Circulating cells and cytokines in arteriogenesis
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1. Summary

This thesis aims at further elucidating the mechanisms of collateral artery growth in both experimental models as well as in humans. Circulating cells and cytokines playing an active role in arteriogenesis constitute the focus of this thesis. Experimental (animal) models provide the basis of arteriogenesis research, identifying novel pathways and factors of potential use in the clinical situation. Methods of assessment of collateral artery growth in the experimental setting are of utmost importance, and a precise distinction is required between the effect on vascular function and the effect on vascular proliferation of putative therapeutic agents. None of the hitherto promising compounds for the stimulation of arteriogenesis have successfully advanced to clinical application yet (also underlined by the negative outcome of the START-study, chapter 5), and there is a number of critical issues in the translation of experimental results to patient care. The lack of appropriate atherosclerotic animal models and the young age of experimental animals are two major obstacles to be taken into account. The paucity of data on the molecular mechanisms of arteriogenesis in man warrants further detailed investigations of human collateral artery growth. Hence, a reversed bench-to-bedside approach in which the pathophysiology of arteriogenesis is first investigated in patients and then validated in an experimental setting may be a new promising option. Clinical data on vascular growth analyzing circulating cells (monocytes) and growth factors in detail are provided in this thesis. In a reverse translational research approach, these results were validated in experimental models. Finally, because most pro-arteriogenic therapies counteract with atherosclerosis, data from a clinical study analyzing monocyte gene expression in atherosclerotic patients are presented.

In chapter 2, the effects of monocyte chemoattractant protein-1 and the adipocytokine leptin on vascular function and growth were analyzed in a rabbit hindlimb model of arteriogenesis. Leptin had previously been shown to be upregulated upon pro-arteriogenic GM-CSF stimulation, and first studies have demonstrated the leptin receptor also on peripheral endothelial cells. Flow measurements during reactive hyperemia confirmed the pro-arteriogenic effect of MCP-1 and also showed increased hyperemic flow after leptin treatment. However, collateral conductance measured by microsphere infusion under conditions of maximal vasodilation could not verify a pro-arteriogenic effect of leptin. These hemodynamic data were confirmed by immunohistochemical analyses demonstrating increased proliferation of the smooth muscle cell layer of growing collateral arteries from the rabbit hind limb after MCP-1 – treatment, while leptin-treatment left cell proliferation unaffected. Most likely, the observed results of leptin treatment were attributable to its well-documented effect on vasodilation and not on collateral artery growth. Unchanged endothelial cell proliferation underlines the specificity of the animal model of collateral artery growth, which is independent of angiogenesis. This study shows the great importance of choosing the right end-point measurements in the study of vascular growth.

It is still unclear if collateral artery growth in the cerebral circulation differs mechanistically from arteriogenesis in the coronary or peripheral circulation. In a rat model of three-vessel occlusion (ligation of both vertebral and one carotid artery), the effects of systemic...
administration of leptin are described in chapter 3. Functional measurements of CO2 reactivity showed improved hemodynamic reserve in leptin treated animals. However, similarly to the results in the peripheral circulation (chapter 2), microsphere perfusion under conditions of maximal vasodilation failed to detect a stimulating effect of leptin on arteriogenesis also in the cerebral circulation. In contrast, the known pro-arteriogenic cytokine GM-CSF enhanced both CO2 reactivity and microsphere-based arteriogenic response. In-vitro experiments with cultured rat carotid rings indicated that leptin attenuated carotid artery constriction via an iNOS – mediated mechanism. These results support a protective role of leptin on vascular function, while again stressing the importance of end point measurements when investigating collateral artery growth. Further studies will have to show if this quality is weakened in pathological conditions of hyperleptinemia and leptin resistance.

Chapter 4 reports on the role of the endothelial glycocalyx in a rabbit hind limb model of arteriogenesis. Because of a previously reported role of the inner-endothelial layer of proteoglycans and glycoproteins in shear-stress sensing, which is essential in arteriogenesis, we hypothesized a direct functional role of the glycocalyx in collateral artery growth. Perturbation of the glycocalyx by local hyaluronidase infusion resulted in an attenuation of arteriogenesis and decreased collateral vessel proliferation. After one week of hyaluronidase infusion, gene expression of shear-stress – dependent, arteriogenesis – regulating eNOS and TGFbeta1 was significantly hampered in growing collateral arteries, indicating a shear-stress sensing role of the glycocalyx in arteriogenesis. However, the number of perivascular macrophages was unaffected, possibly because of decreased shear-stress sensing in the presence of increased endothelial permeability. Diminished diameter and impaired function of the glycocalyx might be one of the reasons of limited arteriogenesis in pathological conditions such as diabetes or hyperlipidemia. Additional studies are required to elaborate on the role of the glycocalyx in these pathological conditions.

