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Role of multidrug resistance-associated protein 1 in airway epithelium

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CHAPTER NINE

Summary and future perspectives

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In this thesis we investigated whether multidrug resistance-associated protein 1 (MRP1) levels in bronchi and parenchymal lung tissue are related to chronic obstructive pulmonary disease (COPD) development. MRP1 is a protective protein against toxic substances and oxidative stress in which glutathione (GSH) plays a central role [1, 2]. MRP1 is highly expressed in lung airway epithelium and might be important with respect to protection against inhaled toxic substances. Development of COPD is highly related to smoking and is, amongst others, characterized by an imbalance between oxidants and antioxidants [3]. Diminished MRP1 function may therefore predispose smokers to a higher susceptibility to COPD [4].

After the general introduction of this thesis, in **chapter two**, we reviewed available knowledge on ATP-binding cassette (ABC) transporters that are expressed in the lung and speculated about their potential role in normal lung function but also in the pathological situation. So far, ABC transporters have hardly been investigated in prevalent lung diseases such as asthma and COPD. Three pulmonary diseases have been described previously to be caused by mutations in ABC transporters, i.e. cystic fibrosis, Tangier disease and fatal surfactant deficiency. Several pulmonary drugs are substrates for ABC transporters, thus, interindividual variation in functional activity of these transporters is likely to affect drug treatment of lung diseases. Amongst 20 different tissues, the lung and trachea were identified to have high transcriptional activity for many ABC transporters [5]. According to this survey of the literature, the most important ABC transporters in bronchial epithelium are MRP1, P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and cystic fibrosis transmembrane conductance regulator (CFTR). Conflicting results exist about MRP2, 3, 4 and 5. MRP1 is also present in bronchoalveolar lavage inflammatory cells (mainly macrophages), goblet cells and possibly in alveolar type I cells. It remains to be determined whether MRP2, 3, 4, 5, P-gp and CFTR are expressed in alveolar type I cells. The most important ABC transporters in alveolar type II cells are ABCA1 and ABCA3 and these proteins appear to play an important role in the production of surfactant. Lung endothelial cells are BCRP positive but results on P-gp are inconclusive. In summary, mutations and polymorphisms in ABC transporters may have important clinical consequences for development of lung diseases. Insight in the function of ABC transporters in the lung may open new ways to facilitate treatment of

Chapter 9

lung diseases.

In **chapter three**, MRP1, P-gp and LRP (lung resistance-related protein) protein expression was determined in lung sections of COPD patients and controls in a semi-quantitative manner. We detected lower levels of MRP1 in bronchial epithelium of COPD patients (n=11) compared to healthy controls (n=8) and also lower MRP1 expression in severe and very severe COPD (n=10) compared to mild and moderate COPD (n=9). P-gp and LRP did not differ between groups. Expression levels did not correlate with lung function parameters or pack-years smoked. These results indicate that diminished MRP1 expression is indeed related to COPD and that lower levels are related to more severe disease. It would be of great interest to investigate a larger group of patients with disease severity from mild to severe to verify our results and to investigate a potential relationship of MRP1 expression levels with inflammation and remodeling markers pertinent to COPD development.

In **chapter four**, we questioned whether MRP1 transcriptional expression levels and functional MRP1 mediated transport differs between bronchial epithelial cells derived from healthy and severe COPD lung tissue. MRP1 expression is higher in bronchial epithelial cells from six healthy donor lungs compared to the native lung from the same individual with severe COPD who underwent lung transplantation. A comparable trend was found for MRP1 activity which was analyzed in four patients. In addition, both MRP1 expression and MRP1 activity correlated well in cells from the native and donor part of the bronchi. This study is in line with the results of chapter 2 in which we describe a higher MRP1 expression in biopsies of healthy individuals compared to COPD patients. This approach provides the unique opportunity to analyze differences in MRP1 expression in healthy and COPD bronchi irrespective of external influences such as current smoke exposure and use of medication. Thus, there are indications that a lower MRP1 activity is related to development of COPD.

