Chapter 9

Summary, discussion, conclusions and perspectives

9.1 Summary

In chapter 2.1-2.4 some methodological problems of obtaining, processing and evaluating bronchial biopsies were described. In chapter 3-5 the inflammatory data of our study on bronchial biopsies in nocturnal asthma were presented. Finally, in chapter 6-7 markers of inflammation in other body compartments were described, i.e. in exhaled air, serum and BAL fluid.

Chapter 2.1 was directed to the problem of obtaining large and intact biopsies from the central airways. It is known that bronchial biopsies are rather small and that optimal biopsy technique is necessary to obtain high quality tissue samples, as sufficient length of intact basement membrane and sufficient depth of submucosal tissue are required. In this study, size and qualitative aspects of bronchial biopsies from non-asthmatic subjects, obtained by forceps of three different sizes (types FB-19C, FB-21C and FB-35C) were compared. We concluded that bronchial biopsies obtained with forceps type FB-35C were the largest, but showed significant damage and crush artifacts. In contrast, biopsies obtained with forceps type FB-21C were only slightly smaller and had more intact basement membrane, more submucosal depth and well preserved morphology. Therefore, forceps type FB-21C was used in further studies of this thesis.

In chapter 2.2 inflammatory cell counts in sections of fresh frozen and glycol methacrylate (GMA)-embedded biopsies were compared. An important advantage of GMA embedding is its better morphology, although the main impression is that its lower antigenicity may be a problem. Indeed lower numbers of CD3-, CD4- and CD8-positive cells were counted in GMA-embedded biopsies as compared to snap-frozen biopsies. In addition, only a weak correlation between CD3-, CD4- and CD8-positive cell counts of both techniques was observed, which we supposed to be caused by an ongoing loss of antigenic properties during storage of plastic-embedded tissue. An additional study confirmed that CD3-, CD4- and CD8-positive cell numbers decrease significantly within a few months after embedding in glycol methacrylate. Therefore, we recommended to process glycol methacrylate-embedded biopsies as soon as possible. Further, we considered frozen tissue to be preferred in the quantitative evaluation of inflammatory cells (like CD3, CD4, CD8, EG2) because of ease of the method and reliable cell counting. On the
other hand, glycol methacrylate-embedded tissue shows superior morphology and seems attractive to study qualitative aspects as cell-cell and cell-matrix relationships.

In chapter 2.3 we evaluated the amount of tissue that is necessary to produce constant cell counts in frozen sections of bronchial biopsies. We formulated this question because immunopositive cells are present in varying densities in lung tissue, even within a section of a bronchial biopsy. CD4-, CD8- and EG2-immunopositive cells in bronchial biopsies of five healthy and five asthmatic subjects were evaluated. Twenty successive areas of 0.1 x 0.1-mm submucosa were counted, and changes larger than 10% were recorded. We demonstrated that the cumulative counting of 10 areas of 0.1 x 0.1-mm along 1 mm intact basement membrane is sufficient to obtain constant cell numbers (per area), provided that a proper selection of a representative part of the biopsy has been made. Also, it appeared that volume artefacts and presence of smooth muscle and glands were responsible for the observed changes in cell number above 10% after counting 1-mm basement membrane.

In chapter 2.4 the semi-quantitative and quantitative way of evaluating inflammatory cells in bronchial biopsies were compared. Fresh frozen airway biopsies of 7 asthmatic and 7 healthy subjects were immunostained for CD3, CD4, CD8, CD25 and EG2 antigen, and examined in a quantitative and semi-quantitative way. This study demonstrated that both methods correlated in the evaluation of CD4-, CD8- and EG2-immunopositive cells. The quantitative method showed a higher density of CD8- and CD25-positive cells in biopsies of asthmatic subjects as compared to healthy subjects, whereas the semi-quantitative method showed a relatively higher density of CD3- and CD4-positive cells. This partial agreement may be explained by the intrinsic features of the two methods. The semi-quantitative method includes the deeper and larger parts of the biopsy, and has the advantage that biopsies of lower morphological quality do not have to be discarded. In contrast, the quantitative method includes only small parts of the biopsy (generally the superficial layers) selected on basis of integrity of the tissue and absence of smooth muscles and glandular epithelium. We concluded that with high cell density the semi-quantitative method is more useful, whereas with low cell density the quantitative method may be preferred.

