Relationship between immunohistochemical assessment of bronchial mucosa microvascularization and clinical stage in asthma

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Abstract
Although hardly ever used in current practice, fibrobronchoscopy may provide interesting histopathological–clinical correlations in patients diagnosed with different stages of asthmatic disease. The aim of the study was to evaluate the correlation between semi-quantitative microvascularization features and the asthma severity assessed according to the GINA classification 2006. Our study group consisted in 21 patients diagnosed with asthma of different stages of severity and two-control patients investigated by fibrobronchscopy with associated biopsy. The tissue fragments underwent standard processing procedures for the immunohistochemical exam, using CD34 as microvascularization marker. The semi-quantitative analysis was based on the “hot spot” method and on a score system that corresponds to the microvessels density. The statistical analysis of the correspondence between CD34 score and clinical parameters was performed using the SPSS 17 software, applying non-parametric correlation tests. The CD34 evaluation showed an increase in blood vessels count in all asthmatic patients in comparison to the control group and a close correlation with the asthma severity, reflected by the FEV1 values. The statistical analysis showed an inverse correlation between PEV1 [%] values and CD34 expression ($r$=0.93, $p<0.01$). Our data concur to other research reports, supporting the hypothesis that angiogenesis initially facilitates the edema development and later on appears to be involved in the bronchial wall thickening, as a component of the chronic inflammatory response, with concomitant distensibility reduction. The bronchial mucosa microvascularization evaluation opens new perspectives for advanced therapies, with beneficial effects for asthmatic patients’ life quality.

Keywords: CD34, microvascular density, immunohistochemistry, asthma.

1 Introduction
The pathogenesis of asthma involves an increased responsiveness of the tracheobronchial tree to several stimuli, that otherwise would have little or no effect on healthy individuals, resulting in a chronic inflammatory disease. The fibrobronchoscopy technique has been uncovering new histopathological findings in the last 20 years since its application as a routine diagnosis tool. The fibrobronchoscopy investigation allows not only direct visualization of the large airways but also the bronchoalveolar lavage and/or the collection of tissue fragments from patients presenting at variable stages of evolutive disease [1, 2].

The chronic mucosal inflammation, accompanied by edema and a moderate increase of blood vessels density appears to be responsible for the increase of distensibility observed in the mild asthma symptoms [3, 4]. The association between the chronic inflammation with the prominent deposition of subepithelial collagen, muscular and glandular hypertrophy and a significant increase in vessel density result in a complex remodeling phenomenon, which is characteristic in severe asthm [5–7].

Few research reports are mainly focused on qualitative and quantitative comparative evaluation of the mucosal vascular component in asthma, and on identifying the involved stimulating factors, which are currently available in literature [8–11].

TNF-$\alpha$, VEGF, and b-FGF are considered important endothelial stimulating growth factors in the bronchial wall [12]. Supplementary, VEGF is closely correlated to mucosal bronchial edema by increasing vascular permeability [10, 11]. The correlation between long-term corticosteroid therapy and bronchial vessel density has been investigated by several research teams [10, 13–15]. The therapeutic benefits of high-dose long-term corticosteroid and of $\beta_2$ agonists’ inhalers are correlated to decreased bronchial vessel density [14, 15].

Despite the continuous research efforts, the correlations between vascularization and asthma pathogenesis, mainly concerning the bronchial remodeling process, remains incompletely elucidated [8, 9].

Our study is included in the current research framework, as a double histopathological and clinical approach, which has the main purpose to assess the correlation between the microvascularization semi-

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Materials and Methods

The study group consisted of 21 asthma patients from the Outpatient Department in the Clinical Pneumology Hospital of Iassy evaluated between January 2007 and August 2008. The control group consisted in two patients without asthma symptoms, which have been investigated for tumor suspicion that has been later dismissed. A standardized working protocol authorized by the Ethics Committees of the Pneumology University Hospital of Iassy and “Grigore T. Popa” University of Medicine and Pharmacy, Iassy, was applied after patients’ informed written consent.

Patients’ history, pulmonary X-ray exam and spirometry evaluation using Forced Expiratory Volume in 1 second (FEV1) resulted in categorization according to GINA classification of 2006 [16]. Consequently, fibrobronchoscopy associated to biopsy from the middle or the right inferior lobar bronchi was performed. According to international recommendations, five to seven tissue fragments were obtained in each case as a guarantee of avoiding the risk of insufficient material or inconclusive result [1, 17].

The collected tissue fragments were fixed for 24 hours in buffered formalin and processed for paraffin embedding. Serial sections of 4 μm were cut, dewaxed and stained. Routine histological examinations followed by immunohistochemistry were performed in all cases.

A series of minimum standard criteria were used in quality evaluation, as following: appropriate specimen collection certified by the absence of degenerated tissue, appropriate fixation and coloration, presence of a subepithelial connective zone of a minimum of 0.3 mm, and absence of hyaline cartilage, mucus, or extensive hemorrhage.

