Mechanisms of arteriogenesis: from cellular adhesion to therapeutic stimulation
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SUMMARY AND CONCLUSIONS
**Summary**

This thesis focused on the basic mechanisms of arteriogenesis and experimental pharmacological modulation of collateral artery growth in different animal models. As outlined in the introduction, particular attention was paid on the process of leukocyte attraction and migration and the extrapolation of these observations to therapeutic stimulation of arteriogenesis. It was also stated that before transferring experimental data from smaller species, two requirements have to be met: The effect of pro-arteriogenetic therapies on the underlying atherosclerotic disease has to be assessed as well as the stimulatory action on arteriogenesis has to be confirmed and reproduced in a model that resembles the human situation more closely.

In chapter 2, the role of the inflammatory cytokine tumor necrosis factor alpha (TNF-α) is examined. For this study, we developed a new murine hind limb model based on microsphere perfusion measurements under maximal vasodilation, while previous studies assessed hind limb flow at rest with Laser-Doppler scanning devices. Microsphere perfusion data revealed that TNF-α is an essential factor for a normal arteriogenic response upon femoral artery occlusion. Furthermore, we identified the TNF-α signaling pathway during arteriogenesis, using two different knock-out strains, lacking either the p55 receptor (TNF receptor 1, TNFR1) or the p75 receptor (TNFR2). As most biological effects of TNF-α are mediated by signaling through the TNFR1, we hypothesized that this is most likely also the case for arteriogenesis. Indeed, we could demonstrate that p55 -/- mice show a comparable deficit in perfusion restoration as the TNF-α -/- mice, whereas mice lacking the p75 receptor displayed a normal arteriogenic response. A direct comparison of Laser-Doppler derived flow measurements with microsphere perfusion measurements in nude mice showed the superiority of the newly developed microsphere method over Laser-Doppler scanning devices.

Chapter 3 confirmed our previous findings about the role of TNF-α during arteriogenesis. Experiments with clinically available compounds for therapeutic TNF-α inhibition in rheumatoid disorders showed that the application of these substances in the rabbit hind limb model after femoral artery ligation significantly inhibited collateral artery growth. Immunohistological data first confirmed specific binding of the drugs to rabbit TNF-α and co-located this chemokine to accumulating monocytes in the perivascular space of growing collateral arteries. Furthermore, quantitative assessment of vascular proliferation and post-mortem angiograms supported our hemodynamic results. These observations might help to unravel the reasons of the unfavorable outcome of clinical trials for the treatment of chronic heart failure with TNF-α antagonists.

In chapter 4, the specific role of different leukocyte subpopulations during arteriogenesis has been investigated. The first reported arteriogenic compound acting via the monocytic pathway was MCP-1. This protein is however not selective for monocytes, but also attracts other leukocytes (e.g. granulocytes). In this study we therefore focused on the effect of granulocyte and lymphocyte attraction on arteriogenesis. Using in-vitro stimulation and immunohistological data after in-vivo application of the different factors (MCP-1 for monocytes, IL-8 and NAP-2 for
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granulocytes, lymphotactin for lymphocytes), we could confirm the efficacy of the human proteins in the rabbit model. Intra-arterial infusion of any of the factors other than MCP-1 into the proximal stump of the occluded femoral artery could however not improve collateral conductance. This study thus confirms previous experiments claiming the key role for monocytes/macrophage for arteriogenesis.

In chapter 5 we investigated the process of monocyte transendothelial migration and assessed the arteriogenic response in the rabbit hind limb model under MCP-1 stimulation and concomitant inhibition of monocyte adhesion with a monoclonal antibody directed against ICAM-1. In addition, we used different transgenic mouse strains derived from different genetic backgrounds to investigate this mechanism in more detail. Blocking the adhesion of monocytes via ICAM-1 antibody led to an almost complete abolishment of the stimulatory action of MCP-1 on arteriogenesis. These data were confirmed in mice lacking functional ICAM-1. Moreover we could demonstrate a comparable deficit in mice lacking the counterpart of ICAM-1, the Mac-1 receptor. The inhibition of the first step of leukocyte adhesion, endothelial rolling mediated by interaction of different selectin isoforms, however showed that this process is not a necessary pre-requisite for collateral artery growth. Mice lacking all three isoforms (i.e. E-, P- and L-selectin) due to a mutation in the fucosyltransferases IV and VII did not display a significant reduction in the arteriogenic response as compared to their healthy wild-type strain.