In chapter 3 we tested the hypothesis that bronchial biopsies of subjects with nocturnal asthma have more inflammatory cells at night than at daytime, in contrast to subjects without nocturnal asthma. We recruited 13 healthy controls, 15 atopic asthmatic patients with PEF-variation ≤ 15% and 10 atopic asthmatic patients with PEF-variation > 15%. Bronchial biopsies were obtained at 16 h and 4 h, with an interval of 1-2 weeks. Bronchial biopsies were used to study inflammatory cells (chapter 3), vascular adhesion molecules...
The number of CD3-, CD4-, CD8-, CD25-, AA1 (tryptase)- and EG2- immunopositive cells in the submucosa did not increase at night in any of the three groups. The number of EG2-positive cells in the two asthmatic groups was significantly higher than in healthy controls, both at 16 h and 04 h. The number of EG2-, CD4- and CD25-positive cells at 04 and at 16 h tended to be higher in asthmatics with a PEF variation > 15% as compared to asthmatics with PEF variation ≤ 15%. We concluded that increased nocturnal airway obstruction is not caused by increased numbers of inflammatory cells in the bronchial submucosa at night.

Because vascular adhesion molecules are thought to be important for the recruitment of inflammatory cells in the process of asthmatic airway inflammation, we hypothesized in chapter 4 that adhesion molecules are expressed more extensively at night than at daytime in subjects with nocturnal asthma. Biopsies were double-immunostained for CD31 in combination with P-selectin, E-selectin, ICAM-1 or VCAM-1. We found no significant day-night differences in expression of adhesion molecules in any of the three groups. The expression of VCAM-1 in biopsies of asthmatic patients was significantly higher than in biopsies of healthy controls: 4.5 vs 2.5 at 16 h and 11 vs 0 at 04 h. In asthma, VCAM-1 expression was positively correlated with the number of EG2- and CD25-positive cells both at 16 h and 04 h, suggesting a role for VCAM-1 in the ongoing airway wall inflammation of asthma.

Because Nitric Oxide (NO) may be involved in the pathogenesis of asthmatic airway inflammation, we hypothesized in chapter 5 that the inducible form of NO synthase (iNOS) is upregulated at night in bronchial biopsies of patients with nocturnal asthma. Biopsies were immunostained with a polyclonal antibody against iNOS. We demonstrated that the expression of iNOS in the bronchial wall was not increased at night in the asthmatic patients with increased nocturnal airway obstruction, nor in the other subjects. Moreover, biopsies of asthmatic patients more frequently contained iNOS positive cells than those of healthy controls, both at 16 and 04 h. In contrast to reports in the literature, iNOS immunoreactivity in the epithelium was very sparse and seemed mainly located in migrating inflammatory cells and not in epithelial cells. Interestingly, epithelial and endothelial iNOS positivity at 04 h was accompanied by a higher degree of submucosal EG2 positivity. We concluded that nocturnal asthma can not be explained by circadian variations in iNOS expression and that increased expression of iNOS and EG2-positive cells probably represent a common underlying mechanism.

In chapter 6 the variable or constant presence of endothelial NO synthase in bronchial biopsies of subjects with and without nocturnal asthma was studied. Biopsies obtained at 16 and 04 h were double-immunostained for eNOS
in combination with a common endothelial antigen (CD31). The degree of immunopositivity for eNOS was evaluated and expressed as the percentage of CD31 positive vessels encountered in complete sections of a biopsy. The mean (SD) 16-04 h changes in % eNOS expression in biopsies of healthy controls, asthmatic patients with mild and severe nocturnal airway obstruction differed significantly: -17 (44), -14 (24) and +13 (27) %. Moreover, the 16-04 h change in eNOS expression in biopsies of asthmatic subjects significantly correlated with PEF variation. We concluded that patients with large swings in airway diameter lack the normally occurring increase in eNOS expression at night.

