The airway smooth muscle in asthma: More than meets the eye
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Chapter 1

General introduction
1. Asthma and airway remodeling

Asthma is a respiratory disease affecting many people worldwide. It is a chronic inflammatory disease of the airways that is characterized by episodic symptoms and airways inflammation. The associated functional changes include variable airways obstruction [1], airway hyperresponsiveness to a variety of inhaled stimuli [2], increased fluctuations of respiratory resistance by forced oscillation technique [3], and impairment of bronchodilation following deep inspiration [4].

One of the critical aspects of inflammation in asthma seems to be the mast cell infiltration of airway smooth muscle (ASM) [5,6]. In addition to inflammation, a process of structural changes called airway remodeling has been observed during the course of asthma. These changes include an increase in ASM mass, increased deposition of extracellular matrix (ECM) proteins in- and outside the ASM layer, goblet cell and glandular hyperplasia, and angiogenesis [7,8].

2. Pathophysiology of asthma

Although the symptoms of asthma are clear and well described, it is nowadays still unknown what the underlying pathophysiologic mechanisms are that actually induce the observed airway functional changes in asthma. Although airway hyperresponsiveness varies with inflammation as induced with e.g. allergens or respiratory virus infection, targeted interventions on (eosinophilic) inflammation are often unable to reduce hyperresponsiveness in asthma [9]. It is likely that airway remodeling contributes to variable airways obstruction in asthma [10], but the associations between aspects of remodeling and hyperresponsiveness are rather weak or absent [11]. Biopsy studies in patients with different asthma severities demonstrated that increases in collagen content and ASM mass were more prominent in patients with severe asthma when compared to moderate and mild asthmatics [12,13]. However, it has been more difficult to link aspects of remodeling in bronchial biopsies to the degree of obstruction or airway hyperresponsiveness in asthma. Recent studies suggest that features of ASM are the exception to this.
3. Airway smooth muscle

3.1. ASM and airway function

ASM and its close environment can be major determinants of impaired airway function in asthma. First, in children with asthma, lung function (FEV₁) appears to be associated with ASM surface area, distance from muscle to reticular basement membrane and with ASM light chain kinase (MLCK) expression [14]. Second, in adult asthmatics, airway hyperresponsiveness increases with mast cell counts within the ASM layer [15]. In addition, we observed that the impaired bronchodilation following a deep inspiration in asthma is also associated with mast cell infiltration in ASM [4]. Third, it appeared that FEV₁, airway hyperresponsiveness, and deep-breath induced bronchodilation were all three associated with ASM protein expression in bronchial biopsy specimens [16]. Hence, there are multiple associations between airway function and ASM characteristics in asthma (Figure 1).

Figure 1 – Overview of factors regulating ASM tone
Multiple factors influence the ASM tone resulting in an altered airway function. These factors not only include structural components e.g. epithelium and extracellular matrix, but also various proteins and inflammatory mediators. ASM: airway smooth muscle; ECM: extracellular matrix; EGF: epidermal growth factor; MLCK: myosin light-chain kinase; MMPs: matrix metalloproteinases; RBM: reticular basement membrane; α-SMA: alpha-smooth muscle actin; TGF-β: transforming growth factor-beta; TIMPs: tissue inhibitors of metalloproteinases; TNF-α: tumor necrosis factor-alpha.
Indeed, the mechanics of ASM are likely to be changed in asthma, due to altered dynamic behavior and increased stiffness [17]. This may be associated with changes in cytoskeleton and actin-myosin cross-bridge cycling induced by reduced tidal stretch, which might be promoted by chronic airways inflammation and/or remodeling [18]. Interestingly, patients with fatal asthma exhibit altered ECM composition within the ASM layer as compared to non-fatal cases [19]. These physiological and pathological observations seem to be consistent, and suggest that ASM structure-function relationships are playing a key role in the clinical expression of asthma.

ASM itself can be an active contributor to airways inflammation and remodeling in asthma. It has been reported that isolated and cultured ASM cells show a synthetic-proliferative phenotype [20]. Therefore, it is likely that ASM phenotype not only contributes to changed airway dynamics [17], but also to the regulation and perpetuation of airways inflammation and remodeling.

3.2. ASM phenotyping

The increase of ASM mass by airway remodeling can be due to hyperplasia and/or hypertrophy [8,21]. It is believed that the mast cell infiltrate [22] and the changes in the composition of ECM proteins in the ASM layer [23] might be involved in this process of ASM growth. Regarding the synthetic property, ASM is capable of producing many cytokines, chemokines, bronchoprotective factors, adhesion molecules, and growth factors [20]. Therefore, based upon the ASM mast cell infiltration [5], and the altered ASM cell structure and function [17,20], it is highly likely that gene expression of ASM in asthma is changed.

