Restoring the balance of the pulmonary endothelium

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Chapter 10

Summary and future perspectives

Pulmonary Arterial Hypertension (PAH) is diagnosed when the mean pulmonary arterial pressure is increased above 25 mmHg and other causes of an increase in pulmonary artery pressure are excluded.(1) Chronic pressure overload leads to dysfunction of the right ventricle and ultimately right ventricular failure and death.(2, 3) The WHO classification of PAH ('chapter 1, table 1), based on etiological perceptions, shows the variety of causes contributing to this condition.(1) Experimental animal models support the notion that PAH cannot be narrowed down to one specific cause. Experimentally, multiple hits are required to mimic the characteristic vascular lesions seen in PAH.(4-8) The same is probably true for human PAH, since even the best-known mutation in the heritable form of PAH, located in the Bone Morphogenetic Protein type 2 Receptor (BMPR2), has a low penetrance of 20%.(9-12) Besides genetic mutations, other hits playing a role in the pathophysiology of PAH are altered blood flow, drugs and toxins, growth factors, infections, inflammation, neurohormonal activation, metabolic changes and vasoactive mediators (Figure 1).(2, 13, 14) The end result is the characteristic vasculopathy of PAH with the appearance of plexiform lesions, muscularization of peripheral arteries, medial hypertrophy of the muscular arteries and neointima formation (chapter 1, figure 2).(13-15) Endothelial dysfunction plays a key role in occlusive remodeling and an increased vascular tone, and is targeted with all currently approved PAH therapies.(16, 17) The pathophysiology of initiating hits and cellular dysfunction is studied extensively, but not yet completely understood (Figure 1). Current research is clouded by a mix of factors that could be either functioning as an initiating factor, or as maintenance factor. Because vascular remodeling itself cannot only be induced by altered blood flow, but can also be responsible for further alterations in blood flow, the pathobiology of PAH can well be described as a vicious circle of remodeling and altered blood flow.

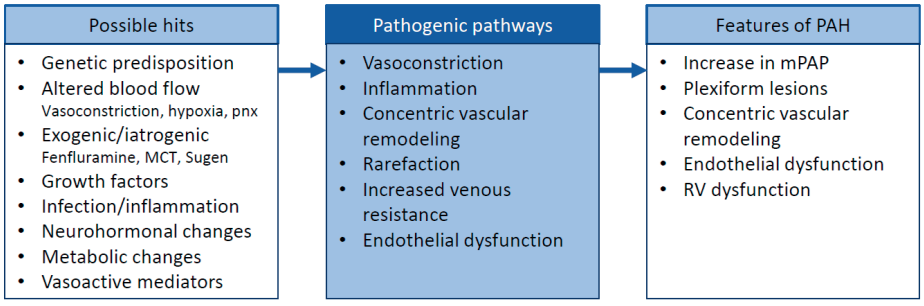

Figure 1 – Pathophysiology of PAH. MCT, monocrotaline rat model; mPAP, mean pulmonary artery pressure; pnx, pneumonectomy; RV, right ventricular.

Effects of altered pulmonary blood flow on vascular remodeling

Endothelial cells (EC) are continuously exposed to the frictional forces that the blood exerts on the vascular wall. The influence of altered blood flow on the pathophysiology of PAH is studied with modeling techniques visualizing these forces and mimicking

this in vitro.(18-20) In **chapter 3** we investigated the effects of altered pulmonary blood flow induced by pneumonectomy in rats. In **chapter 4** we compared findings in pneumonectomized rats with human lung tissue of patients that underwent pneumonectomy because of lung cancer. In both studies, pneumonectomy alone was related to only minor structural alterations. A couple of patients showed thrombotic arteriopathy, a common pathological finding in PAH.(14, 21-23) Although proliferation of EC after pneumonectomy is upregulated, there was no change in intimal wall thickness in rats or patients. The rat model shows that a combination with growth factor inhibition is necessary as a secondary hit to induce both proliferative and pro-apoptotic signaling leading to severe angio-obliterative pulmonary hypertension. This shows that altered blood flow alone is not sufficient to induce structural changes in the pulmonary vasculature, but acts as a hit contributing to the final common pathway.