In **chapter five**, we aimed to determine whether absence of MRP1 and P-gp plays a role *in vivo* in cigarette smoke induced emphysema and pulmonary inflammation. We found that the pulmonary inflammatory response is reduced in lungs of Mrp1/Mdr1a/Mdr1b triple knockout (TKO) mice after six months of smoke exposure compared to wildtype mice. This was reflected by a reduction of inflammatory cells and reduced IL-8 levels in lungs of TKO mice with a trend for reduced GM-CSF. It remains to be investigated whether this effect is positive or negative with respect to lung

diseases because inflammation contributes to the development of COPD, but inflammation is also necessary for lung repair. Six months smoking did not induce lung emphysema in neither wildtype nor Mrp1/Mdr1a/Mdr1b deficient mice. This may be due to a low susceptibility of this mice strain (FVB mice) to develop lung emphysema. In further studies, more than six months of smoke exposure would be recommended to induce emphysema in these mice. Otherwise, it is suggested to backcross the TKO mice to a mouse strain that is more prone to develop emphysema. In addition, smoke exposure of the Mrp1 single knockout mice and the P-gp (Mdr1a/1b) knockout mice should be performed to unravel the contribution of the separate efflux pumps by comparing the effects to wildype mice.

Since our data suggest that MRP1 expression is associated with COPD, we set out to test whether cigarette smoke extract (CSE) affects MRP1 mediated activity **(chapter six).** The *in vitro* effects of cigarette smoke exposure have been analyzed in the bronchial epithelial cell line 16HBE14o⁻. The main results were that CSE inhibits MRP1 functional activity and that cell survival after CSE incubation is decreased when MRP1 activity is blocked. In contrast, inhibition of P-gp and BCRP did not affect cell survival. These data strongly suggest that MRP1 function is affected by smoking and that MRP1 is important for cell survival to resist cigarette smoke toxicity.

If MRP1 is cytoprotective for lung function, it is of clinical interest to investigate the effect of pulmonary drugs prescribed to COPD patients on MRP1. **Chapter seven** describes the effects of COPD treatments on MRP1 functional activity in the bronchial epithelial cell line 16HBE14o⁻. Budesonide inhibited MRP1 activity, whereas formoterol had no significant effects. Interestingly, simultaneous incubation of budesonide and formoterol resulted in a lower MRP1 inhibition than budesonide alone. Clinical studies have shown that treatment of COPD patients with a combination of corticosteroids (e.g. budesonide) and long-acting beta-mimetics (e.g. formoterol) are capable to reduce the number of exacerbations. Further research is necessary to investigate whether the effects on MRP1 function contribute to these observations that are beneficial to COPD prognosis. With N-acetylcysteine we found stimulation of MRP1 activity in a concentration dependent manner and a similar tendency for ipratropium bromide. These results indicate that with respect to MRP1, a combination of budesonide and formoterol could be beneficial for treatment of COPD patients compared to single treatment with budesonide. In addition, the stimulation of MRP1 activity with ipratropium bromide and N-acetylcysteine might be positive as well for treatment of

Chapter 9

COPD. Each investigated drug has its particular proven positive effects for COPD patients; the results of our study on MRP1 function give additional information on putative (side)-effects of these drugs, which should be taken into account.

In **chapter eight**, it was studied if incubation with indomethacin, a non-steroidal anti-inflammatory drug, can circumvent drug resistance in an MRP1 overexpressing small cell lung cancer cell line. Small cell lung cancer initially responds well to chemotherapy but often become resistant at recurrence. Indomethacin is an inhibitor of MRP1 function. Surprisingly, cells with low MRP1 expression were more resistant to indomethacin than cells with high MRP1 levels, as determined with cell survival and apoptosis assays. We found that indomethacin decreased GSH levels in MRP1 overexpressing cells. This was accompanied by a decrease in mitochondrial membrane potential, and altogether made these cells less resistant against cellular stress induction. This mechanism could potentially be exploited for treatment of lung cancer in which either intrinsic or extrinsic multidrug resistance caused by MRP1 overexpression plays a role.

