On the functional consequences of bronchial basement membrane thickening

MANLIO MILANESI, EMANUELE CRIMI, ANTONIO SCORDAMAGLIA, ANNAMARIA RICCIO, RICCARDO PELLEGRINO, G. WALTER CANONICA, AND VITO BRUSASCO

Dipartimenti di Scienze Motorie e Riabilitative e di Medicina Interna, Università di Genova, 16132 Genova; and Fisiopatologia Respiratoria, Azienda Ospedaliera S. Croce e Carle, 12100 Cuneo, Italy

Received 29 November 2000; accepted in final form 9 April 2001

THICKENING OF THE RETICULAR basement membrane (RBM) has been described as a characteristic feature of airway remodeling in asthma (26), although it has also been observed in subjects with asymptomatic airway hyperresponsiveness (8, 19). Modeling studies (17, 27) have suggested that RBM thickening may represent an increased load on airway smooth muscle, thus limiting its shortening and opposing airway narrowing. On the other hand, RBM thickening may reduce the number of mucosal folds that form when ASM shortens, which would favor airway narrowing (30). The available experimental data (3, 9, 10) seem to support the latter prediction, although studies relating RBM thickening to severity of disease are conflicting (9, 10, 11). The assessment of the functional effects of RBM thickening in asthma may be complicated by the concomitant effects of several confounding factors, such as presence of inflammatory cells and mediators in the airways, atopic status, and allergen exposure.

In this study, we reasoned that, if RBM prevalently acts as a load contrasting airway smooth muscle shortening in vivo, then its thickening should reduce airway narrowing and occurrence of air trapping on exposure to a bronchoconstrictor agent. This hypothesis was tested in four groups of patients affected by bronchial asthma, perennial rhinitis, seasonal rhinitis, and chronic obstructive pulmonary disease (COPD), conditions in which airway hyperresponsiveness and airway remodeling may be variably associated.

METHODS

Subjects. Thirty-three subjects participated in the study (Table 1). Of these, 11 were affected by mild perennial asthma, 8 by perennial rhinitis without asthma, 5 by seasonal rhinitis, and 9 by COPD. Allergic sensitization was assessed by a standard skin prick test panel (Laboratorio Farmaceutico Lofarma, Milan, Italy) and radioallergosorbent test (RAST; Pharmacia, Uppsala, Sweden), including mites, cladosporium, alternaria, grasses, parietaria, olive, cypress, and cat and dog dander. All subjects with perennial rhinitis or asthma were sensitized to house dust mite allergen only (RAST 3rd- to 4th class), whereas those with seasonal rhinitis were only sensitized (RAST 4th class) to either grass (n = 2) or parietaria (n = 3) and were studied during pollen season. Rhinitic subjects never experienced symptoms that may be related to asthma or airway hyperresponsiveness (6), whereas asthmatic subjects were variably affected by concomitant rhinitis. Diagnoses of asthma and COPD were made according to the definitions given by the American Thoracic Society (1, 2). All subjects classified as COPD had a history of chronic bronchitis, and in none of them did the administration of 200 µg of salbutamol increase 1-s forced expiratory volume (FEV1) by ≥12% of control and >200 ml. Subjects with reduced, single-breath CO diffusion capacity were not included. At the time of the study, all patients were in a stable clinical condition, and none of them was taking...
regular treatment with steroids, cromolyn, or bronchodilator drugs. Asthmatic and COPD subjects were using short-acting $\beta_2$-agonists on demand, which were avoided for 12 h before the studies. Seasonal rhinitic subjects were using oral and local antihistamine on demand, which were avoided for 5 days before studies. As they were studied during pollen season, treatment avoidance was associated with occurrence of mild symptoms of rhinitis. The study was approved by the institutional ethics committee, and each subject gave written, informed consent.

Protocol. All subjects attended the laboratory on 2 consecutive days to have airway responsiveness to methacholine (MCh; day 1) and bronchial biopsy (BB; day 2) performed. The eight subjects with perennial rhinitis and nine of those with asthma were also studied after an allergen inhalation challenge, the results of which are the object of a companion paper (12).

Lung function. All measurements were obtained by a Vmax 6200 system (SensorMedics, Yorba Linda, CA). On each occasion, the highest of three FEV$_1$ and forced vital capacity (FVC) within 100 ml was retained. In two asthmatic and one COPD subject, FVC values were not retained because forced expiration was not sustained for $>6$ s. Forced expiratory maneuvers without a sharp peak flow were discarded.