Contribution of pulmonary vascular remodeling to pulmonary vascular resistance.

Vasoconstriction is the most accepted contributor to increased pulmonary vascular resistance (PVR) in PAH, although vasodilating therapies have limited effect on PAH disease progression. The rat model combining the VEGF inhibitor Sugen with pneumonectomy instead of the more frequently used combination of Sugen with hypoxia (SuHx), shows that hypoxic vasoconstriction is not an obligatory hit for the development of pulmonary hypertension. Concentric remodeling of the pulmonary vasculature is thought to contribute to increased PVR because only a minority of PAH patients exposed to acute vasodilator challenges show substantial pressure decreases.(24) Current information on pulmonary vascular remodeling in PAH is often limited to an assessment of the wall thickness of all small lung vessels taken together. In **chapter 5** we aimed to objectify the remodeling between different vessel orders, to compute the influence of vascular remodeling on resistance. While it is generally assumed that the increase in vascular resistance in PAH is explained by severe structural changes in most, if not all, small pulmonary vessels, our measurements did not support this assumption. First, we found that the majority of vessels (70%) was not affected through a change in inner diameter. Second, the size of the affected vessels and the degree of diameter change varied greatly. Third, our computations showed that remodeling of 30% of the pulmonary vessels could maximally explain a 1.4 fold increase in PVR, resulting in ≈140 dynes·cm^{-5} when multiplied by the maximal normal PVR limits of 99 dynes·cm^{-5}. This is still far from the PVR of about 857 dynes·cm^{-5} measured in our patient group. We suggest two other factors, besides vasoconstriction and concentric remodeling, contributing to increased vascular resistance. First, vascular rarefaction, a phenomenon that is not only possible in the pulmonary vasculature but is already shown to be involved in RV-dysfunction in PAH(25-28) . Second, there could be a substantial venous involvement in PAH.(29, 30) Different contributors to increased pulmonary vascular resistance is of importance for new therapeutic strategies.

Targeting endothelial dysfunction in PAH

The expanding knowledge on the pathophysiology of PAH might indicate novel treatment strategies.(31) With the main focus on endothelial dysfunction we aimed to explore relevant therapeutic targets. An example of flawed sensitivity in EC can be appreciated from cilia, sensory antennas that integrate signaling and fine-tune EC responses. Cilia dysfunction in different cell types shows the role of cilia in the response to injury, regulating cell sensitivity and cell differentiation. Cilia are known as mechanosensors for fluid shear stress.(32-35) We show in **chapter 6** that the cilia on healthy pulmonary EC are responsive to inflammatory cytokines by elongation, as their length is inhibited by IL-10 and NFkB inhibitors. EC of PAH patients, on the other hand, have elongated cilia unresponsive to pro- and anti-inflammatory treatment. This could be explained by ongoing cytokine production or a contributing mechanism independent of inflammatory signaling, like metabolic changes. Shear stress, of which the importance in PAH is discussed above, even further elongates the cilia that are randomly arranged on the EC surface, indicating defective mechano-responses. Previous studies show that exposure of cilia to shear stress leads to activated TGF-beta signaling, possibly leading to endothelial-to-mesenchymal transition (endo-MT).(35-37) Endo-MT is a process recently recognized to contribute to vascular remodeling seen in PAH.(38-40) The role of changed cilia length in pulmonary EC and their contribution to endothelial dysfunction in the pathogenesis of PAH warrants further research.

Since the discovery of the BMPR2 mutations in 2000 in the context of hereditary PAH, a lot of research has been done on this pathway.(9-12) In different subtypes of PAH increased TGF-beta and decreased BMP-signaling contribute to endothelial dysfunction.(38, 41-46) The complexity of this pathway, described in **chapter 7**, is emphasized by contrasting study results, indicating BMP9 missense mutations as a cause of pulmonary hypertension versus the protective effect of BMP9 knock down in animal models.(45, 47, 48) Not all hits are interchangeable with one another and some hits are more potent than others to induce or worsen PH. In **chapter 8** we studied phenotypic effects and the potential therapeutic role of BMP9 in different types of EC (peripheral blood derived, pulmonary artery and microvascular EC). Microvascular endothelial cells of PAH patients showed the strongest response to BMP9 stimulation, with increased and sustained activation of TGF-beta signaling due to loss of EC suppressor function. This process is also regulated through inflammatory cytokines, shown by prevention of these effects by interleukin-6 (IL-6) inhibition. Caution is warranted in therapeutic use of BMP9 in PAH because of the loss of the antagonistic effects of TGF-beta and BMP signaling and may need to be combined with therapy directed to IL-6.