Future perspectives

This is the first study in which MDR proteins were investigated with respect to their role in COPD development. The results of our patient studies, *in vitro* experiments and animal studies, show a potential role for MRP1 (and maybe P-gp as well) in COPD development and in the handling of cigarette smoke. Further investigation is required to determine the underlying mechanisms.

Polymorphisms in the *MRP1* gene locus have been identified and this might lead to different MRP1 functional activity and thus to different responses to certain drugs between individuals [6]. Until now, the pharmacological implications and phenotypical consequences of individual variations in MRP1 function are speculative [7, 8]. Recently, it was reported that polymorphisms in *MRP1* are associated with increased risk of ovarian cancer [9]. It can be hypothesized that functional polymorphisms in *MRP1* are related to development of COPD. Thus, it would be of great interest to determine polymorphism frequencies in COPD populations compared to individuals without pulmonary diseases. Another aspect is that interaction with (protection against) oxidative stress factors can be studied. It is for instance known that reduced fruit and/or vitamin intake is associated

with lower lung function, a characteristic of COPD. Thus gene-environment interaction could be studied as to polymorphisms in MRP1 and dietary habits. Furthermore the possibility can be explored whether MRP1 activity is related to disease susceptibility, but also whether its function is related to disease severity and/or progression. This is possible by association with lung function decline, stage of COPD (airway versus parenchymal disease) and inflammatory and remodeling markers. In addition, single nucleotide polymorphisms in other MDR proteins such as P-gp and BCRP would be of interest in this respect because these transporters are also present in high to moderate levels in human lungs. Other enzymes important in detoxification processes are members of the glutathione S-transferase (GST) family. The function of GSTs is detoxification of a broad range of substances by conjugation to GSH, and many of these GSH-conjugates are transported out of the cell by MRP1 [1]. Polymorphisms in *GST* genes are associated with COPD and a rapid decline in lung function of smokers [10, 11]. Because MRP1 and GST function are strongly related, it would be of great interest to study whether polymorphisms in *MRP1* are associated with development of COPD and/or decline of lung function compared to individuals without a history of pulmonary diseases and whether functional interaction between GST and MDR genes enhances the effects of polymorphisms in these individual genes.

At present, techniques used in genomics or proteomics are still improving and can be of great use for further research. With a micro-array, the expression of all 48 ABC transporters can be determined at the mRNA level in one assay. In addition, mRNA expression levels of genes that are involved in detoxification, such as enzymes involved in oxidative stress (e.g. GSH metabolism proteins) or enzymes from the cytochrome P450 family can be measured. Patient materials (biopsies, primary cells) can be analyzed with these techniques to compare mRNA or protein expressions with material from healthy individuals. Using *in vitro* models by incubating lung cells with smoke extract, identification is possible of the genes (or proteins) that are differentially expressed. The bronchial epithelial cell line 16HBE14o$^-$ is very suitable for drug transport studies when compared to other cell lines since its permeability properties are comparable to those reported in native lung epithelial cells [12]. For example, 16HBE14o$^-$ cells form tight junctions and have the ability to develop cilia under certain culture conditions. It was already known that these cells express CFTR [13], and functional expression of P-gp and LRP was studied as well [14]. In this thesis, the 16HBE14o$^-$ cell

