Therapeutic arteriogenesis: from experimental observations towards clinical application [cum laude]
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SUMMARY AND CONCLUSIONS
CHAPTER 13

Summary
This thesis comprises studies on both basic mechanisms and experimental pharmacological modulation of arteriogenesis as well as clinical arteriogenesis. In Chapter 2, a review is presented concerning current concepts on collateral artery growth. Differences as well as parallels with the two other forms of vessel growth, angiogenesis and vasculogenesis, are outlined. Several pathways of stimulating arteriogenesis are presented, sorting an effect either via monocytes/macrophages or via compounds acting directly upon the vascular wall. Particular attention is paid to the hurdles encountered from experimental findings towards clinical application, such as the extrapolation of results from small animal models towards larger species. Potential negative side-effects like atherosclerosis are outlined and recommendations for assessment of clinical efficacy are given.

In Chapter 3, the role of TNF-α signaling is studied. In this chapter we used a newly developed mouse-model of microsphere perfusion measurements in the murine hindlimb after femoral artery ligation. Applied in several knockout strains we could demonstrate that TNF-α is a prerequisite for a normal arteriogenic response upon vascular occlusion. Moreover, we were able to show that this effect is mediated via the p55 receptor, whereas the p75 receptor is not directly involved. In this chapter we also present data, showing the superiority of microsphere-based measurements over Laser-Doppler derived measurements of collateral flow.

In Chapter 4, the role of CD44 during arteriogenesis is described for the first time. This study shows that the normal arteriogenic response is reduced in CD44 knockout mice to a level that is even below that of TNF-α knockout mice as described in Chapter 3. This reduced response is the result of both a defective leukocyte trafficking as well as a decreased stability of vascular growth factors. The technique of laser microdissection is introduced in this field of research. With this technique individual collateral arteries are collected from tissue sections and analyzed for their RNA content.

In Chapter 5 the rabbit hindlimb model is described. Central issue of this chapter is the pro-arteriogenic efficacy of MCP-1. Moreover, the concept of pruning is presented, i.e. the decrease over time of the number of visible collateral vessels towards a few large-diameter vessels with high capacity. This chapter also includes a detailed description of the rabbit hindlimb model of femoral artery ligation that is also implemented in chapters 6 and 7.

In Chapter 6 a study on the effects of GM-CSF on collateral artery growth is presented. In-vitro evidence is provided that GM-CSF inhibits apoptosis of monocytes/macrophages as a potential mechanism for its pro-arteriogenic effects. Also the additive effects of combined treatment with GM-CSF and MCP-1 are described.
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In Chapter 7 the pro-arteriogenic potential of TGF-β1 was assessed. It is shown that the expression of the active isoform of this cytokine is increased around proliferating collateral arteries. Moreover, a sevenfold increase in collateral conductance upon exogenous application of TGF-β1 was documented. It is shown that these effects of TGF-β1 are not mediated via increased angiogenesis but rather via true collateral artery growth, i.e. arteriogenesis. With several in vitro systems it was shown that the pro-arteriogenic effects of TGF-β1 are most probably monocyte-mediated.

Chapter 8 involves the description of a newly developed porcine model of arteriogenesis. This model enables the quantification of resistance and conductance of the collateral circulation in the pig hind limb. Therefore, a model was designed to simultaneously measure peripheral pressures, central pressure and limb volume flow. Assessments were made under controlled conditions of pressure and flow via the instrumentation of the animal with a shunt system and a pump-driven extracorporal circulation. This large animal model was applied as a prerequisite for extrapolation of the experimental findings in mice and rabbits, outlined in the previous chapters, towards the clinical situation in humans.

In Chapter 9 the pig hind limb model was applied to study the efficacy of MCP-1 in a large-sized species. The infusion of MCP-1 upon femoral artery occlusion reduces the resistance of the collateral circulation in a larger-sized species and increases total capacity.

Chapter 10 deals with the balance between arteriogenesis and atherosclerosis. In this Chapter we evaluated the effects of MCP-1 in a model of hyperlipidemia and atherosclerosis. It is shown that MCP-1 can still induce an arteriogenic response under conditions of hyperlipidemia in the Watanabe heritable hyperlipidemic rabbit, albeit to a much lesser extent as compared to normal New Zealand White rabbit. The question whether MCP-1 influences the development of atherosclerosis could not be addressed unequivocally due to a large standard deviation in plaque surface assessments.

Therefore, in Chapter 11, the potential pro-atherogenic effect of MCP-1 was studied in an additional animal model of ApoE -/- mice. MCP-1 is shown to have a strong and protracted reducing effect on the collateral vascular resistance. This is however accompanied by systemic effects on atherosclerotic plaque development. Under influence of MCP-1 treatment, we found increased neointima formation, atherosclerotic plaque progression and modulation of cellular content of plaques. These findings stress the delicate balance between the induction of collateral vascular growth and the stimulation of atherosclerosis.

