Chapter 1

General introduction and aim of the thesis
Chapter 1

The vascular system, disease and treatment

The vascular system of the human body is an extensive network of arteries, capillaries and veins through which blood is pumped by the heart. It performs an essential role in homeostatic regulation of the human body, transporting oxygen, nutrients and cellular and metabolic waste\(^1\). The integrity of the endothelium, a single layer of endothelial cells covering the vascular lumen, is fundamental for the homeostasis of the vascular system. The endothelium plays a pivotal role in regulation of coagulation, blood pressure, immunological and inflammatory processes and vascular remodeling through the production of autocrine, paracrine and endocrine compounds\(^2\)-\(^5\). The pathogenesis of various diseases including hypertension, atherosclerosis, arterial restenosis, diabetes mellitus and nephropathy has been associated with dysfunction of the endothelium. Endothelial dysfunction is associated with a decreased synthesis of vascular nitric oxide (NO), and an altered responsiveness of the blood vessel to important hormones, including angiotensin II (AngII) and transforming growth factor beta (TGF-\(\beta\))\(^6\)-\(^9\).

NO induces vasodilation and possesses anti-inflammatory, anti-coagulant, anti-proliferative and anti-inflammatory properties\(^10\), \(^11\) and counteracts the vascular actions of endogenous Ang II\(^12\). Ang II induces vasoconstriction by acting on the vascular smooth muscle cells and is critically involved in the regulation of blood pressure. In addition to its hemodynamic actions, Ang II promotes cell proliferation and migration as well as extracellular matrix deposition in the vascular wall. Therefore it is not surprising that Ang II is a key mediator of vascular remodeling, which is a close interplay of changes between vascular tone and structure.

The effects of the cytokine transforming growth factor beta (TGF-\(\beta\)) on the cardiovascular system are ambiguous. On the one hand, TGF-\(\beta\) acts as an anti-atherogenic and plaque-stabilizing factor\(^13\), but on the other hand it has been demonstrated that TGF-\(\beta\) participates in the development of vascular fibrosis and vascular remodeling\(^14\). TGF-\(\beta\) affects all cell types of the vessel and regulates various aspects of cellular homeostasis, including proliferation, differentiation, migration and cell death. In addition to direct signaling via the TGF-\(\beta\) receptors and downstream effectors (smads), crosstalk of TGF-\(\beta\) signaling with other major signaling pathways such as the mitogen-activated protein kinases (MAPKs) is involved in the final cellular response to TGF-\(\beta\). This characteristic of TGF-\(\beta\) signaling is probably responsible for the pleiotropic and multifunctional nature of its cellular responses, which makes it strongly dependent on contextual factors, such as ligand concentration, cell type, differentiation status and presence of other hormones\(^15\)-\(^17\).

Given their key function in vascular homeostasis, established and experimental therapeutic approaches in cardiovascular disease target NO, angiotensin II and TGF-\(\beta\) signaling. Nitroglycerin, which is believed to use the same signaling pathway as NO, is the most commonly used anti-ischemic drug in the last century. Unfortunately, upon chronic treatment with nitroglycerin its vasodilatory effect diminishes rapidly\(^18\). Furthermore, to treat hypertension, myocardial infarction, stroke, renal disease and heart failure, interference with the angiotensin II signaling cascade through inhibition of its production (ACE inhibitors) or
blockade of the Angiotensin II type I receptor (angiotensin receptor blockers: ARB) represent the most effective therapeutic strategies. However, treatment with ACE inhibitors as well as ARBs is only effective in a part of the patient population.

Besides NO and Angiotensin II, TGF-β signaling may be a potential target of therapy. Currently, several strategies are under investigation, including scavenging of the TGF-β ligand by TGF-β1 neutralizing antibodies, or selective inhibition of intracellular signaling transduction by targeted overexpression of either Smad7 or dominant-negative receptor mutants. However, as the action of TGF-β is tissue specific and dependent of the stage of the disease, interference with the TGF-β pathway must be well controlled in a spatio-temporal manner.

Gene Therapy
The emerging field of gene therapy is recognized as a potential additional therapy in cardiovascular disease, particularly in cases in which patients are resistant to current approaches. The vascular system, especially the endothelium, is an attractive target for gene therapy because of its accessibility, its importance in vascular (patho)physiology and its involvement in a wide range of diseases. Gene therapy comprises of the cellular delivery of oligonucleotides (DNA or RNA) in an attempt to modify the expression of specific gene(s), or to correct abnormal genes by providing copies of the healthy gene. Modification of gene expression may constitute of upregulation by administration of DNA encoding for the gene of choice or downregulation by interference at the post-transcriptional level employing gene specific synthetic antisense oligonucleotides, such as oligodeoxynucleotides (ODNs) or siRNA.

More recently, microRNAs, which are endogenous antisense oligonucleotides, are discovered to play an important role in the regulation of gene expression in normal as well as pathological conditions. To date, several studies have indicated that specific microRNAs or mutation in the target mRNA sequence play a role in vascular inflammation and disease. These and future identification of the mechanism and targets of miRNAs may offer new gene therapeutic strategies to treat vascular diseases.