Chapter 9

line was characterized for expression of MRPs and BCRP. We found that MRP1 expression was very high in these cells, like in primary bronchial cells. It would be of special interest to analyze expression of all known ABC transporter proteins in these immortalized cells and to investigate which proteins are affected by cigarette smoke by exposing cells to cigarette smoke extract. For comparison, cancer cell lines and primary lung epithelial cells can be used to validate the model. Thus, exposing these cells to cigarette smoke, isolation of protein and RNA, and performing microarrays and real-time PCR will give insight in which (MDR) proteins, besides MRP1, play a role in cigarette smoke detoxification. Many substances in cigarette smoke are carcinogenic or mutagenic. Therefore, the potential protective role of MRP1 in development of lung cancer would be of significant interest besides its role in COPD. Smoke exposure can be performed using cigarette smoke extract (liquid smoke), as was applied in our study, or exposure to gaseous cigarette smoke in an air-liquid interface experimental setup. Using 16HBE14o$^-$ cells in this setup would be a good model because these cells are known to develop cilia when they are grown in an air-liquid transwell system resembling the *in vivo* natural situation [12].

RNA interference was used in the current thesis to downregulate MRP1 expression in 16HBE14o- cells to study modulation of cigarette smoke toxicity and effects on MRP1 functional activity. Another approach to unravel the role of MRP1 and/or P-gp in detoxification mechanisms of cigarette smoke would be to isolate primary bronchial epithelial cells of lungs of Mrp1/Mdr1a/1b triple knockout mice or the single knockout mice and incubate these cells with cigarette smoke extract for analysis with flow cytometry. The advantage of this experiment is that these mice totally lack expression of MRP1 instead of transient downregulation with RNA interference. In this way, contribution of MRP1 can be studied by e.g. adding back MRP1 by transfection with MRP1 expression constructs. A major disadvantage is that it is difficult to culture these primary cells for more than three to five passages. Also, it has to be taken into account that the permanent absence of MRP1 (instead of a decreased function) may induce a non-physiological situation by alterations of cellular detoxification pathways and compensation mechanisms. To investigate the direct and indirect effects on MRP1 expression of cigarette smoke, experiments with luciferase constructs for the MRP1 promoter are invaluable. This system is also useful to determine the effects of expression of trancription factors that regulate MRP1, such as nuclear factor-E2 p45-related factor (NRF2).

Animal studies are necessary to analyze the *in vivo* effects of smoking on the cellular level in a controllable way. COPD animal models have been developed to this aim. We used a model with mainstream, nose only smoke exposure for 6 months, which has been succesfully applied in other studies to investigate emphysema development [15]. Significant alterations in inflammation were detected in the lung but we were unable to objectivate emphysema development with the commonly applied linear mean (Lm) intercept method. This could be due to resistance of this mouse strain (FVB) to develop emphysema, since it is well known that large inter-strain differences exist with regard to COPD development. For further studies, it is suggested to backcross MRP1 knockout mice to a mouse strain that is more prone to develop emphysema such as C57Bl6/J or A/J mice to assess whether emphysema can be induced in these mice. In this setting, it is feasible to study treatment with drugs that e.g. intervene with oxidative stress or stimulate MRP1 expression.

Not much is known about effects of other pulmonary drugs on expression or activity of MRP1 or other ABC transporters [16, 17]. The anti-inflammatory drug montelukast (a leukotriene receptor antagonist) blocks the action of leukotrienes and relieves the symptoms of asthma. Interestingly, montelukast functionally and structurally resembles the leukotriene antagonist MK571, a very potent MRP1 inhibitor. In recent studies, montelukast has been used for treatment of COPD patients in clinical trials. This treatment had a positive anti-inflammatory effect and beneficial effects on hypertonic saline-induced airway responsiveness in COPD patients [18, 19]. Since montelukast is an MRP1 inhibitor, chronic treatment with this drug in an experimental (animal) setting might further elucidate the relation between MRP1 function and COPD (and possibly asthma [20]) development.

In conclusion, lower MRP1 expression is related to COPD and pulmonary inflammation. Cigarette smoke and several pulmonary drugs affect MRP1 activity. Further studies are required which might lead to therapy based on genetic profiling to optimize treatment and to reduce negative (long-term) side effects. However, to quit smoking is until now still the best therapy that can be prescribed by pulmonologists in order to change the course of disease in COPD.

Chapter 9

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Chapter 9