MCh challenge. Solutions of MCh were prepared by adding distilled water to dry powder MCh chloride (Laboratorio Farmaceutico Lofarma). Aerosols were delivered by SM-1 Rosenthal breath-activated dosimeter (SensorMedics) driven by compressed air (30 psi) with 1-s actuation. The aerosol output at the mouth was 10 µl per actuation. Aerosols were inhaled during quiet tidal breathing. After 20 inhalations of saline as a control, the subjects inhaled double-increasing doses of MCh from 20 µg, until FEV$_1$, measured 1 min after each dose, decreased below 80% of control value or the maximum dose of 1,200 µg was achieved. The double increments of dose were obtained by using two MCh concentrations (1 and 10 mg/ml) with appropriate numbers of breaths. A 3-min interval was allowed between dose increments. Airway responsiveness to MCh was estimated from the noncumulative dose causing a 20% reduction in FEV$_1$ from control (PD$_{20}$), calculated by interpolation between two adjacent points of the log dose-response curve. In subjects without a 20% reduction of FEV$_1$, the last MCh dose was retained as the PD$_{20}$.

BB. After premedication with atropine (0.5 mg im) and prometazine hydrochloride (50 mg im), oxibuprocaine (4% solution) was instilled into the nostrils. A fiber-optic bronchoscope (type 1T20, Olympus BF) was then passed through the nose or the mouth without endotracheal tube and, after local anesthesia of pharynx and airways, into the bronchi. Four biopsy specimens were taken from the right upper lobe distal bifurcation.

Tissue specimens were fixed in 10% formaldehyde at 4°C for 4 h, embedded in paraffin, cut at 5 µm with a rotative microtome, and stained with hematoxylin and eosin and toluidine blue. Biopsy specimens were discarded if they were too small or thin, incorrectly oriented, or had the RBM disrupted. For cell counts (eosinophils and mast cells), bronchial mucosa was examined to a depth of 100 µm from the basement membrane over adjacent, non-overlapping high-power fields (at ×500 magnification with the aid of an eyepiece graticule) until all of the available area was covered. For each quantification, three sections of each specimen were examined, and cells were expressed as number per square millimeter of submucosa. Mast cells were examined at ×1,000 magnification to detect ongoing degranulation (granules surrounding the cell or crossing the cell membrane). The intraobserver mean coefficient of variation for three repeated measurements was 9% for eosinophils and 8% for mast cells. In two COPD cases, analysis to a depth of 100 µm was not possible, and inflammatory cells were not valuable. The thickness of RBM was measured on hematoxylin- and eosin-stained preparations by light microscope image analysis (Q 500, Leica, Cambridge, UK) at ×400 magnification from the base of bronchial epithelium to the outer limit of the reticular lamina, at regular intervals (20 µm), until at least 40 readings were obtained. The intraobserver mean coefficient of variation for three replicate measurements was 3%.

Data analysis. Occurrence of air trapping during airway narrowing was inferred from the linear regression of the FVC values recorded at each step of the challenge against the corresponding FEV$_1$ values. In this analysis, the slope value quantifies the amount of air trapping associated with airway narrowing, with a value of zero indicating airway narrowing with no air trapping.

Differences among groups were assessed by ANOVA with Duncan post hoc comparisons when values were normally distributed and by Kruskal-Wallis ANOVA by ranks when they were not normally distributed. The relationships between airway responsiveness and air trapping to RBM thickness were assessed by calculating Pearson’s coefficient of correlation. Statistical significance was considered at $P$ values <0.05. Data are presented as means ± SD, geometric means, or medians with upper and lower quartiles.

RESULTS

Lung function and BB data are presented in Table 2. In asthma, baseline FEV$_1$ as percentage of predicted and FEV$_1$/FVC were significantly lower ($P < 0.05$) than in perennial rhinitis and higher than in COPD ($P < 0.01$) but not significantly different from seasonal rhinitis ($P > 0.1$ for both comparisons). Furthermore, there were no differences between perennial and sea-

| Age, yr | 26 ± 3 | 24 ± 3 | 29 ± 7 | 59 ± 15 | 59 ± 15 |
| Height, cm | 176 ± 7 | 175 ± 5 | 176 ± 5 | 166 ± 8 | 166 ± 8 |
| Weight, kg | 80 ± 7 | 71 ± 8 | 78 ± 6 | 68 ± 14 | 68 ± 14 |
| Smoking habits, pack·yr | 0 | 0 | 0 | >30 | >30 |
sonal rhinitis ($P > 0.1$ for both comparisons). In COPD, baseline FEV$_1$ percentage and FEV$_1$/FVC were significantly less than in any other groups ($P < 0.01$ for all comparisons).

