Time course of arteriogenesis following femoral artery occlusion in the rabbit

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Abstract

Objective: We examined the time course of arteriogenesis (collateral artery growth) after femoral artery ligation and the effect of monocyte chemoattractant protein-1 (MCP-1).

Methods: New Zealand White rabbits received MCP-1 or phosphate buffered saline (PBS) for a 1-week period, either directly or 3 weeks after femoral artery ligation (non-ischemic model). A control group was studied with intact femoral arteries and another 1 min after acute femoral artery ligation.

Results: Collateral conductance index significantly increased when MCP-1 treatment started directly after femoral artery ligation (acute occlusion: 0.94±0.19; without occlusion: 168.56±15.99; PBS: 4.10±0.48; MCP-1: 33.96±1.76 ml/min/100 mmHg). However, delayed onset of treatment 3 weeks after ligation and final study of conductance at 4 weeks showed no significant difference against a 4-week control (PBS: 79.08±7.24; MCP-1: 90.03±8.73 ml/min/100 mmHg). In these groups increased conductance indices were accompanied by a decrease in the number of visible collateral vessels (from 18 to 36 identifiable vessels at day 7 to about four at 21 days).

Conclusion: We conclude that the chemokine MCP-1 markedly accelerated collateral artery growth but did not alter its final extent above that reached spontaneously as a function of time. We show thus for the first time that a narrow time window exists for the responsiveness to the arteriogenic actions of MCP-1, a feature that MCP-1 may share with other growth factors. We show furthermore that the spontaneous adaptation by arteriogenesis stops when only about 50% of the vasodilatory reserve of the arterial bed before occlusion are reached. The superiority of few large arterial collaterals in their ability to conduct large amounts of blood flow per unit of pressure as compared to the angiogenic response where large numbers of small vessels are produced with minimal ability to allow mass transport of bulk flow is stressed.

Keywords: Arteries; Blood flow; Collateral circulation; Macrophages; Microcirculation

1. Introduction

In the adult, blood vessels can grow either via the process of angiogenesis or via the process of arteriogenesis [1–4]. Angiogenesis refers to the sprouting of endothelial cells from pre-existing vessels, thereby forming new capillary networks. One of the main stimulants of this process is hypoxia, increasing the transcription of angiogenic growth factors such as vascular endothelial growth factor (VEGF), which is a known but weak mitogen for endothelial cells [5], via the oxygen dependent regulation of the nuclear protein HIF (hypoxia inducible factor) [6]. Arteriogenesis in contrast occurs independent from ischemia and refers to the proliferation of pre-existing arteriolar connections into functional collateral arteries. When a main artery develops a hemodynamically relevant stenosis, causing a fall in intravascular pressure in the dependent vasculature, blood flow is re-distributed through these interconnecting arterioles, significantly increasing shear stress, which is in turn leading to activation of the endothelium and upregulation of cell adhesion molecules (ICAM, VCAM, selectins) [7–11]. Circulating mononuclear blood cells are attracted by the activated endothelium.
attach to the CAMs and migrate into the vessel wall, giving rise to the production of various cytokines and growth factors (e.g. bFGF, TNF-α, GM-CSF and MCP-1) [2,9,12,13], finally leading to proliferation and growth of the collateral vasculature. Monocyte chemoattractant protein-1 (MCP-1) has been shown to stimulate the process of arteriogenesis [14] via an increased attraction of circulating monocytes to sites of proliferating collateral arteries.

In comparison to angiogenesis, stimulation of arteriogenesis is probably the more efficient process to replace an occluded artery, because Newtonian flow is related to the fourth power of the vessel’s radius, which means that small changes in the diameter of a collateral vessel result in large changes in blood flow. Thus, the increase in blood flow to potentially ischemic tissue, as caused by the development of two or three large collateral arteries, cannot be equaled by newly formed capillaries, however numerous.

One of the most important questions with regard to therapeutic arteriogenesis is that of responsiveness to the agent applied. This first increases but rapidly decreases with time after arterial occlusion. Blood flow measurements in rats showed that 2 weeks after femoral artery ligation the increase in collateral blood flow had stopped. When treated with basic fibroblast growth factor (bFGF), collateral flow increased during the first week about two-fold but did not change significantly in the following weeks [15] although full vasodilatory reserve was not yet reached. These findings and those of Unger [16] and our present results clearly show that therapeutic arteriogenesis has only a limited time-window. Therefore knowledge of the time course of arteriogenesis in any given species is of great importance in order not to miss the responsive cycle of the time window.

