation in order to separate effects of substances on vascular proliferation from effects on vasodilation.

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Keywords: Angiogenesis; Blood flow; Collateral circulation; Cytokines; Growth factors

1. Introduction

Arteriogenesis, i.e. the growth of collateral arteries from pre-existent anastomoses, is a naturally occurring process, serving to restore blood flow to territories of the heart or the peripheral musculature that are devoid of blood supply due
to arterial obstruction [1,2]. Numerous well-documented cases are known of patients with severe triple-vessel disease but normal left-ventricular function by virtue of a well-developed collateral circulation. However, in the majority of patients with occlusive arterial disease, the increase in capacity of the collateral circulation via arteriogenesis is not sufficient to regain normal tissue perfusion and hence function. Thus, strategies that might induce the process of arteriogenesis are currently intensively studied. The methods applied to evaluate such newly developed strategies in the experimental setting vary significantly. The calculation of perfusion per mass tissue by microspheres is considered the gold standard in the experimental field [3]. This method is, however, cumbersome, time-consuming and expensive, and in recent years only a minority of reports on the stimulation of collateral artery growth has presented such microsphere-based data. A more frequently used method is capillary counting to detect angiogenesis. Also in-vivo hemodynamic measurements of pressure and/or flow have been applied. In the present paper we compared different methodologies with the gold-standard microsphere technique and tested the accuracy of these methods to detect changes in the capacity of the collateral circulation. These methodologies were applied in the well-documented model of rabbit femoral artery ligation. Animals were treated with either monocyte chemoattractant protein-1 (MCP-1), leptin or phosphate buffered saline (PBS). MCP-1 is one of the best documented and most potent accelerators of arteriogenesis, functioning most probably via the attraction of monocytes which are known to play a prominent role during arteriogenesis [4–6].

Leptin has been reported to stimulate angiogenesis [7] but has not been evaluated previously for its arteriogenic properties. This adipocytokine exerts stimulatory effects on monocytes, increasing inflammatory cytokine and cell surface marker production [8,9] and enhances monocyte survival via anti-apoptotic effects [10]. These mechanisms have been shown to play a key role during arteriogenesis [11], prompting us to test the effects of leptin on collateral artery growth in the rabbit hind limb model.

2. Materials and methods

2.1. Flow cytometric analysis of monocyte inflammatory markers

To test the biological activity of recombinant human leptin on rabbit tissue, whole blood samples of rabbits were collected in heparinized tubes. Samples were incubated for 2 h at 37 °C and 5% CO₂ in the presence of either PBS, 400 ng/ml recombinant human MCP-1 (Peprotech, London, UK), 400 or 800 ng/ml recombinant human leptin (R&D Systems, Wiesbaden, Germany) or 200 ng/ml LPS (Sigma, Deisenhofen, Germany) as a positive control. After stimulation, the samples were stained for the monocyte marker CD14 (PE-conjugate, Dako, Glostrup, Denmark) and the monocyte activation marker CD11b (FITC-conjugate, Research Diagnostics, Flanders, NJ, USA). Monocyte activation was quantified using flow cytometry. Blood from human healthy volunteers was handled in the same manner to compare the effects on rabbit monocytes with those on human monocytes.

2.2. Animal model

The present study was performed with the permission of the State of Baden-Wuerttemberg, Regierungspraesidium Freiburg, according to Section 8 of the German Law for the Protection of Animals. It conforms 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).

Forty-two New Zealand White Rabbits were randomly assigned to receive either recombinant human MCP-1 (0.5 μg/kg/day, n=16), recombinant human leptin (0.1 mg/kg/day, n=12) or PBS (n=14) as control after occlusion of the right femoral artery. Substances were administered via an intra-arterial catheter implanted into the proximal stump of the occluded artery and connected to an osmotic minipump (2 ML-1, delivery rate 10 μl/h, Alzet, Cupertino, CA, USA) that was placed subcutaneously in the right lower abdomen. Animals were anesthetized with an injection of ketamine hydrochloride and xylazin. Under sterile conditions, the femoral artery was exposed and cannulated with a sterile polyethylene catheter. The tip of the catheter was placed distal to the deep femoral artery as well as the femoral circumflex artery to ensure substance delivery into feeding arteries of the collateral circulation. After careful suturing of the incision, animals were housed individually with free access to chow and water.