MCh-PD$_{20}$ was significantly less in asthma than in perennial rhinitis ($P < 0.01$), indicating greater airway responsiveness in the former. A FEV$_1$ decrease $>20\%$ of control was achieved at Mch doses $<1.2$ mg in all asthmatic subjects but in only two subjects with perennial rhinitis. No other comparison of MCh-PD$_{20}$ was statistically significant among groups ($P > 0.06$ for all comparisons). The slope of FVC vs. FEV$_1$ (Fig. 1 and Table 2) was significantly steeper in COPD than in asthma and perennial rhinitis ($P < 0.05$ for both), whereas the intercept was less than in perennial and seasonal rhinitis ($P < 0.05$ for both).

The thickness of RBM was not significantly different ($P > 0.3$) between asthma and perennial rhinitis. In both groups, however, RBM was thicker than in COPD and seasonal rhinitis ($P < 0.01$ all comparisons) and above the reported normal range (28).

In asthma, RBM thickness positively correlated with MCh-PD$_{20}$ ($r = 0.77, P < 0.01$; Fig. 2) and negatively with the slope of FVC vs. FEV$_1$ ($r = -0.73, P < 0.03$; Fig. 3). These relationships suggest that the thicker the RBM, the less the airway responsiveness and the less the occurrence of air trapping. In perennial rhinitis, there was a narrow range of near-normal MCh-PD$_{20}$ values, despite a wide range of increased RBM thickness values. By contrast, in seasonal rhinitis and COPD, MCh-PD$_{20}$ values were widely variable and occasionally (3 rhinitic and 3 COPD subjects) below the normal range, despite a narrow range of near-normal RBM thickness values.

Eosinophil numbers were not significantly different among groups ($P > 0.5$ for all comparisons). Mast cell number was higher in asthma and perennial rhinitis than in COPD and seasonal rhinitis ($P < 0.01$ for all comparisons). The percentage of degranulating mast cells, i.e., with granules surrounding or crossing cell membrane, was greater in seasonal rhinitis than in perennial rhinitis and in asthma ($P < 0.01$). No significant correlation was found between RBM thickness and inflammatory cell numbers in bronchial mucosa in any group.

**DISCUSSION**

The major findings of the present study are that 1) RBM thickening is not unique to bronchial asthma, as it also occurs in subjects with perennial rhinitis without asthma, and 2) when present, RBM thickening is associated with less airway narrowing and air trapping.
in response to inhaled MCh. These findings would support the opinion that RBM thickening represents an additional load on airway smooth muscle.

Comments on methodology. Our analysis of the effects of MCh on airway narrowing and air trapping is based on the assumption that total lung capacity does not change during the challenge (24), and, therefore, any decrease in FVC reflects an increase in residual volume and, by inference, air trapping (13). In contrast, a decrease in FEV1 is assumed to reflect airway narrowing at the level of the flow-limiting segment, or an increase in frictional losses at high-lung volume, or both (21). It is possible that changes in FVC and in FEV1 are determined by mechanisms occurring at different levels along the bronchial tree. Therefore, their comparison with results of bronchial biopsies taken in large airways assumes that airway remodeling in these airways is also representative of remodeling in more distal airways (25). Furthermore, in this study, biopsy specimens were taken at a single site and assumed to be representative of airway remodeling throughout the lung. This assumption seems to be justified by the findings of Bradley et al. (4) and Jeffery et al. (15).

Comments on results. The occurrence of subepithelial fibrosis in asthma was first reported by Roche et al. (26) more than a decade ago and ever since has been considered as a characteristic feature of bronchial asthma (9, 16). The results of the present study indicate that RBM thickening is not unique to bronchial asthma, as it also occurs in subjects with perennial allergic rhinitis who never suffered from asthma symptoms. The findings of RBM thickening in both perennial asthma and rhinitis, but not in seasonal rhinitis and COPD, would suggest that subepithelial fibrosis may result from IgE-mediated airway inflammation with prolonged exposure to the sensitizing allergen. This is in agreement with a recent animal study (23), in which long-standing exposure to the sensitizing agent was necessary to cause collagen deposition in the airways.