Furthermore, weaknesses exist in the experimental evaluation of the time factors as they are influenced by pharmacological agents because of the paucity of sensitive and quantitative methods to study changes as a function of time. Methods developed to provide functional measurements are Laser-Doppler imaging, infrared thermography and oxymetry. In the rabbit hindlimb model these measurements are subjected to considerable method- and operator variability. Furthermore, these techniques measure only derivatives of hindlimb perfusion like flow- or erythrocyte velocity, skin circulation, or skin oxygenation, instead of total hindlimb perfusion.

The only well established and described method to measure directly hindlimb perfusion in vivo after femoral artery occlusion is the use of radioactive tracer microspheres [17,18]. Since their introduction, fluorescent microspheres successfully replaced radioactive tracers in many experimental settings [19–21] and have been shown to be superior in handling, accuracy and reproducibility [22].

In the rabbit hindlimb four different microspheres were used to study the effects of MCP-1 on arteriogenesis in an ex vivo model [14]. In the present study, we measured hindlimb perfusion after femoral artery occlusion as a function of time using six differently labeled fluorescent microspheres at different perfusion pressures at maximal vasodilation. In addition we will describe our experience with a high resolution, low keV X-ray angiography in the rabbit hindlimb model of arteriogenesis.

2. Methods

2.1. Animal model

The present study was performed with the permission of the State of Hessen, Regierungspresidium Darmstadt, according to Section 8 of the German Law for the Protection of Animals. It conforms to 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).

Seventy-two New Zealand White Rabbits (NZWR) were randomly assigned to one of six groups (n=12 each). After ligation of the femoral artery, two groups received either phosphate buffered saline (PBS) or monocyte chemoattractant protein-1 (MCP-1) (0.2 μg/kg/day) locally via an osmotic minipump for 1 week. To study the long-term development of collateral arteries, animals of groups 3 and 4 were treated with either PBS or MCP-1 for a 1-week period, 3 weeks after ligation. In the fifth group of 12 animals, the femoral artery was ligated directly before the final experiment. To obtain the normal conductance index value and angiographic appearance of the vascular tree of the rabbit hindlimb a sixth group was evaluated without femoral artery ligation. For the implantation of the osmotic minipumps (Model 2 ML 1, Alza Corp., Palo Alto, CA, USA), the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight) and xylazine (8 mg/kg body weight). Supplementary doses of anesthetics (10–20% of the initial dose) were given intravenously as needed. All surgical procedures were performed under sterile conditions. After incision of the skin, the femoral artery was dissected, exposed and cannulated with a sterile polyethylene catheter (inner diameter: 1 mm; outer diameter: 1.5 mm). The catheter was placed with the tip positioned approximately 1 cm distal to the branches of the artery circumflexa femoris and the artery profunda femoris, pointing upstream in order to deliver the substances continuously and during first-pass into the collateral circulation. The incision was sutured carefully to prevent self-mutilation of the animals. Animals received 0.5 ml of intramuscular tetracycline as an antibiotic prophylactic. After the surgical procedure, the animals were housed individually with free access to water and chow and were allowed to move freely. There were no signs of any gross impairment or...
necrosis. For final experiments animals of each group were randomly assigned to either angiographic or hemodynamic measurements.

2.2. Post-mortem angiograms

After the treatment period the animals were sacrificed. The distal descending aorta was cannulated (inner diameter: 2.2 mm; outer diameter: 2.5 mm) and briefly perfused with a buffer containing adenosine at a concentration of 1 mg/kg to achieve maximal vasodilation. To prevent premature gelling, all solutions and the animal itself were kept in a water-bath at 37°C. The rinsing procedure was followed by an 8-min infusion with a contrast medium based on bismuth and gelatin [23] at a pressure of 80 mmHg. Subsequently, the contrast medium was allowed to gel by placing the hindlimbs on crushed ice for 60 min. Angiograms of each single hindlimb were taken at two different angles in a Balteau radiography apparatus (Machlett Laboratories) at 30 keV accelerating voltage using a single-enveloped Structurix D7DW film (Agfa). For quantification of visible collateral arteries the resulting angiograms were assessed in a single blinded fashion under stereoscopic viewing in three dimensions. Only vessels indubitably showing a defined stem-, mid- and re-entrant zone according to the Longland classification [24] were counted and marked to make sure that no vessel was counted twice.