2.3. Flow and pressure measurements

After 7 days, animals were anesthetized again and underwent tracheotomy for artificial ventilation and continuation of narcosis by volatile anesthetics (isoflurane). One hour before the start of parameter recording, the catheter of the osmotic minipump was ligated to stop compound infusion. The right common carotid artery was cannulated with a polyethylene catheter (inner diameter 1.0 mm, outer diameter 1.5 mm) for pressure measurements. Similarly, both anterior saphenous arteries were cannulated with catheters (inner diameter 0.58 mm, outer diameter 0.96 mm). Catheters were connected to pressure transducers (Spectramed, Statham Instruments, Oxnard, CA, USA). After median laparotomy the aorta and the bifurcation of the common iliac arteries were dissected and exposed, and perivascular flow probes (1 mm, 1 RB, Transonic, Ithaca, NY, USA) were placed around the common iliac arteries for flow analysis. Pressure and flow were measured under resting conditions. Reactive hyperemia, i.e. vasodilation,
was achieved by clamping the aorta 1–2 cm cranial of the bifurcation for a period of one minute, completely blocking flow to the lower extremities during this period. Hemodynamic parameters were analyzed directly after opening of the clamp during a period of 5 s under maximal reactive hyperemia. Parameters were continuously recorded on a computerized system (PowerLab, ADInstruments, Colorado Springs, USA). All measurements were repeated three times and a mean value was used for further analysis. Flow ratio of right versus left hind limb was calculated. Ankle-brachial index (ABI) was calculated by dividing peripheral pressure (PP), which was derived from the saphenous artery, and systemic pressure (SP), which was derived from the carotid artery. Flow/pressure-deficit ratio (FPDR) was defined as the extrapolated iliac flow at a pressure deficit over the stenosis of 100 mm Hg. Formulas are shown in Table 1. Flow–pressure curves and conductance measurements under pump-controlled adenosine-induced maximal vasodilation were performed as previously described, using fluorescent microspheres [5].

2.4. Immunohistochemistry

For immunohistochemistry, animals were sacrificed and tissue was harvested from the hind limbs. Tissue was directly frozen and stored at −80 °C. 5 µm sections were cut with a cryomicrotome (Leica, Bensheim, Germany) and fixed in acetone. For quantitative analysis, a total of 20 sections from the M. vastus intermedius (M. quadriceps) were cut for analysis of vessel proliferation, while 36 sections were cut from the peroneus muscle as well as quadriceps muscle for capillary counts. The muscles were divided into four equal parts, with 5 µm sections taken from each part and with a 15 µm distance between sections. Sections were placed on cationic coated slides (Superfrost Plus, MJ Research, Waltham, MA, USA). To detect proliferation, slides were incubated with a primary mouse anti-rat antibody against Ki-67 (clone MIB-5, Dako), after antigen retrieval with citrate buffer (pH=6.0), and a mouse anti-rat antibody against Ki-67 (clone 1 A4, FITC-labeled, Sigma). The nuclear protein detected by the Ki-67 antibody is only expressed in proliferating cells and plays a role in DNA synthesis [12]. Immunohistochemical quantification of Ki-67 has been shown to be a valuable technique for the assessment of proliferation [13]. For capillary countings, slides were incubated with a mouse anti-human antibody against CD31 (DAKO, clone JC 70A), Goat anti-mouse Cy3 (Amersham Biosciences, Freiburg, Germany) was used as secondary antibody. Nuclei were stained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA). Negative controls were performed by omitting the first antibody. Microscopic imaging was done with a Leica camera at a magnification of 200× (capillary counts) or 400× (Ki-67). All quantitative analyses were performed in a blinded manner. Proliferation indices of endothelial and smooth muscle cells of collateral arteries were calculated. For capillary counting, both total number of capillaries per area (mm²) as well as capillary/fiber ratio were calculated.