Thus far, more than 1300 human gene therapeutic trials have been performed worldwide. However, gene therapy has still not been approved for regular clinical use. In about 70% of the clinical trials, recombinant viruses have been used as a gene delivery vector. As viruses have the innate ability to infect host cells, they are efficient vectors for gene delivery. However, the drawbacks of viral gene transfer are the possible immunogenic, inflammatory, cytotoxic and in the case of retroviruses, oncogenic responses. Furthermore, the costs of large-scale production of such viruses are generally high. For these reasons, non-viral based delivery systems for DNA or RNA have received considerable attention. A wide variety of non viral methods are developed ranging from intramuscular injection of plasmid DNA to specified systems that are devised to enhance cellular delivery like liposomes and polyplexes. These non-viral vectors have the potential to be relatively safe, due to their low inflammatory, non-infectious properties and may be produced at a large scale with relatively low costs.
low costs. However, the main drawback of non-viral vectors is their limited efficiency, restricting their clinical use\textsuperscript{30}. To accomplish efficient delivery of oligonucleotides to the vascular wall, several biological barriers have to be overcome. First, oligonucleotides need to be transported to the endothelium. In the bloodstream the (delivery systems with the) oligonucleotides will encounter degrading enzymes, such as DNAses and RNAses, and immune cells. Both may result in the degradation of the oligonucleotides prior to reaching the endothelium. Furthermore regarding safety issues, since the delivery system encounters immune cells, it needs to be low immunogenic\textsuperscript{31}. The first physical barrier comprises of the plasma membrane, which needs to be crossed to enter the cytosol. The entrance process may be facilitated by fusion of the delivery system to the plasma membrane, or by pore formation and/or endocytosis. When entering the cell via endocytosis, the delivery system also needs to facilitate endosomal escape in order to deliver the oligonucleotides in the cytosol\textsuperscript{31}. Finally, in many approaches the delivered genes have to migrate to the nucleus and overcome the barrier of the nuclear envelope to result in expression of the transgene\textsuperscript{30, 32-34}.

**Ultrasound and Microbubble Targeted Therapy**

Microbubbles were originally developed as ultrasound (US) contrast agents and are administered intravenously to the systemic circulation to enhance the scattering of blood in echocardiography. Microbubbles consist of a gas core stabilized with an encapsulation, and range from 1 to 10 \( \mu \)m in diameter\textsuperscript{35}. Nowadays, an important aspect of research is the therapeutic application of US and encapsulated microbubbles in gene therapy and targeted delivery of drugs, due to their low toxicity and immunogenicity, local application and cost-effectiveness. Moreover, molecular imaging and therapeutic compound delivery may be performed simultaneously, in an efficient way\textsuperscript{36}.

To date, US and microbubble mediated gene therapy targeting the vascular system has already been successfully applied in several experimental disease models to promote angiogenesis\textsuperscript{37-39}, attenuate vascular sclerosis\textsuperscript{40}, reduce neointima formation\textsuperscript{41-43} and augment endothelial function\textsuperscript{44}. It is of importance to note that virtually all of these studies, which used transgene expression instead of gene silencing, used plasmids encoding potent paracrine factors, limiting the need for a highly efficient vector able to transfect the majority of all target cells.

To fully exploit the therapeutic possibilities of ultrasound and microbubble mediated therapy it is necessary to understand all facets of how ultrasound and microbubble mediated drug and gene therapy is facilitated. Despite studies demonstrating that ultrasound and microbubble targeted gene delivery may be a promising technique for gene therapy, there is limited data on the parameters of UMTD of oligonucleotides that influence transfection efficiency in endothelial cells. Furthermore the exact mechanism of cellular uptake of therapeutics after ultrasound and microbubble targeted delivery (UMTD) is also not fully understood, though one of the principal mechanisms is thought to be induction of cell membrane pores\textsuperscript{45, 46}. To modulate vascular function through ultrasound and microbubble
targeted gene therapy both plasmids encoding transgenes or siRNA’s mediating gene silencing may be used. Although siRNA and plasmid DNA are both oligonucleotides, they differ substantially in size (~15 kDa vs ~3500 kDa), which may strongly influence the rate of diffusion. Furthermore, oligonucleotides for gene silencing are effective in the cytoplasm whereas plasmid DNA needs to be transported to the nucleus for transcription. These characteristics may influence the efficacy of ultrasound and microbubble targeted gene therapy, however a direct comparison between these two strategies has not been made.

Intravenous injection of microbubbles is the most convenient route of administration in vascular ultrasound and microbubble mediated therapy. However when the microbubbles disperse over the total blood volume, the concentration of microbubbles drops dramatically. Furthermore, microbubbles and drugs or nucleotides quickly separate after intravenous injection if both are not directly coupled. For this reason, most in-vivo studies relied on microbubble infusion directly upstream of the target organ. The development of organ- or cell-targeted microbubbles that bear drugs or nucleotides, will not only help to identify the diseased target area and locally increase the concentration of the therapeutics, but may also decrease side effects and protect the therapeutics from degradation.

**Aim of the thesis**

The aim of the first part of this thesis was to determine the optimal parameters of UMTGD and to determine if induction of gene expression or gene silencing is the most efficient method of modifying gene expression with UMTD. Therefore in chapter 2, ultrasound and microbubble targeted gene delivery parameters were systematically changed and its effect on gene delivery to endothelial cells was determined. In chapter 3 we studied the modulation of the expression of the moderately expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cultured endothelial cells after UMTD with plasmid encoding transgenes (to increase its expression) or siRNA (to reduce its expression). The aim of chapter 4 was to establish the mechanism(s) of UMTD. For this, we studied uptake of dextran molecules ranging in size of 4-500 kDa by endothelial cells after exposure to ultrasound and microbubbles. The aim of the second part of the thesis was to identify possible targets of intervention and to explore novel administration techniques in vivo. In chapter 5, we studied the interaction between TGF-β1 and AngII signaling in vascular smooth muscle cells. Finally the possibility of targeting microbubbles to TGF-β expressing cells was explored (chapter 6).
References
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