2.3. Hemodynamic measurements

For hemodynamic measurements, the animals were again anaesthetized using the same concentrations as listed above. Animals were heparinized with a bolus injection of 5000 Units heparin. The animals were ventilated after tracheotomy and the anesthesia was deepened with a continuous infusion of pentobarbital (12 mg/kg/h). Therefore the jugular vein was dissected and cannulated with a polyethylene catheter (inner diameter: 1 mm; outer diameter: 1.5 mm). For installation of a pump-driven shunt, the carotid artery was cannulated (inner diameter: 2.2 mm; outer diameter: 2.5 mm). The arteria saphena magna which corresponds to the anterior tibial artery in humans and is the main arterial supply to the lower limb and foot in the rabbit, was exposed just above the ankle and cannulated with a polyethylene catheter (inner diameter: 0.58 mm; outer diameter: 0.96 mm). For sampling of the microsphere reference, the left femoral artery was exposed and cannulated with a polyethylene catheter (inner diameter: 1 mm; outer diameter: 1.5 mm). The distal abdominal aorta was cannulated (inner diameter: 2.2 mm; outer diameter: 2.5 mm) and a pump-driven shunt between the carotid artery and the aorta was installed to perfuse both hindlimbs. Catheters of the saphenous arteries and the cannula of the aorta were connected to Statham P32DC pressure transducers (Statham, Spectramed). A cannulating ultra-sound flow-probe was installed to measure total flow to both hindlimbs. Pressures and total flow were continuously recorded on a computerized recordings system (MacLab, MacIntosh) from which they were later retrieved for further analysis. To achieve maximum vasodilation adenosine (Sigma Chemical Company, St. Louis, MO) was continuously infused (1 mg/kg/min) into the shunt system.

2.4. In vivo pressure-flow relations

After stabilization of peripheral and systemic pressures both legs were perfused at six different systemic pressure levels between 45 and 75 mmHg, using a roller pump (Stoeckert) installed in the above-mentioned shunt between carotid artery and abdominal aorta for maintenance of stable flow levels between 53 and 110 ml/min (Fig. 1). At each resulting different pressure level, differently labeled fluorescent microspheres (either scarlet, crimson, red, blue-green, orange, yellow-green; diameter: 15 μm; Molecular Probes, Eugene, Oregon, USA) were injected into a mixing
chamber installed in the shunt system. From the left femoral artery a blood sample was withdrawn for 3 min at a rate of 0.6 ml/min as a flow reference for each single microsphere.

2.5. Counting of microspheres

The following muscles were dissected from the leg: quadriceps, adductor longus, adductor magnus, gastrocnemius, soleus, plantaris and peroneal muscles. Each muscle was divided into three consecutive samples (~0.5 g) from the proximal to the distal end. The whole muscle and afterwards each muscle sample were weighted. The samples were then homogenized and placed loosely in 12×75 mm tubes (Becton Dickinson, Lincoln Park, NJ). To each of the tissue samples and to the blood flow reference samples the following was added: 3 ml of a proteinase/SDS solution [SDS stock solution: 1% SDS, 0.5% sodium azide (both Sigma Chemical Company, St. Louis, MO) and 0.8% Tween-80 (Fisher Scientific, Fairlawn, NJ) in 50 mM pH 8 Tris buffer (Sigma Chemical Company, St. Louis, MO)] and 1 mg/ml proteinase K (Boehringer Mannheim Corp.). Blue microspheres (4000/ml, diameter: 15 µm; Molecular Probes, Eugene, Oregon, USA) were used as an internal standard. Each tube was capped and secured in a shaking water bath at 50°C for 24 h. All samples were then centrifuged at 1000×g for 30 min, the supernatant was pipetted off and the pellet was resuspended in 1 ml CellWash (Becton Dickinson, Lincoln Park, NJ). Directly before FACS analysis the probes were rigorously shaken. For microsphere counting, a flow-cytometer (FACSCalibur) equipped with a second laser and a detector for a fourth fluorescence was used. After FACS analysis each single microsphere was classified and counted with a computerized analysis system (Becton Dickinson, Lincoln Park, NJ). Flow for each sample was calculated from the number of microspheres in the sample (n), the respective microsphere count in the reference sample (nrs), the internal standard microsphere count in the sample (ISr) and in the reference sample (ISrs), the weight of the reference sample (w) and the time during which the reference sample was withdrawn (t) using the following equation:

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\text{Flow (ml/min)} = \frac{m_r \cdot IS_{rs} \cdot w}{IS_r \cdot n_r \cdot t}
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The left hindlimb was processed in the same manner as described above and served as an internal validation group.