2.5. Quantitative polymerase chain reaction on collateral arteries

To visualize collateral arteries in the quadriceps and adductor muscle, undiluted warm (37 °C) latex (Chicago Latex Products no. 563, Crystal Lake, IL, USA) was infused into the distal abdominal rabbit aorta at a constant pressure of 120 mm Hg. This contrast agent enables visual identification of the vessels; its elastic properties allow a gentle excision without damaging the artery. Three collateral arteries from the vastus intermedius muscle, a muscle which normally contains two large collateral anastomoses, and the adductor muscle, where dissected and immediately snap frozen in liquid nitrogen to prevent RNA degradation. Dissected vessels were then immersed into RLT (Qiagen, Hilden, Germany) buffer solution, mechanically homogenized and incubated in proteinase K solution. RNA was isolated with minispin columns according to the manufacturer’s instructions (Qiagen). An additional DNase digestion step was introduced to remove DNA contaminations: DNase (Qiagen) (27.3 Kunitz units) was solved in RNase-free water, directly added onto the spin-column membrane and

### Table 1
Hemodynamic parameters one week after right femoral artery occlusion and treatment with PBS, MCP-1 or leptin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS</th>
<th>MCP-1</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (at rest)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flow right [ml/min]</td>
<td>8.1±0.7</td>
<td>9.4±1.1</td>
<td>9.30±1.0</td>
</tr>
<tr>
<td>flow left [ml/min]</td>
<td>10.8±0.7</td>
<td>12.3±1.3</td>
<td>12.1±1.0</td>
</tr>
<tr>
<td>flow ratio no unit</td>
<td>0.75±0.06</td>
<td>0.76±0.03</td>
<td>0.77±0.05</td>
</tr>
<tr>
<td>Reactive Hyperemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flow right [ml/min]</td>
<td>17.4±1.0</td>
<td>23.1±1.1</td>
<td>21.6±0.8</td>
</tr>
<tr>
<td>flow left [ml/min]</td>
<td>31.2±0.9</td>
<td>30.0±1.4</td>
<td>31.3±1.3</td>
</tr>
<tr>
<td>flow ratio no unit</td>
<td>0.56±0.07</td>
<td>0.77±0.06</td>
<td>0.69±0.04</td>
</tr>
<tr>
<td>ABI no unit</td>
<td>0.27±0.02</td>
<td>0.34±0.02</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>FPDR [ml/min/100 mm Hg]</td>
<td>39.5±3.6</td>
<td>51.8±2.8</td>
<td>52.13±2.36</td>
</tr>
</tbody>
</table>

Whereas no effects were apparent at rest, MCP-1-treatment resulted in a significant increase in flow, flow ratio, ABI and FPDR measured during reactive hyperemia. Leptin-treatment also resulted in a significant increase in flow, flow ratio, FPDR, but not in ABI. Flow ratio=flow right iliac artery/flow left iliac artery. Ankle-brachial-index (ABI)=right peripheral pressure (PP)/systemic pressure (SP). FPDR=100×flow right iliac artery/(SP-PP) (p-values as compared to PBS).
incubated for 15 min at room temperature. Quantity of obtained RNA was assessed by spectrophotometric analysis with a RiboGreen® reagent (excitation ~480 nm, emission ~525 nm, Molecular Probes, Eugene, OR, USA). Total RNA (1 μg) was mixed with 1 μg of random primer (Promega, Madison, WI, USA) and heated at 70 °C for 10 min. Then samples were stored on ice, centrifuged, and reverse-transcribed in the presence of 1 μl per 2 μl reaction volume PowerScript® Reverse Transcriptase (Clontech, Palo Alto, CA, USA). Reactions were carried out at 42 °C for 30 min in 20 μl buffer consisting of 5× first-strand buffer (Clontech), 2 μl 100 mM DTT, and 2 μl 10 mM dNTP mixture (Peqlab, Erlangen, Germany). The reaction was stopped by heating the samples for 15 min at 70 °C, and the resulting cDNA was diluted 1:50 with sterile water.