The role of CD44 during arteriogenesis was the topic of chapter 6. This transmembrane receptor exerts three different functions that are all of relevance to the process of arteriogenesis: it serves as an adhesion molecule for leukocytes, it stabilizes growth factors (e.g. bFGF, PDGF) and it activates TGF-β. Here, we could show that mice deficient for CD44 show a strong reduction in microsphere perfusion. Furthermore, single collateral arteries were isolated from tissue sections using Laser microdissection and analyzed for their RNA content of different growth factors. Using this technique, we could show that indeed CD44 serves as a stabilizer of growth factors during arteriogenesis.

In chapter 7 the rabbit hind limb model of femoral artery ligation and subsequent analysis of the efficacy of MCP-1 by collateral conductance measurements based on fluorescent microsphere perfusion was described in more detail. This model was also used in chapters 4, 5, 8-10 and 13. Parts of this study confirmed in-vivo data from a previous observation of an increase in collateral conductance from an ex-vivo model. Furthermore, in this study we present data on the effect of MCP-1 in a chronic model in which treatment was initiated three weeks after occlusion to better mimic the clinical situation. In addition we could show that over time angiographic appearance of collateral arteries inversely correlates with the improvement in collateral blood flow, a process called pruning: few large collateral arteries remain whereas the majority of the initially appearing small collateral arteries regress.

In chapter 8 we examined the effect of another factor acting on arteriogenesis via the monocytic pathway, GM-CSF. In-vitro data showed that GM-CSF inhibits monocyte/macrophage apoptosis, which probably represents the mechanism of its
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pro-arteriogenic effects, since it does not exert a strong chemoattractive action. Furthermore, we investigated the additive effects of a combination of GM-CSF and MCP-1 on collateral conductance.

In chapter 9 the pro-arteriogenic potential of TGF-β1 was assessed. Immunohistological analysis showed an increased expression of the active isoforms of TGF-β1 around growing collateral pathways. Functional measurements revealed a significant increase in collateral conductance. This effect was not mediated via an increased angiogenic but rather via a stimulated arteriogenic response. Several in vitro experiments supported our hypothesis that the observed effects are most likely mediated via monocytes/macrophages.

Chapter 10 focuses on the systemic atherogenic properties of a local application of MCP-1 for stimulation of collateral artery growth. Together with chapter 11, it deals with the balance between pro-arteriogenic and pro-atherogenic effects of growth factors and cytokines. In this chapter, Watanabe heritable hyperlipidemic rabbits were used to assess plaque formation due to treatment with MCP-1. This study showed that MCP-1 is still able to induce a strong arteriogenic response under hyperlipidemic conditions, although the increase was not as marked as in healthy New Zealand White rabbits. Due to a large standard deviation in plaque surface area in the Watanabe rabbits, the question whether MCP-1, given locally, induces atherogenesis could not be confirmed or denied without doubt.

Therefore, a comparable model was used to answer the question of potential unwanted side-effects of therapeutic MCP-1 infusion in chapter 11. This study again confirmed that MCP-1 is a strong inducer of arteriogenesis, even in atherosclerotic individuals. However, our observation after MCP-1 application in ApoE-/- mice revealed a systemic side-effect on atherogenesis. Intra-arterial infusion enhanced neointima formation, plaque progression and modified plaque composition towards a more unstable phenotype. Thus, our findings stress out the importance of assessing the atherogenic properties of factors before applying them in a clinical setting, even in small pilot studies.

Finally, in chapter 12 we describe an experimental study that examined the effect of clinically used drugs for the treatment of occlusive artery diseases, Aspirin and clopidogrel. We could demonstrate that Aspirin treatment inhibited leukocyte migration and activation, vascular proliferation and thus collateral artery growth. Clopidogrel which is used for the same indications but acting via a different pathway proved to act neutral and did not affect any of the assessed parameters.

**Interpretation and conclusions**

Arteriogenesis, i.e. the growth of pre-existing interconnecting anastomoses to functional collateral conductance arteries, is the most efficient process to restore blood flow upon arterial occlusion to jeopardized tissues. It represents a natural escape mechanism to reduce the negative effects of arterial stenosis. The following conclusions can be drawn from the studies presented in this thesis.
1. TNF-α as well as CD44 signaling are important and essential pathways during arteriogenesis. A lack or a functional deficit of one of these factors significantly hampers the natural process of arteriogenesis. The negative outcome of clinical studies that were performed using TNF-α inhibitors for the treatment of chronic heart failure demonstrated the importance of TNF-α in various processes. Our studies with application of TNF-α inhibitors after femoral artery ligation confirmed our previous data from transgenic mice stressing the essential role of TNF-α signaling via its p55 receptor. As chronic heart failure often depends on coronary heart disease and subsequent ischemia, our results may give new insights into the potential reasons why these clinical trials failed.