Although it must be kept in mind that a significant correlation does not prove a causal relationship, the relationships of MCh-PD20 and FVC vs. FEV1 slope to RBM thickness observed in asthma would suggest that subepithelial fibrosis may play a protective role against airway hyperresponsiveness and air trapping. This would be in keeping with the theoretical prediction that increased collagen deposition tends to increase airway wall stiffness, thus providing additional internal load on airway smooth muscle and limiting its linear shortening (7, 17). It has been theoretically predicted that maximal shortening of unloaded airway smooth muscle would result in complete airway closure (22). It has also been proposed that maximal airway smooth muscle shortening does not occur in vivo because of the elastic load imposed by lung elastic recoil (20). The negative relationship between air trapping (FVC vs. FEV1 slope) and RBM thickness found in asthma suggests that subepithelial fibrosis may be an additional, important factor limiting airway closure or extreme flow limitation. The flat relationships between RBM thickness and functional measurements (MCh-PD20 and FVC vs. FEV1 slope) observed in perennial rhinitis suggest that any degree of RBM thickening is sufficient to counteract the force developed by nonasthmatic airway smooth muscle. By contrast, the significant relationships found in asthma suggest that large RBM thickening is necessary to efficiently oppose the greater force generated by airway smooth muscle.

Another theoretical prediction was that RBM thickening may influence the pattern of mucosal folding on airway smooth muscle shortening (30), with a thicker
RBM corresponding to a reduced number of folds, thus resulting in a greater airway narrowing. However, according to a recent study, the number of mucosal folds is determined by the number of tethers between RBM and the smooth muscle layer, rather than RBM thickness itself (27). The finding of a different MCh-PD<sub>20</sub> despite similar RBM thickening in asthma and perennial rhinitis, does not support the hypothesis that subepithelial fibrosis may contribute significantly to enhance airway narrowing. Others have reported a positive, although weak, correlation between airway responsiveness and RBM thickness in asthma (3, 10, 14). This inconsistency might be due to the confounding effect of additional mechanisms modulating airway narrowing, independent of the RBM thickening. Inflammatory cells and their mediators may be variably present in allergic subjects, depending on allergen exposure, thus possibly causing an increased sensitivity of airway smooth muscle to contractile stimuli. Furthermore, subepithelial fibrosis may be associated with the presence of myofibroblasts (5), whose contribution to force development is, however, undetermined.

Airway responsiveness was occasionally increased in seasonal rhinitis but was always near normal in perennial rhinitis. Although the percentage of degranulating mast cells was greater in seasonal than in perennial rhinitis, their absolute numbers were similar. If the number of degranulating mast cells reflects the amount of mediators released in the airways and the airway smooth muscle of seasonal and perennial rhinitic subjects produces similar forces, it is possible that the lower airway responsiveness in the latter is somehow related to the protective effect of increased RBM thickness. This interpretation would be consistent with an animal study (23) in which airway hyperresponsiveness occurred after the first 2 wk of exposure to the sensitizing allergen, in association with an increase in airway inflammation, but waned in the subsequent 10 wk, when fibronectin and collagen deposition occurred in the airway wall.

In COPD, the inhalation of MCh caused greater air trapping than in asthma, suggesting enhanced airway closure or extreme airflow limitation. Modeling studies (18) have shown that airway smooth muscle force is not a major determinant of airway narrowing in COPD. In these subjects, airway hyperresponsiveness may result from airway wall thickening (29) or reduced loads on airway smooth muscle (20). In the present study, the elastic recoil of the lung could not be determined. Therefore, possible effects of differences in the external elastic load on airway smooth muscle cannot be evaluated. Pulmonary emphysema was, however, unlikely to be present in our subjects, as those with reduced CO diffusion capacity were not included. It may be speculated that the enhanced air trapping in COPD is the result of an increased thickness of total airway wall but not of RBM.

The apparent negative relationship between airway responsiveness and RBM thickening observed in asthma might have been due to a concomitant increase in lung elastic recoil in response to MCh. In a previous study, however, inhaled MCh did not affect the static pressure-volume curves of mild asthmatic subjects (24). Furthermore, in six subjects in whom the effect of deep inhalation was evaluated during the challenge (12), there was no significant correlation between the ability to dilate constricted airways by lung inflation and the thickness of RBM.

In conclusion, even if it cannot be excluded that RBM thickening may limit the amount of inhaled bronchoconstrictor agents reaching airway smooth muscle, the findings of the present study seem to support modeling studies (17, 27), suggesting that RBM thickening may protect against airway narrowing by loading airway smooth muscle.

This study was supported in part by grants from Ministero dell’Università e della Ricerca Scientifica e Tecnologica (Rome, Italy) (to V. Brusasco and G. W. Canonica) and by Associazione per la Ricerca delle Malattie Immunologiche ed Allergiche (Genoa, Italy).

REFERENCES


12. Crimi E, Milanesi M, Oddera S, Mereu C, Rossi GA, Riccio A, Canonica GW, and Brusasco V. Inflammatory and me-