Real-time PCR was performed as recently described [14]. In short, diluted cDNA (9 μl) was pipetted into a single well of a 96-well reaction plate (Applied Biosystems, Weiterstadt, Germany) for PCR with the PRISM-7700 (Applied Biosystems). Samples were mixed with 11 μl SYBR-green PCR master mix (Applied Biosystems) and 1 μl each of gene-specific primer pairs (5 pM). Primers were designed using Primer-3 [15] with a melting temperature of 59 °C to generate products of ~80 bp sizes. The following primers were used: murine/human Ki-67 [forward 5′-CAA CTT TGG TGA TTC CAT TA-3′, reverse 5′-TTA GGA GGC AAG TTT TCA TC-3′]; rabbit histone 3 [forward 5′-GGG TGA AGA AAC CTC ATC GT-3′, reverse 5′-CGA ATC AGA GCG TCA GTG GA-3′]; rabbit 18srRNA [forward 5′-CGG ACA GGA TTG ACA GAT TG-3′, reverse 5′-CAA ATC GCT CCA CCA ACT AA-3′]. A typical PCR protocol took ~2.5 h to complete, including a 10 min denaturation step followed by 40 cycles (95 °C denaturation for 1 min, 60 °C annealing and extension step for 1 min). The quantification data were analyzed with sequence detection software (ABI PRISM-7700 Version 1.7). Specificity of the primers to generate a single product was tested routinely by agarose gel electrophoresis and ethidium bromide staining.

2.6. Evaluation of nitric oxide production

Human umbilical vein endothelial cells (HUVECs, Promocell, Heidelberg, Germany) were cultured in endothelial cell growth medium (Promocell) containing 0.4% endothelial cell growth supplement/heparin, 2% fetal calf serum (FCS), 0.1 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 1 ng/ml basis fibroblast growth factor, 50 ng/ml amphotericin B, and 50 μg/ml gentamicin at 10% CO₂ and 37 °C until cell confluency. Cells were washed with PBS and starved with endothelial cell basal medium containing 0.5% FCS and 1% penicillin/streptomycin for 24 h.

After stimulation with 800 ng/ml leptin for 1 h or 16 h, 10 μmol/l of the cell-permeable diaminofluorescein-2 diacetate (DAF-2DA, AG Scientific, San Diego, CA, USA) was added and cells were further incubated in red phenol-free Dulbecco’s modified Eagle medium (Promocell) for 30 min. DAF-2DA reacts with nitric oxide (NO) forming a green-fluorescent triazolofluorescein, quantified by flow cytometry [16].

2.7. Statistical analysis

Results are expressed as mean±SEM. Comparisons between different groups were performed using one-way analysis of variance (ANOVA) test after testing for normal distribution. A p-value <0.05 was considered statistically significant.

3. Results

The study protocol was completed in all animals. We observed no gross impairment or necrosis in the right leg or foot during the days following the operation.

3.1. Monocyte inflammatory marker expression

In-vitro stimulation of rabbit blood showed an increased expression of CD11b on monocytes upon both MCP-1 as well as leptin stimulation (Fig. 1a): [arbitrary fluorescence

![Fig. 1. Monocyte Mac-1-expression (heterodimer CD11b/CD18) increased dose-dependently in both rabbit (A) as well as human (B) tissue after leptin and MCP-1 in-vitro stimulation as shown by flow cytometric analysis of the expression of the αM subunit CD11b [arbitrary fluorescence units].](https://academic.oup.com/cardiovascres/article-abstract/64/2/356/322303)
3. Hemodynamic parameters

Flow measurements at rest as assessed with ultrasonic flow probes did not show any difference between the treatment groups. Both total inflow into the right extremity as well as flow ratio (PBS 0.75±0.06; MCP-1 0.76±0.03; leptin 0.77±0.05) remained unchanged.