Heat-induced epitope retrieval technique was performed using a Target Retrieval Solution with a pH 9 (code S3308, Dako, Denmark) for immunohistochemistry. After blocking the endogenous peroxidase and the non-specific binding, the sections were incubated overnight at 4°C with the anti-CD34 antibody (clone QB End 10, CD34 antibody (clone QB End 10, Dako, Denmark) with a dilution ratio of 1:100. The immune reaction was amplified by using the Dako EnVision Peroxidase System (code M 7165, Dako, Denmark) for immunohistochemistry.

The semi-quantitative evaluation of the microvascular density (MVD) used the “hot spots” identification in five vascularized areas at 40x magnification, in each case [18]. After CD34-positive elements were counted in each area, using greater magnification (×200), the average value found in each case was associated to the following scoring system:

- a mild increase of the blood vessels count or mild hypervascularization (MVD<25) – score 1 (+);
- a moderate increase of blood vessels count or moderate hypervascularization (MVD from 25 to 35) – score 2 (++);
- a strong increase of blood vessels count or severe hypervascularization (MVD>35) – score 3 (+++).

SPSS 17 software, applying non-parametric correlation tests (Spearman, Kruskal–Wallis, and Newman–Keuls) was used to perform the statistical analysis.

Results

After evaluation of the duration and the degree of pulmonary tests impairment, the patients (10 men and 11 women, with age range between 22 and 70 years) were divided into clinical-based study groups (Table 1), according the degree of asthma’s severity (GINA 2006), as following: four cases of intermittent asthma (stage 1), six cases of mild persistent asthma (stage 2), eight cases of moderate persistent asthma (stage 3), and three cases of severe persistent asthma (stage 4).

Table 1 – General characteristics and functional parameters of the patients included in our study

<table>
<thead>
<tr>
<th>Patients with asthma (n=21)</th>
<th>Mean values ± DS / n</th>
<th>Min.</th>
<th>Q25</th>
<th>Median</th>
<th>Q75</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average persistence of asthma [years]</td>
<td>16.6±9.7</td>
<td>1</td>
<td>12</td>
<td>20</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>FEV1 [% of ideal]</td>
<td>79.3±20.3</td>
<td>45</td>
<td>62</td>
<td>79.8</td>
<td>95</td>
<td>116</td>
</tr>
</tbody>
</table>

The microscopic evaluation of the specimens showed scattered lamina propria microvessels in the control patients (Figure 1) and abundant microvascularization in asthmatic patients (Figure 2).

The microvascularization pattern evaluation resulted in the following classification:

- the microvessels densification in the superficial zone (immediately subjacent the basement membrane) (Figure 3), characteristic of asthma stages 1–2;
- the constant and uniform microvessels distribution in the entire lamina propria, with a perpendicular trajectory on the surface epithelium instead of a parallel route (Figure 4), in asthma stages 3–4.

The application of semi-quantitative analysis in the study group resulted in the following characteristics (Table 2):

Table 2 – Distribution of cases according to the microvascularization scoring system vs. severity degree of asthma

<table>
<thead>
<tr>
<th>Type of asthma</th>
<th>No. of cases</th>
<th>MVD (mean, st. dev)</th>
<th>Score value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>4</td>
<td>19.25±2.21</td>
<td>1</td>
</tr>
<tr>
<td>Mild persistent</td>
<td>6</td>
<td>21.16±2.56</td>
<td>1</td>
</tr>
<tr>
<td>Moderate persistent</td>
<td>8</td>
<td>30.87±2.53</td>
<td>2</td>
</tr>
<tr>
<td>Severe persistent</td>
<td>3</td>
<td>41.66±1.52</td>
<td>3</td>
</tr>
</tbody>
</table>

- a slight increase in the number of blood vessels (19.25±2.21) was noted in all cases diagnosed with intermittent asthma, and scored as 1;
- the mean vessels’ count value in the lamina propria was similar in patients with mild persistent asthma (21.16±2.56) compared with that registered in those diagnosed with intermittent asthma, being also scored as 1 (Figure 5);
- a moderate vessels density increase was registered
in all cases of moderate persistent asthma (30.87±2.53), scored as 2 (Figure 6);

- a significantly increased vessels density in all cases diagnosed with severe persistent asthma was registered (41.66±1.52), being scored as 3.

The non-parametrical statistical analysis performed by Spearman test showed a significant correlation between the disease severity evaluated by FEV1 values and microvessels density revealed by CD34 immunoexpression \( r = 0.93, p < 0.01 \) (Figure 7).