As Nitric Oxide in exhaled air may reflect airway inflammation we hypothesized in chapter 7 that subjects with nocturnal asthma show a circadian rhythm in exhaled Nitric Oxide, inverse to the circadian rhythm in airway obstruction. We measured NO concentration together with FEV₁ at 12, 16, 20, 24, 4, 8 and 12 h in 6 healthy controls, 8 asthmatics without and 6 asthmatics with increased nocturnal airway obstruction. Exhaled NO did not show a significant circadian variation in any of the three groups as assessed by cosinor analysis (in contrast to the FEV₁ in both asthma groups). However, exhaled NO concentration at 4 h was higher than at 16 h in subjects with nocturnal asthma; otherwise values at all time points were similar. Mean NO concentration was significantly higher in subjects with nocturnal asthma than in subjects without nocturnal asthma and higher again than in healthy controls, at all time points. Moreover, mean NO levels over 24 hours correlated with 16-4 h PEF variation in the asthmatic group. We concluded that subjects with nocturnal asthma exhale higher NO levels during the whole day, suggesting more severe airway wall inflammation than subjects without nocturnal asthma.

In chapter 8 we studied the relationship between important cytokines in serum and BAL fluid and clinical manifestation of atopic asthma. IL-4, IL-5 and IFN-γ levels of serum and BAL fluid (obtained at 16 and 04 h) were determined in 17 atopic asthmatics and 8 non-atopic healthy subjects, participating in another study on nocturnal asthma. Serum IFN-γ increased significantly at night in asthmatic subjects. Serum IL-4, IL-5 and IFN-γ levels in asthmatic subjects were significantly higher than in healthy controls, both at 16 and 04 h. In asthmatic subjects, serum IFN-γ at both time points correlated negatively with PC₂₀ methacholine and positively with the mean 16-04 h PEF variation. In contrast, no relationship was found between the serum levels of IL-4 and IL-5 and the parameters of clinical manifestation of asthma. BAL fluid levels of IL-4, IL-5 and IFN-γ were frequently below the level of detection, may be as a consequence of dilution. We concluded that serum IFN-γ levels may indicate the severity of airway wall inflammation in subjects with atopic asthma. We suggested a.o. epithelial cells to be the cellular source of serum interferon-γ.

9.2 Discussion

Findings

The findings from this research support the hypothesis that nocturnal asthma is related to airway inflammation at night. Subjects without nocturnal asthma not show a relevant increase in exhaled Nitric Oxide from mucosa (CD31 positive vessels in epithelial cells in epithelium, adhesion molecules like selectin). Our study shows no signs of a circadian rhythm in central airway obstruction. With respect to cytokines, subjects with nocturnal asthma have higher serum levels of pro-inflammatory cytokines like IFN-γ compared to non-nocturnal asthmatics and healthy controls, which may lead to a relatively small increase in exhaled Nitric Oxide and, therefore, decreased in PEF variation at night, implying a circadian change in airway obstruction less likely.

Our findings from this research support the hypothesis that nocturnal asthma is related to airway inflammation at night. The levels of IFN-γ in serum are higher in nocturnal asthmatics than in those without nocturnal asthma, indicating a possible link between airway inflammation and clinical manifestation of asthma. We suggest that the systemic levels of IFN-γ may be a marker for the severity of airway inflammation in atopic asthma, with epithelial cells possibly being the source of the cytokine.

Selection of participants

This study included 17 atopic asthmatics and 8 non-atopic healthy controls. The research was divided into two groups, with different criteria for participation. The first group included all subjects with asthma, participating in the study on PEF variation > 15%. The second group included participants with airway obstruction > 15% per day, during the study period. We concluded that the level of airway obstruction may be an important factor in the manifestation of nocturnal asthma. We used a combination of clinical symptoms and laboratory findings to assess the severity of airway inflammation in subjects with atopic asthma.