2. Monocytes and their migration into the peri-vascular space are essential for a proper arteriogenic response, whereas other leukocyte subpopulation do not seem to be significantly involved. Lack of functional ICAM-1 respectively its counterpart on the leukocyte membrane, Mac-1, results in a strong reduction in collateral artery growth. Interference with monocytic rolling did not inhibit arteriogenesis.

3. MCP-1 is the most potent pro-arteriogenic factor to date. It induces arteriogenesis in the rabbit and the porcine hind limb. Its efficacy is preserved under hyperlipidemic conditions in Watanabe rabbits and ApoE -/- mice. However, even the local application of MCP-1 leads to systemic side-effects worsening the formation and composition of atherosclerotic plaques.

4. TGF-β1 is a potential alternative factor for therapeutic stimulation of arteriogenesis, as well as GM-CSF. Both factors have not been related to pro-atherogenic side-effects. TGF-β1 is known for its plaque-stabilizing effects. Furthermore, TGF-β1 does not enhance angiogenesis, which has been observed to worsen atherogenesis. For GM-CSF, previous studies have even reported an anti-atherogenic property. Thus these two factors are potential candidates for clinical studies, either as a single factor treatment or in combination with other pro-arteriogenic factors.

5. Substances used for the treatment of occlusive arterial diseases should be examined for the arteriogenic properties. Aspirin significantly reduced the arteriogenic response upon femoral artery occlusion in the rabbit, whereas clopidogrel acted neutrally. As large clinical trials showed, clopidogrel displays several advantages over Aspirin. Our data might help to explain the favorable effect of clopidogrel over Aspirin during the CAPRIE-trial. A clinical study to assess differences in the outcome of patients suffering from peripheral artery disease after Aspirin or clopidogrel treatment is planned to further elucidate this interesting mechanism.

Future perspectives
The first description that collateral artery growth is a process of active proliferation and that monocytes/macrophages accumulate in the vascular wall of growing collateral arteries originated from the 1970s. Although huge efforts were made to find factors that therapeutically stimulate and accelerate this process, there is still no
pharmacological compound approved for use in the clinical setting of coronary and peripheral artery disease. A variety of case-reports have been published to promote "therapeutic angiogenesis" as a potential solution for patients not eligible for mechanical revascularization techniques. This hype finally ended with the negative results of the first placebo-controlled randomized clinical trials. This was not further surprising as the half-life of comparable hypotheses in modern medicine is very short and almost every time ends in a new hype that replaces the preceding one. Nowadays, this new hype is constituted by stem cell research for neovascularization, vasculogenesis and myogenesis in the adult. It has to be hoped that this very interesting new concept is of longer duration than therapeutic angiogenesis.

Most likely the reason for the discouraging results of the clinical trials was the lack of appropriate experimental data and the fact that collateral artery growth is a very complex mechanism that involves various different cell types (e.g. monocytes/macrophages, smooth muscle cells, fibroblasts, endothelial cells etc.) and numerous known and unknown factors. Thus, it is unlikely that the application of a single growth factor will significantly promote arteriogenesis in patients that suffer from occlusive artery disease already over a longer period of time. Cellular components as therapeutic targets will potentially help to resolve this dilemma. Monocytes/macrophages are essential for a normal arteriogenic response and can be used to stimulate this process as they deliver and produce a variety of growth factors and chemokines in a time dependent manner, meaning that these cells release the appropriate factor at the appropriate location and time.

The present thesis has focused on the basic mechanisms of arteriogenesis. For a proper replacement of an occluded artery, arteriogenesis serves as the most efficient mechanism, since it provides the growth of large conductance arteries. In contrast, angiogenesis refers to the growth of small caliber vessels, capillaries. Although important for tissue nutrition and oxygen supply, capillaries cannot replace a large feeding artery. For further clinical studies on therapeutic collateral artery growth, experimental models have to get closer to the "authentic" human situation. Therefore, data not only about the stimulatory effects of pro-arteriogenic compounds in different disease models (e.g. ApoE -/- or diabetic mice) but also their potential side-effect profile on the underlying disease have to be obtained. Furthermore it is necessary to test the efficacy of therapeutic substances in "pruning" models, as the usual patient normally suffers from a long-lasting progression of his atherosclerotic disease and has thus already started growing collateral arteries.

Further studies will have to elucidate whether this patient population is eligible for a pro-arteriogenic therapy, based on single factors, combinations of different substances or cell based pathways.