2.6. Calculation of conductance indices

In our model, collateral arteries span from the arteria circumflexa femoris and the arteria profunda femoris to the arteria genualis and the arteria saphena parva. After femoral artery occlusion, these vessels provide the blood supply to the lower limb and the distal part of the adductor. Systemic pressures (SP) at the distal part of the abdominal aorta and peripheral pressures (PP) in the saphenous artery were measured. PP is the pressure in the re-entrant region and is identical to the pressure head of the circulation in the lower leg. Collateral flow is equal to the sum of blood flows to the tissues of the distal adductor muscle plus the flow to the tissue of the lower leg. Resistance of the collateral artery network was defined as the pressure difference between SP and PP divided by the collateral blood flow. Conductance is defined as the reciprocal value of vascular resistance and is a recognized parameter in vascular physiology. Because even after maximal vasodilation a positive pressure intercept is observable, all conductance indices were calculated from the slope of the pressure–flow relations.

2.7. Statistical analysis

Results are presented as means±standard deviation. Comparisons between two means were performed using the unpaired Student’s t-test. Analysis was performed using a computerized software package (Sigma Stat, Jandel Scientific). P<0.05 was considered to be statistically significant.

3. Results

No animals were lost during or after femoral artery ligation. We also did not observe any gangrene or gross impairment of hindlimb function after femoral artery occlusion. The body weights and body temperature within the different groups did not show any significant difference. There were no detectable differences in the plasma values of total protein, albumin, glutamic oxaloacetic transaminase and glutamic pyruvic transaminase.

3.1. Angiographic findings

Fig. 2a shows an angiogram of a rabbit hindlimb without femoral artery occlusion. Post-mortem angiograms immediately after occlusion of the femoral artery showed pre-existing interconnecting arteries spanning from the arteria profunda femoris and the arteria circumflexa femoris to the arteria genualis and the arteria saphena parva. These vessels did not show any corkscrew-like formation characteristic for collateral arteries (Fig. 2b). The ligation of the femoral artery led to a proliferation and growth of these pre-existing arterioles. One week after

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2The method of using fluorescent microspheres that were counted after tissue digestion in a FACS cytometer was developed by Dr Bruce Ito who shared his experience with us but did not publish it. The description of this method is done with his consent.
Fig. 2. Postmortem angiograms of rabbit hindlimbs without (a) and after acute femoral artery ligation (b). One week after femoral artery ligation, several collateral arteries spanning the occlusion site can be detected (c). Continuous infusion of MCP-1 for the same time period significantly increases collateral vessel density (d). Animals treated for a 1-week period 3 weeks after femoral artery occlusion showed no significant difference between PBS (c) and MCP-1 (f) infusion.
PBS infusion the number of visible collateral arteries significantly increased, showing the typical corkscrew formation (Fig. 2c). MCP-1 treatment for the same time period further increased the collateral vessel count (Fig. 2d). The high amount of relatively small collateral arteries declined during the remodeling process resulting in a lower number of vessels with a relatively large diameter both in the control group as well as in the MCP-1 treated group (Fig. 2e and f). Quantification of visible collateral arteries under stereoscopic viewing verified the radiographic appearance (number of angiographically visible collateral arteries: without occlusion: 6.66±1.17; after acute occlusion: 8.27±1.12; 1 week PBS: 16.16±1.40; 1 week MCP-1: 30.16±1.96; 3 weeks occlusion plus 1 week PBS: 10.15±0.98; 3 weeks occlusion plus 1 week MCP-1: 11.07±0.87) (Fig. 3).

3.2. Haemodynamic parameters

The normal conductance index of the arterial vessel bed in the non-occluded rabbit hindlimb was 168.56±15.99 ml/min/100 mmHg. One week after femoral artery ligation the collateral conductance index increased about 4-fold in comparison to the acute occlusion (0.94±0.19 ml/min/100 mmHg; 1 week PBS: 4.10±0.48 ml/min/100 mmHg). MCP-1, given intraarterially as a continuous infusion, significantly increased the collateral conductance index as compared to the PBS-treated group (1 week MCP-1: 33.96±1.76 ml/min/100 mmHg). Four weeks after ligation blood flow was further restored towards normal (3 weeks occlusion plus 1 week PBS: 79.08±7.24 ml/min/100 mmHg). At this time point the collateral conductance index of the MCP-1 treated group did not differ significantly from the control group (3 weeks occlusion plus 1 week MCP-1: 90.03±8.73 ml/min/100 mmHg) (Fig. 4). Conductance index measurements of the left leg, acutely occluded by the catheter from which the reference sample was withdrawn, showed no significant differences between any group (left acute occlusion...
hindlimb using fluorescent microspheres in-vivo. In contrast to the measurement of only one collateral blood flow value, collateral conductance indices were calculated from blood flows and corresponding blood pressures at six different pressure levels between 45 and 75 mmHg. Thereby not only information is obtained about collateral blood flow at a specific pressure but also about the ability of collateral arteries to conduct blood to dependent regions. We analyzed each single sample after specific tissue digestion with proteinase leaving the microspheres unaffected which were subsequently counted for fluorescence intensity by FACS analysis. Each microsphere was classified using its specific spectrum in all four identifiable fluorescence ranges, allowing the use of as many as seven different labels for tissue perfusion measurements. The total number of the different microspheres per measured sample was between 200 and 1000. This number has been shown to allow precise flow quantification [25]. The data provided by the measurements with the fluorescent microspheres are true functional data. However, the unit of conductance (ml/min/mmHg) as used in our model cannot be extrapolated directly to other models since it is specific for the rabbit hindlimb and experimental conditions. We therefore think it is more appropriate to use percentages of normal conductance as shown in Fig. 5.