Results from experimental studies showed that the colony-stimulating factor GM-CSF is an efficient pro-arteriogenic cytokine that enhances monocyte concentration and prevents monocyte apoptosis. We therefore conducted the START-trial (STimulation of ARTeriogenesis), a pilot study on the stimulation of arteriogenesis in the clinical setting, which is described in chapter 5. Patients with peripheral vascular disease received subcutaneous injections of GM-CSF or placebo for two weeks, and maximum walking distance as well as ankle brachial index and laser-Doppler flow of the microcirculation were measured at day 14 and day 90. A strong placebo effect was found in the primary end point (maximum walking distance), which was not significantly different in the GM-CSF therapy group. The negative results of this first clinical arteriogenesis trial in peripheral vascular disease underline the problems arising when translating successful experimental studies into clinical application, and demonstrate, with the strong placebo effect, the difficulties of clinical endpoints to accurately detect collateral artery growth. Because a comparable study in coronary arteriogenesis had provided positive results, potential differences of GM-CSF in the coronary and peripheral circulation identify once again the complexity of arteriogenesis.
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In light of the difficulties to translate experimental findings into clinical practice, we initiated a patient study investigating monocyte gene expression. Inter-individual variation in the collateral response upon coronary artery obstruction led us to the hypothesis that monocyte mRNA expression might differ between patients with sufficient versus insufficient collateral artery development. A comprehensive study, presented in chapter 6, showed stimulated monocytes from patients with insufficient coronary collateralization (“non-responders”) to more strongly express interferon-beta and interferon-beta – regulated genes. Indeed, application of interferon-beta in a mouse model of collateral artery growth resulted in an inhibition of adaptive arteriogenesis. As described in an accompanying editorial, this study for the first time provides evidence of genetic variation in the collateral vascular response. Interestingly, not upregulation of a pro-arteriogenic gene expression profile, but decreased expression of the anti-arteriogenic interferon-pathway was found in patients with well developed coronary collateral arteries.

Following transcriptome differences observed in the clinical trial, we conducted an experimental study investigating the mechanisms of interferon-beta induced inhibition of arteriogenesis. As described in chapter 7, interferon-beta induced monocyte apoptosis in-vitro. Stimulated monocytes from knockout-mice lacking the interferon-receptor showed strong downregulation of the interferon-pathway. This inhibition of interferon-beta signaling resulted in a significantly increased arteriogenic response. With immunohistochemistry identifying the interferon-receptor on the vascular media, we conducted in-vitro experiments showing that interferon-beta directly inhibited smooth muscle cell proliferation by interfering with the cell cycle. Inhibiting interferon-signaling in-vitro using RNA-interference techniques, proliferation of smooth muscle cells was stimulated. In a reversed bench-to-bedside approach, this experimental study validates the role of interferon-beta in arteriogenesis. For the first time, inhibition of cytokine signaling was shown to positively influence collateral artery growth.

Insights into the process of collateral artery growth in man are mandatory to explore potential therapeutic approaches for stimulation of arteriogenesis. In chapter 8, local coronary collateral metabolic and cytokine profiles were studied in a large group of patients with subtotal or total coronary occlusions. Oxygen gradient between collateral and systemic blood was found to negatively correlate with invasively determined collateral flow index. Locally secreted pro-arteriogenic cytokines were found in highest concentrations in patients with a less matured collateral circulation. Already increased cytokine levels in insufficiently developed collateral arteries suggest that further growth factor application might not be the optimal therapeutic approach at this stage. Instead, downregulation of anti-arteriogenic cytokines selectively in the collateral circulation suggests inhibition of anti-arteriogenic signaling as an alternative treatment strategy.

Monocyte activation and vascular inflammation are processes that occur in both arteriogenesis and atherosclerosis. Chapter 9 reports on a transcriptome analysis of circulating mononuclear cells in patients with severe atherosclerotic coronary artery disease compared to patients without coronary artery disease on angiography. While signs of enhanced vascular inflammation were found in atherosclerotic patients, circulating monocytes from
these patients showed decreased inflammatory gene expression, mediated by enhanced expression of transcription factors involved in negative regulation of inflammatory gene expression. Inter-individual variation of gene expression was large, particularly in macrophages, T-cells, and stimulated monocytes, thus preventing the use of individual genes as biomarkers. This study presents evidence that circulating monocytes from severely atherosclerotic patients seem to be de-activated by the inflamed vessel wall. Information on altered circulating monocyte function in atherosclerosis sheds a new light on the molecular cause of hampered collateral artery growth in atherosclerosis.