In contrast, flow during reactive hyperemia significantly increased after 7 days treatment with MCP-1. Accordingly, flow ratio (PBS 0.56±0.07 vs. MCP-1 0.77±0.06, no unit, p < 0.001) as well as ABI increased. Finally, FPDR increased significantly in the MCP-1 treated animals. With the gold-standard method of microsphere-based conductance measurements under adenosine-mediated vasodilation the effects of MCP-1 were most clearly detectable (PBS 50.8±2.1 vs. MCP-1 225.8±8.8 ml/min/100 mm Hg, p < 0.001, Fig. 2). Leptin administration also led to a significant increase in total inflow into the right hind limb as well as to an increase in flow ratio (leptin 0.69±0.04, p < 0.05 vs. PBS) during reactive hyperemia. Change in ABI was not significant. FPDR increased in leptin-treated animals. In contrast, microsphere-based conductance measurements did not show any difference between leptin and PBS treated animals (leptin 54.7±5.1 ml/min/100 mm Hg, p = ns vs. PBS, Fig. 2) (see also Table 1 for hemodynamic data).

3.3. Immunohistochemistry

Ki-67 staining revealed a significant increase in proliferating smooth muscle cells in collateral arteries of MCP-1-treated animals (PBS 24.7%±1.8%; MCP-1 32.0%±1.0%, p < 0.01). Leptin-treatment, however, did not lead to an enhanced proliferation index when compared to PBS (leptin 22.7±0.6 %, p = ns vs. PBS, Fig. 3). Proliferation of collateral artery endothelium rose accordingly after treat-
ment with MCP-1, but not with leptin (PBS 20.4%±1.1%; MCP-1 24.5%±0.9% (p<0.05 vs. PBS); leptin 20.0% ±1.4% (p=ns vs. PBS)).

Capillary/fiber ratios were not significantly different between the control and the treated groups, neither when assessed in the peroneus muscle (PBS 1.4±0.1, MCP-1 1.5±0.1, leptin 1.3±0.1 capillaries per muscle fiber, p=ns for all comparisons between groups), nor when assessed in the quadriceps muscle (PBS 2.3±0.1, MCP-1 2.1±0.1, leptin 2.0±0.1 capillaries per muscle fiber, p=ns for all comparisons between groups, Fig. 4). Assessment of absolute capillary numbers instead of capillary/fiber ratios provided similar results (data not shown).

3.4. Gene expression analysis

Using real-time PCR, an 8.8±3.1-fold (p<0.01) increase in mRNA content of Ki-67 was found in proliferating collateral arteries as compared to arteries harvested from the sham-operated side. Treatment with MCP-1 led to a 25.5±8.1-fold (p<0.005) increase in Ki-67 mRNA as compared to control (Fig. 5). Histone 3-mRNA, which was analyzed as a second marker of proliferation, could not be found upregulated after 7 days of collateral vessel growth (ratio proliferating/resting vessel 1.4±0.5 (p=ns), ratio MCP-1/no treatment 0.9±0.2 (p=ns)).

3.5. Nitric oxide production

Stimulation of HUVECs with leptin for 1 h did not result in a change in NO production as assessed by quantification of triazolofluorescein in FACS analysis: [arbitrary fluorescence units] PBS 214.2±4.8; 400 ng/ml leptin 202.3±14.1; 800 ng/ml leptin 200.5±6.2 (p=ns for comparison between all groups). A longer incubation period (16 h) did not result in an increase in NO production either (data not shown).

4. Discussion

In vitro application of leptin dose-dependently increased CD11b expression on rabbit monocytes, proving evidence of the biological activity of recombinant human leptin in the rabbit. Hemodynamic pressure–flow measurements in reactive hyperemia showed increased collateral flow in both the MCP-1 as well as the leptin treated groups. However, in contrast to the MCP-1 treated animals, collateral conductance as measured with the gold standard microsphere technique was not increased in leptin treated animals, and immunohistochemistry showed no increased mitogenic activity of vascular wall cells. Thus, whereas the effects of MCP-1 on collateral flow can be attributed to active proliferation of collateral arteries, the effects of leptin on collateral flow are not related to vascular growth. As known from the literature, leptin exerts beneficial effects on endothelial function, providing an alternative explanation for the observed increase in hyperemic collateral flow.