Disrupted signaling found in PAH EC is also of importance for treatments focused on growth factors, as discussed in **Chapter 9**. Although proliferation of healthy EC is inhibited by nintedanib, a TKI-inhibitor targeting VEGF, PDGF, FGF and TGF-beta

signaling, this effect is not seen in cells from PAH patients. There were no effects on vascular remodeling in lung tissue of SuHx rats, but unexpectedly we found improvement on RV dilatation possibly through inhibition of fibrosis in the heart. This study suggests that nintedanib, approved for idiopathic pulmonary fibrosis (IPF), may be safely used in the context of pulmonary hypertension associated with IPF.(49-52)

Future perspectives

The pulmonary artery pressures are increased in one third of the patients after pneumonectomy.(53-55) As described in chapters 3 and 4, we found only minor alterations in the pulmonary vasculature in our experimental rat model and lung tissue of patients after major lung resection. In combination with other hits, the rat model is of importance to study which component of the pathogenesis can be attributed to altered blood flow. In particular the influence on endo-MT and specific interactions between proliferation and apoptosis of EC would be of interest. To further explore which additional hits are important to shear stress, the lung tissue of patients after pneumonectomy that do develop increased pulmonary artery pressures should be further studied.

In chapter 5 the heterogeneity of the vascular remodeling in PAH became clear. The high pulmonary artery pressures found in PAH cannot be explained by vasoconstriction and concentric remodeling of the pulmonary vasculature alone. Rarefaction is an often debated phenomenon in the field of PAH. There is proof of rarefaction in the heart of PAH patients, but its occurrence in the PAH lung remains controversial.(25-28) An important future study would be to visualize the entire diseased pulmonary circulation all the way down to the capillaries. This approach, to date only attempted with micro-CT in rats, will likely encounter technical difficulties due to tissue properties and limitations in image resolution.(56-58) In addition, it will be important to study the possibility that a profound venous pathology contributes to the increase in PVR in PAH, as it contributes to pulmonary hypertension due to left heart disease. It has been demonstrated that capillary pressures are increased in PAH, suggesting a high venous resistance.(30) Venous resistance is also involved in chronic thromboembolic pulmonary hypertension.(29) We hypothesize that in addition to other forms of pulmonary hypertension venous resistance also has a role in PAH.

The significance of the pro-inflammatory environment of microvascular endothelial cells in PAH is emphasized in chapters 7 and 8. In the context of cilia located on the endothelium, we showed a non-responsiveness of cilia length to inflammatory cytokines or inhibition with IL-10 and NFkB. Also the response of the endothelium to novel therapeutic agents, like BMP9, is altered in PAH by inflammation. EC of PAH patients stimulated with BMP9 showed induction of transcription factors for endo-MT, that could be inhibited with an IL-6 capturing antibody. IL-6, a pro-inflammatory cytokine that is increased in PAH and correlates with prognoses, seems to be a

promising therapeutic target for PAH.(41, 59-62) Recent research shows that BMPR2 mutant rats that develop spontaneous pulmonary hypertension, can be distinguished from the ones that don't by pulmonary IL-6 overexpression.(63) Future studies should explore the precise mechanism via which IL-6 influences the TGF-beta/BMP pathway and the effects of modifying this on the pulmonary vasculature.

Pulmonary arterial hypertension is a complex group of diseases that is caused by a combination of hits leading to a final common pathway that cannot be stabilized or cured by one treatment option alone. Future treatment strategies should focus on targeting multiple pathogenic pathways at once in which combination therapy seems inevitable.

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