2. INTERPRETATION AND CONCLUSIONS

Experimental research has provided a large body of data on vascular growth in general and collateral artery growth specifically. A number of issues remain, however, unsolved, and the implementation of pro-arteriogenic strategies in clinical practice is still awaited. This thesis provides further information on the molecular mechanisms of arteriogenesis in experimental and clinical models. The following conclusions can be drawn from the studies presented:

After the leptin-receptor had been discovered on endothelial cells, studies that followed showed a stimulating role of leptin on angiogenesis. Yet, two studies from our group in the peripheral and the cerebral circulation demonstrate in a rat and a rabbit model that leptin does not affect collateral artery growth. The beneficial effects on vascular tone and function, however, which were seen in both studies, identify a protective role of the adipocytokine in vascular function. The role of adipocytokines in vascular biology is not yet completely elucidated. Relative leptin-resistance as observed in pathological conditions may be one explanation for vascular (endothelial) dysfunction in patients with hyperleptinemia, diabetes or hyperlipidemia.

While vascular (endothelial) function is of great importance in cardiovascular disease, improved vasodilatory capacity is not the same as genuine proliferation of a collateral artery. Proper detection methods are required in both experimental as well as clinical research to reliably test if a putative pro-arteriogenic therapy has the desired effect. A distinction between capillary sprouting (angiogenesis) and collateral artery growth (arteriogenesis) needs to be made.

The translation of stimulation of arteriogenesis from the experimental model to patient care is still problematic. Further detailed knowledge on the molecular mechanisms of arteriogenesis in man is needed. Our study underlines the absence of ischemia in growing collateral arteries in man. Data from intracoronary blood sampling indicate that decreased local oxygen concentrations are related to enhanced cytokine signaling in immature collateral arteries, and that further growth factor delivery might therefore be ineffective. This may be one of the reasons why growth factor therapy has been unsuccessful in patients with insufficient collateralization. Instead, inhibition of anti-arteriogenic signaling might be an alternative treatment strategy.
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Inter-individual variation in collateral arterial growth in humans is reflected by differential transcriptomes between patients with sufficient or insufficient coronary collateralization. Data from patient studies imply a hitherto underestimated role of anti-arteriogenic signaling, whose inhibition can successfully stimulate collateral artery growth. Thus, inhibition of inflammatory cytokine (interferon-beta-) signaling is capable of stimulating arteriogenesis without enhancing vascular inflammation and probably without negative effects on atherosclerosis.

The search for biomarkers reliably separating atherosclerotic patients from well-matched controls is hampered because of large inter-individual variation. Out of four different mononuclear cell types, only resting monocytes show differentially expressed genes and reveal a biological pathway upregulated in atherosclerotic patients compared to controls. Compensatory upregulation of negative regulators of inflammatory gene expression in atherosclerotic patients may be the result of vascular inflammation in this group. Possibly, de-activated circulating monocytes in atherosclerotic patients are defective in their arteriogenic response. On the other hand, this de-activation of monocyte inflammatory state may damp progression of atherosclerosis, which is annihilated by pro-arteriogenic cytokine therapies, resulting in aggravated atherosclerosis.

3. Future Perspectives

After the first reports on coronary collateral arteries by Fulton [1] and Schaper [2], research on the stimulation of arteriogenesis moved to the focus of a number of research groups. The clinical impact of a sufficiently developed collateral circulation has only recently been underlined in a 10-year follow-up study showing decreased mortality in patients with adequate coronary collateralization [3]. Yet, successful clinical application of pro-arteriogenic therapies is still awaited. Many reasons for these difficulties in bringing arteriogenesis from bench to bedside have been discussed, some of which are alluded to in this thesis. Profound knowledge of the molecular mechanisms of collateral artery growth in humans will help guide future steps in the development of pro-arteriogenic compounds. Expanding one’s view beyond collateral arteries to the whole cardiovascular continuum, the full spectrum of regenerative medicine moves into the focus. In the failing heart lacking adequate oxygen supply, improvement of perfusion by vascular growth is one area of interest. Heart failure patients will additionally need regenerative myocardial growth. For both arteriogenesis and myocardial repair, complex approaches using multiple (growth) factors appear promising, as underlying pathophysiological processes are complex and may require interventions at several stages. This issue has made stem and progenitor cell based strategies so interesting [4], and novel approaches now utilizing cardiac and embryonic stem cells are continuously being reported on [5].

Clinical results presented in this thesis [6] indicated that modulation of anti-arteriogenic signaling may be a promising alternative to the application of pro-proliferative cytokines that are mostly inflammatory. This thesis also suggests de-activation of circulating monocytes in atherosclerosis as one of the causes of the lack of success of pro-arteriogenic...
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strategies in clinical studies. With the inherent “Janus phenomenon” [7] of pro-collateral therapies, the “holy grail” remains the therapy which stimulates collateral growth without aggravating atherosclerosis. Future pro-arteriogenic therapies will have to account for the complex interaction of circulating cells, vascular biology, atherosclerosis and vascular growth. The right intervention with inflammatory signaling must eventually favorably modulate the vascular environment, stabilizing atherosclerotic plaque progression and stimulating vascular growth.
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4. REFERENCES