A few gene expression studies using freshly obtained human ASM tissue have been performed in the past [24-26]. These studies performed real-time or semiquantitative PCR as a method of profiling ASM gene expression, and focused primarily on specific genes, for instance myosin light chain kinase (MLCK) and smooth muscle-specific myosin heavy chain (MCH) isoforms. To our knowledge, gene expression profiling of ASM phenotype in patients with asthma using next-generation high-throughput gene sequencing methods has not been performed yet.

3.3. ASM as therapeutic target

Glucocorticoids are presently still the most effective maintenance and exacerbation therapy in asthma leading to improvements in the clinical status and lung function of patients [27].
However, it is not clear whether these benefits are solely based on anti-inflammatory properties or whether glucocorticoids also exert other local actions within the airways [28]. Evidence shows that glucocorticoids may alter gene transcription in ASM cells [29], which leads to altered transcription of contractile elements, cytoskeleton, cell surface molecules, cytokines and mediators [30]. Indeed, glucocorticoids are influencing the contractile properties and glucocorticoid receptor-related genes of ASM in vitro [31,32], and airway dynamics in vivo [33]. This suggests that ASM phenotype is in close interaction with glucocorticoids [34]. Hence, there is a need to examine the effect of glucocorticoids on ASM transcriptomic profile in asthma.

4. Gene sequencing

Genetic information in cells of human and other pro- and eukaryotes are stored in the form of DNA. This DNA is transcribed into RNA, which in turn is translated into proteins that regulate various biochemical processes of a living cell. With gene sequencing the sequence of the nucleotide bases that form the DNA is determined. Already in 1977 gene sequencing was introduced by Frederick Sanger [35]. This first-generation sequencing method is also called the dideoxy or chain termination sequencing. In short, the template of the DNA molecule is copied repeatedly. Modified nucleotides, also called chain terminators, are added to the reaction, which will terminate the copy process when incorporated in the copied DNA. Due to the fact that copying begins at a fixed location of the template DNA, but terminates randomly through the incorporation of chain terminators by chance, copy DNA with various lengths will be obtained. By comparing these DNA fragments also known as reads, the original DNA nucleotide sequence can be determined.

4.1. Next-generation high-throughput gene sequencing

Since its first introduction in 1977, gene sequencing has undergone many technological advances resulting in the development of next-generation high-throughput gene sequencing techniques, which embroider on the Sanger method. The main advantages are a significant increase in sample numbers that can be simultaneously sequenced, and decrease in costs and time needed to perform a sequencing run [36]. One of the most powerful recent next-generation gene sequencing techniques is transcriptome sequencing (RNA-Seq), which allows a detailed characterization of gene expression profiles at the tissue level [37]. Particularly in complex disorders such as asthma this
technology has the potential to discover gene expression profiles that are characteristic of the disease and thus can improve our understanding of the cellular and molecular pathways involved in this disease [38,39]. In contrast to microarray chips, RNA-Seq allows an unbiased analysis of the transcriptome as it is not dependent on predefined probe sets and is therefore not limited to a selection of known genes or nucleotide sequences [36]. Consequently, RNA-Seq facilitates the discovery and characterization of novel, disease-related genes.

In our gene expression studies, RNA-Seq was performed using the GS FLX system by 454/Roche, which is based on pyrosequencing [40; Figure 2]. In short, the four DNA nucleotides adenine, cytosine, guanine, and thymine are added one at a time to the DNA template. When a nucleotide is incorporated with the DNA template, light is produced through an enzymatic reaction, which is captured by a CCD camera (Figure 2A). Additionally, the intensity of the light signal is proportional to the amount of nucleotides incorporated during one flow. For example, if the light intensity during a flow of adenine is 6, it means that 6 adenine nucleotides were incorporated consecutively to the DNA template. The order and intensity of the light signals obtained during a sequencing run are assembled into ‘pyrograms’, which reveal the DNA sequence (Figure 2B).

Figure 2 - Pyrosequencing by GS FLX
Light is emitted through an enzymatic reaction, when a nucleotide is incorporated (A). The order and intensity of light signals are visualized in a ‘pyrogram’ (B). The DNA sequence, depicted above the ‘pyrogram’, is determined by reading the intensity peaks of the nucleotides from left to right.
Adapted from: Metzker et al. Nat Rev Genet 2010;11:31-46
4.2. Sequencing, mapping, and analyzing

The workflow of a typical gene sequencing process and the method used for the subsequent analysis of the sequence data are highly dependent on the research question. An overview of the workflow for RNA-Seq, which was used in our gene expression studies described in this thesis, is shown in Figure 3.