Severe obstruction or occlusion of conductance arteries leads to a drop in tissue perfusion and organ function. The development of a collateral circulation can compensate for this loss in perfusion and hence constitutes a potential target for pharmacological modulation as a treatment for peripheral or coronary artery disease. The experimental detection of tissue perfusion via microsphere injection remains the gold standard in several preclinical studies on the stimulation of collateral artery growth [3,5]. However, the detection...
of perfusion with the microsphere-method is cumbersome, time-consuming and expensive. In the present study we evaluated the effects of MCP-1 and leptin on collateral flow, using both hemodynamic parameters measured during reactive hyperemia as well as microsphere-based calculations of collateral conductance employing an extracorporal pump-driven shunt.

This study shows the paramount importance of the methodology applied when testing substances for their influence on collateral flow and stimulation of collateral artery growth. While no changes could be detected at rest, hemodynamic measurements during hyperemic flow permitted the detection of the pro-arteriogenic influence of MCP-1. Leptin also increased hyperemic collateral flow. However, using the microsphere-based method, no effects were found for leptin on conductance of the collateral circulation, indicating that the maximal capacity of the collateral circulation, as measured during pharmacologically induced vasodilation and externally driven circulation, is not influenced by leptin. Direct effects on vascular smooth muscle cell proliferation by leptin were excluded by immunohistochemical data, showing no increase in Ki-67 expression in collateral arteries of leptin-treated animals. On the endothelial cell level (vessel intima), in-vivo application of leptin did not increase expression of Ki-67 either. Thus, while the adipocytokine has been reported to stimulate angiogenesis [17], it has no mitogenic properties during collateral artery growth and the leptin-induced effects on blood flow are related to mechanisms other than angiogenesis. The increase in hyperemic collateral flow after leptin treatment without vascular proliferation rather indicates improvement of endothelial function. Endothelial function can be assessed via pharmacologically induced vasodilation [18]. Alternatively, endothelial function can be examined by enhancing flow after release of an arm cuff, which leads to an increase in shear stress and a release of nitric oxide, and finally results in vasodilation [19,20]. Thus, assessment of reactive hyperemia as applied in the present model permits the evaluation of the vasodilatory reserve and endothelial function.

The leptin receptor has not only been found on central nervous tissue but also on endothelial cells [21]. Previous studies have elucidated the role of leptin in the modulation of vascular tone [22–26]. In-vitro vasorelaxation of aortic rings after leptin application can be detected [23]. In-vivo observations in both the coronary and the peripheral circulation prove vasodilation after infusion of leptin in patients [24,26]. The mechanisms leading to enhanced vasodilation after application of leptin are controversially discussed. Experimental in-vivo studies indicate enhanced production of nitric oxide after leptin application as assessed by quantification of NO metabolites [27,28]. In-vitro analyses also indicate NO-dependent arterial relaxation as vasodilatory effects of leptin were abolished after application of L-NAME, a strong inhibitor of nitric oxide synthesis [29]. Increased Akt-phosphorylation of endothelial nitric oxide synthase is suggested as a mechanism leading to augmented NO production [30]. In contrast, Lembo et al. [23] conclude that leptin-induced vasodilation at the level of resistance vessels is NO-independent and regulated by endothelium-derived hyperpolarizing factor (EDHF). This is further supported by observations in patients where leptin-induced vasodilation remained unchanged after administration of L-NAME [24,26]. Brenetti et al. demonstrated that leptin stimulates prostaglandin E2 and F2alpha and did not have an effect on NO production [31]. Our findings support the hypothesis that application of leptin results in enhanced vasodilation via enhanced production of EDHF, prostaglandin E2 or F2alpha, but not via increased endothelial cell production of nitric oxide.