Finally, in Chapter 12 we describe the design of a patient trial, the START trial. This trial is conducted in the Academic Medical Center in Amsterdam, the Rijnland hospital in Leiderdorp and the University Clinic of Freiburg. A total of 40 patients
with peripheral artery disease is treated for two weeks with subcutaneous injections of GM-CSF. Efficacy of the treatment is assessed both at day 14 as well as 3 months after initiation of the treatment. The primary endpoint is treadmill walking time and in addition we measure ankle-brachial index and Laser-Doppler flux. Measurements of volume flow are performed with the use of MRI in a sub-group of the patients.

**Interpretation and conclusions**
Arteriogenesis or the development of large collateral conductance arteries constitutes a natural escape mechanism to ameliorate the negative effects of arterial occlusion or narrowing.

The following conclusions can be drawn from the studies presented in this thesis.

1. Both TNF-α as well as CD44 signaling constitute important pathways during arteriogenesis. A defective functioning of these pathways leads to a strongly decreased arteriogenic response upon arterial occlusion. The CD44 receptor is not only required for leukocyte trafficking during arteriogenesis but also stabilizes pro-arteriogenic cytokines like b-FGF and PDGF. The clinical relevance of this pathway is reflected in the correlation between the development of the coronary collateral circulation and the CD44 response of monocytes upon stimulation. The identification of these pathways opens new options, targetting these pathways for therapeutic arteriogenesis.

2. MCP-1 is a potent pro-artcriogenic factor, inducing arteriogenesis in both rabbits and pigs. The efficacy of MCP-1 is preserved under conditions of hyperlipidemia, however in atherosclerotic ApoE -/- mice, the exogenous application of MCP-1 induced atherosclerotic plaque formation and changed cellular composition of plaques.

3. TGF-β1 constitutes an alternative target for arteriogenic therapy. TGF-β1 showed arteriogenic effects in the rabbit hindlimb model that are similar to MCP-1. Moreover, in contrast to arteriogenesis, angiogenesis was left unaffected by TGF-β1 treatment, reducing the risk for negative side-effects like carcinogenesis and atherosclerosis that are both propelled by angiogenesis specifically. In combination with the reported plaque-stabilizing effects of TGF-β1, further investigations of the potential of TGF-β1 as a clinical compound for stimulation of arteriogenesis are justified.

4. GM-CSF also has been attributed anti-atherogenic properties. The design of the START-trial, presented in this thesis, was the logical consequence of our experimental data and the reported data on the positive effects in patients with coronary artery disease. The results of this trial may provide new therapeutic options in patients with peripheral arterial disease.
Recent developments and future recommendations
In 1970 it was described for the first time that collateral artery growth is a process of active proliferation rather than passive dilatation and at that point in time it was also recognized that this process might be modulated pharmacologically as a new strategy to treat cardiovascular disease. In the next 25 years, a huge effort was made to unravel the basic mechanisms of collateral artery growth leading to several valuable insights. The apparent real clinical breakthrough came in the mid-nineties with the first reports on the potential of growth factors like VEGF and FGF-1 to modulate collateral artery development pharmacologically. However, although significant progress has been made, up till now no pharmacological compound is registered either in the US or in Europe, aiming at the treatment of obstructive arterial disease via an increase of the capacity of the collateral circulation. In fact, none of the few conducted placebo-controlled randomized studies could convincingly show a positive effect of such compounds. This illustrates the complex nature of collateral artery growth and the concept of a single growth factor inducing a clinical relevant increase in collateral artery growth can be considered as an oversimplification of this process.

The dynamics of the reaction of the scientific community towards new approaches are repetitive. The sudden rise and fall of angiogenesis seems not to have tempered enthusiasm for the next "highly promising" approaches and now therapeutic vasculogenesis and myogenesis are embraced as the new saviors from cardiovascular disease. Although intriguing and elegant, it should be notified that these approaches are highly experimental and fail robust knowledge on mechanistic background. A diligent approach is required and it can only be hoped for that the life-cycle of these concepts is less explosive and of somewhat longer duration than that of therapeutic angiogenesis.

The present thesis has focused on the concept of arteriogenesis. Nowadays it is recognized that angiogenesis, i.e. the growth of capillaries, does not have the potential of adequate flow restoration while arteriogenesis, i.e. the growth of large collateral conductance arteries, is better suited for this purpose. However, also for arteriogenesis, a single-factor approach most probably is too good to be true. Therefore cell-based therapy to stimulate arteriogenesis is so attractive since native cells and especially monocytes/macrophages are capable to produce the battery of required factors at the right place and at the right time. That this concept might work in patients has been shown recently in the TACT trial were patients with peripheral artery disease were treated successfully with autologous bone-marrow transplantation to the affected limb. It should be noted though that this study is in need of confirmation by larger controlled studies.

Finally, as president F.D. Roosevelt once said: "It is common sense to take a method and try it: if it fails, admit it frankly and try another. But above all, try something." This is most probably indeed the best approach (as long as it is implemented in the pre-clinical arena) since the need for alternative strategies to treat arterial occlusive disease and its often devastating consequences on patients' quality of life is still present and will expand even further in the coming decades.