1. Sequencing

Sequencing the cDNA of the samples will yield sequence reads of various base pair (bp) lengths. In step 2, these reads are mapped to the reference DNA, which in this case is the human genome. This enables identification of the specific genes that were present in the sequenced samples in step 1. Furthermore, it allows a quantification of those identified genes, because the more reads of a specific gene are present, the more that gene is expressed. Mapping of the reads follows a sequence census approach, which is in its concept similar to the serial analysis of gene expression (SAGE) method [38]. In short, each sequence read is aligned with the reference genome. However, a sequence read may align to multiple locations of the reference genome due to their short bp length compared to the reference. To identify the actual site of origin in the reference genome, the sequence read is put together with other sequence reads that contain overlapping DNA

Figure 3 - Workflow RNA-Seq and subsequent analysis
The workflow employed in our gene expression studies can be divided into 3 steps. First, the samples were sequenced by the GS FLX system (454/Roche). In the second step, the sequence reads were mapped against the reference DNA. Afterwards, analyses of the sequence data were performed by determining the functions of the individual gene, and by identification of gene networks.
sequences to undergo multiple alignments. This will result in the formation of a contig, which is a contiguous sequence constructed from many clone sequences [41]. The eventual bp length of a contig will thus be determined by the length of the sequence tags with overlapping DNA sequences. With an overall longer bp length than the individual sequence tags with which it was constructed, the contig will point towards the actual site of origin when aligned to the reference genome.

After it has been ascertained which genes were expressed in the sequenced samples, statistical analyses can be performed to narrow these genes down to those that were differentially expressed between the study groups, which in our studies consisted of asthma patients and healthy controls. First, the function of an individual gene can be determined and associated with the disease under investigation, e.g. asthma. Second, the set of differentially expressed genes can be used to identify gene networks by pathway analyses. These gene networks may clarify what biochemical processes are differently regulated leading to the manifestation of asthma. For the pathway analyses we used the commercially available Ingenuity Pathway Analysis (IPA) software application [42]. IPA uses its own repository of biological and functional annotations, which is based on results found in literature and other major databases including NCBI (EntrezGene, RefSeq, OMIM), Gene Ontology, and KEGG amongst others. Each identified gene network is assigned a network score by IPA, which is displayed as the negative log of the p-value of that specific network. This network score gives the likelihood that the set of genes in this network could be explained by chance alone. Consequently, networks with a score ≥2 have at least 99% confidence that it is not generated by chance.

5. Aims of this thesis

The aims of the current thesis are as follows:

1. To examine the difference in ASM phenotype between asthma patients and healthy controls by gene expression profiling and histopathology
2. To unfold what biochemical processes in the ASM layer are differently regulated in asthma patients compared to healthy controls that may explain the pathophysiology in asthma
3. To clarify whether the beneficial effects of glucocorticoids on the clinical status and airway function in asthma are solely based on their anti-inflammatory properties or whether they also exert other local actions within the airways
6. Outline of this thesis

1. In chapter 2, RNA-Seq was used to investigate the difference in transcriptomic profile of whole endobronchial biopsy specimens between asthma patients and healthy control subjects.

2. The transcriptomic profiles of the ASM layer obtained from endobronchial biopsies were compared between atopic asthma, and both atopic and non-atopic healthy control subjects using RNA-Seq in chapter 3. Additionally, we investigated the association between the ASM transcriptomic profiles and airway function.

3. To investigate the effect of glucocorticoids on the gene expression of the ASM layer, we compared in chapter 4 the ASM transcriptomic profile of asthma patients that have taken prednisolone or placebo for 14 consecutive days. Furthermore, we investigated the association between the change in ASM transcriptomic profile by glucocorticoids and the change in airway function.

4. The relationship of various ECM proteins in the ASM layer of asthma patients and healthy control subjects with the dynamics of airway function is elucidated in chapter 5.

5. Chapter 6 describes a novel and non-invasive imaging technique called Fibered Confocal Fluorescence Microscopy (FCFM), which was used to visualize in vivo elastic fiber patterns within the airway wall in asthma patients and healthy control subjects. We aimed to establish the concordance between the bronchial elastic fiber pattern in histology and FCFM. Additionally, the elastic fiber patterns within the airway wall were associated with airway function.

6. To conclude, the findings are summarized and discussed, and implications for future research are given in chapter 7.
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