In the clinical setting, the assessment of endothelial function is important in patients with atherosclerotic disease. Improving endothelial function and thus reactive hyperemia is one of the major therapeutic goals in atherosclerotic patients, since endothelial dysfunction is closely linked with arterial occlusive disease and atherosclerosis [32,33]. Notwithstanding the importance of endothelial function, stimulation of collateral artery growth via vascular proliferation has been the subject of most experimental and clinical studies aiming at the improvement of collateral flow. Collateral flow is strongly influenced by parameters like blood pressure, total inflow and vascular tone. Vasodilation is important for reliable comparison of different pro-arteriogenic substances as it rules out errors due to differences in vascular tone. To reach absolute vasodilation in the experimental setting, pharmacological modulation with adenosine or papaverine is required. However, dosages applied must be higher than the animal’s circulatory mechanisms of compensation would tolerate, making the installation of an extra-corporal circulation mandatory [5]. In this situation of absolute vasodilation, the increase in blood flow due to an improved endothelial function by substances that act primarily via vasoactive effects, for example prostaglandin-analoga, cannot be evaluated. However, when focusing on genuine collateral vessel proliferation, pump-driven application of microspheres under maximal vasodilation and calculation of conductance has to be considered the gold standard.

Other studies have relied on investigations of capillary density as a parameter to detect increased collateral flow. Capillary number per unit area is subject to uncontrollable variability, as large differences can be observed in different muscle tissue [34] (red vs. white, slow vs. fast twitch muscle), even within the same muscle [35]. A somewhat better parameter is the ratio of capillaries per muscle fiber. It corrects for differences in the angle at which tissue has been sectioned and for changes due to atrophy or edema, but it should be noted that the above mentioned shortcomings still apply. We assessed both capillary number per area and capillary/fiber ratio in the lower leg (peroneus muscle) and the thigh (quadriceps muscle). Our results, in accordance with Hershey et al. [36], indicate a dissociation between...
arteriogenesis and capillary growth: despite the demonstration of increased collateral growth after MCP-1 treatment, no increase in capillary numbers in either the upper or lower leg was found. Similar results were obtained when calculating capillary/fiber ratio instead of total capillary numbers. It has to be noted that the experimental model used is not ischemic, thus lacking hypoxia as the most important stimulator of angiogenesis [37].

In contrast to capillary counting, the proliferation marker Ki-67 was sensitive enough to detect the pro-arteriogenic effects of MCP-1. However, this might not be the case for compounds exerting only a weak pro-arteriogenic action. We also report here for the first time the possibility of detecting increased Ki-67 mRNA expression in growing collateral arteries by real-time PCR. Histone 3 gene expression was not found to be significantly upregulated after 7 days. Although this parameter is also used to detect proliferation, e.g. in tumor cells [38], post-transcriptional regulation of gene expression can cause varying results [39]. Moreover, for such gene expression analyses, the time point of measurement will significantly influence results due to the large variation in mRNA expression over time during the process of arteriogenesis. In this respect it is of interest to note that Ki-67 mRNA persists for a relatively long time in proliferating cells [12].

Mac-1 is required for monocyte adhesion and thus this receptor plays a central role during arteriogenesis [40]. Indeed, hitherto known pro-arteriogenic substances increase expression of CD11b, as part of the Mac-1 integrin receptor, on monocytes [41,42]. According to previous reports as well as our own findings, leptin also increases CD11b expression on rabbit and human monocytes [43,44], which suggested pro-arteriogenic properties. However, as shown in the present study, leptin displays no effects on arteriogenesis. These findings once again stress the importance of functional in-vivo studies for complex processes such as vascular growth.

5. Conclusion

Using hemodynamic flow and pressure measurements during reactive hyperemia, previous findings on the stimulatory effect of MCP-1 on arteriogenesis were confirmed. Leptin treatment after femoral artery occlusion also resulted in increased hindlimb flow. In contrast to MCP-1, however, these effects were not related to active proliferation of collateral arteries in the rabbit hindlimb model. Our data show that increased collateral flow does not necessarily indicate active proliferation of collateral arteries. To unequivocally establish pro-arteriogenic effects of a given compound, flow-pressure measurements in reactive hyperemia in combination with histological studies using proliferation markers, or pump-controlled microsphere-based measurements of collateral flow under conditions of absolute vasodilation are required.

References