4.1. Time course of arteriogenesis

Our main finding is that collateral artery growth proceeds in two phases: an early phase with recruitment of numerous pre-existent arterioles which significantly increase conductance within 7 days and a sub-acute phase where conductance rises more markedly in the subsequent 3 weeks because of the selective growth of a few large caliber vessels to the disadvantage of numerous small ones that regress again by a process called ‘pruning’. These late large collateral vessels show a 10-fold increase in diameter as compared to the pre-existing arteriolar connection from which they developed. Fig. 6a shows the relationship comparing PBS treatment and MCP1 treatment, indicating that MCP1 at first accelerates the process of arteriogenesis. However, at later time-points both curves come together again and will most likely have the same end-point. As shown in Fig. 6b an inverse relationship is observed between the number of collateral vessels and the conductance index of the collateral circulation. This might seem paradoxical, however it reflects the remodeling of the hindlimb circulation where small vessels are ‘pruned’ away to the advantage of the larger few that conduct blood more efficiently with lesser energy losses, indicating the advantage of arteriogenesis over angiogenesis for flow restoration after arterial occlusion.

It is of note that even in the young healthy animals of our study maximal dilatory reserve is restored to only 50% of the value before occlusion. Four weeks after ligation, collateral conductance index had increased to 47% of the...
physiologic values, which is almost 20-fold higher than 1 week after ligation and about 80 times as high as after acute occlusion. However, it is intuitively clear that the result in atherosclerotic patients with multiple risk factors is probably less favorable. Although we show here that the time course of collateral development can be significantly accelerated by infusion of the chemokine MCP-1, the final conductance index value reached under the influence of the chemokine is not superior to the spontaneous development if sufficient time was allowed to elapse. Once the spontaneous development had come to an end, a late treatment with the arteriogenic chemokine had no significant effect. The ultimate goal of all further experimental efforts will be the restoration ‘ad integrum’ by collateral arteries of the maximal conductance of the artery before occlusion.

4.2. Clinical relevance

In case of chronic arterial occlusion, the human body is capable to build own natural bypasses by collateral artery growth (arteriogenesis). However, due to largely unknown mechanisms the process of arteriogenesis usually falls short of complete restoration of maximal conductance giving rise to clinically observable limitations of organ function, especially under loading conditions. This group of patients will most probably benefit from the stimulation of arteriogenesis and some promising results with substances promoting arteriogenesis in an experimental setting were published over the last few years [14,15,26]. However, our data indicate that growth factor treatment may only be effective during a relatively narrow time-window as was already suggested by reports on coronary collateral arteries in the dog and femoral collateral arteries in the rat [15,16]. It remains to be demonstrated if the gain in time is of sufficient relevance in a clinical setting where most often the time of occlusion is either not known or has occurred some time ago.
5. Conclusion

The development of collateral conductance over time shows that maximal conductance values, as in non-occluded femoral arteries at maximal vasodilatation, are not reached by growth of pre-existing collateral arteries within the time frame of four weeks. Within that time only 47% of normal maximal blood flow is restored, compared to 1% acutely after ligation. The exogenous application of MCP-1 acutely after femoral artery ligation in the rabbit can significantly accelerate the process of collateral artery formation reaching the final outcome several weeks earlier, probably meaning an important gain of time in the treatment of occlusive artery disease. However, when applied sub-acutely (3 weeks after occlusion) no further positive effect was observed indicating a narrow time-window for growth factor responsiveness, which might be prolonged by combination of different factors.

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