Our statistical results were confirmed by other two tests. CD34 expression was significantly correlated to FEV1 values in Kruskal–Wallis test \( (p < 0.01, F = 69.3, 95\% \text{ CI}) \) (Figure 8). The post-hoc analysis performed by Newman–Keuls test showed significant statistical differences between the three studied groups, defined in relation to (i) the asthma severity degree according to FEV1 and (ii) the CD34 expression, as follows:

- mild vs. moderate, \( p = 0.0001 \) (95\% CI);
- mild vs. intense, \( p = 0.0001 \) (95\% CI);
- moderate vs. intense, \( p = 0.01 \) (95\% CI).

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**Figure 1** – *Scattered lamina propria vessels: control group (IHC, anti-CD34, ×100).*

**Figure 2** – *Significant increased lamina propria vessels count: moderate persistent asthma (IHC, anti-CD34, ×100).*

**Figure 3** – *Microvessels positioned in the upper lamina propria: mild persistent asthma (IHC, anti-CD34, ×100).*

**Figure 4** – *Microvessels observed in the entire lamina propria: severe persistent asthma (IHC, anti-CD34, ×200).*

**Figure 5** – *Slightly increased lamina propria vessels count: intermittent asthma (IHC, anti-CD34, ×200).*

**Figure 6** – *Moderately increased lamina propria vessels count: moderate persistent asthma (IHC, anti-CD34, ×200).*
Discussion

The first histological studies of the bronchial mucosa in asthmatic patients are dated in the 60’s [19] but the fibrobronchoscopy implementation is considered the reference moment in understanding the asthma pathogenic substrate and its direct morphological reflection. Although its’ incontestable scientific value results fibrobronchoscopy is not a common investigation in asthma. Consequently, a reduced number of studies available in the mainstream publications are focused on morphological lesions (approximately 200 in 20 years) [1] in comparison to the number of studies designed for different perspectives in asthma approach. Strictly referring to the microvascular component of the bronchial mucosa, the growth and expansion of these blood vessels is still of maximum interest, these changes contributing to the thickening of the bronchial wall, directly resulting in bronchial obstruction [20]. Thus, many research groups have been continued to quantify microvascularization and to better understand the molecular mechanisms, which make this phenomenon, appear and develop in the last decade [5, 8–12, 21–24].

Our study provided an evaluation of the bronchial mucosa hypervascularization in asthmatic patients diagnosed with different degrees of asthma (GINA 2006), by microscopically analyzing tissue fragments collected through fibrobronchoscopy.

The microvascular elements, identified by a strong CD34 immunostaining, showed a different lamina propria distribution in the mild asthma as opposed to the severe asthma. Thus, a microvessel density subjacent the epithelial basement membrane, in the upper lamina propria was observed in intermittent and mild persistent asthma. On the other hand, cases of moderate to severe persistent asthma were characterized by a substantial increase of the microvascular bed within the thickness of the lamina propria. Furthermore, a striking change in microvessels orientation was observed, from an initial parallel direction with the surface of the epithelium to a perpendicular direction with the epithelial surface. According to these qualitative changes, we may postulate that the microvascularization amplification is initiated in the superficial lamina propria and then is extended to the entire thickness of the bronchial mucosa. Supplementary, the connective tissue alterations attributable to the added chronic inflammatory infiltrate concur to a substantial thickness increase of the bronchial mucosa.

The semi-quantitative analysis of the microvascular elements has confirmed the qualitative changes observed in our study group. A progressive amplification of microvessels density was noted corresponding to the severity of asthma stages. The numerical data obtained were translated into a scoring system corresponding to mild, moderate, and severe qualitative categories. The statistical analysis of the correlation between the degree of microvascularization and the clinical stage was based on the new scoring system that we have recently implemented. A significant difference between clinical severity of the disease and the microvascularization morphology resulted from nonparametric statistics, as a prominent amplification of the vascular bed was noted in severe persistent asthma in comparison to mild asthma.

Although our methodology was different, our study complies with other researches and our results concur with reported data. The numerous counting methods might generate technical difficulties added to the well-recognized anti-endothelial antibodies non-specificity [25]. The commonly used method is of blood vessels counting after identifying the “hot spots” using a 200× magnification. Chalkley counting technique, with a rotating grid, which has 25 randomly positioned points, can quantify the number of points that are intersecting blood vessels, using a 250x magnification [25]. The patients’ selection criteria, the number of hot spots evaluated, as well as the dimensions of the selected areas (depending on the magnification amplitude) can further generate different results [25]. The following examples are relevant. The results of one of the first quantitative studies investigating the lamina propria microvascularization in asthma compared to control patients showed that hypervascularization occurs even in mild persistent asthma (738±150 microvessels/mm², total area of vascularization 19.4 mm² vs. 539±276 microvessels/mm², total area of vascularization 12.7 mm²) [23], Fragments of bronchial mucosa collected by fibrobronchoscopic examination from control and asthmatic patients have recently been used in another morphometric study using a Chalkley grid, with microvessels density quantification in a 1-mm² area [5]. The authors have predominately
identified different caliber capillaries and post-capillary venules and seldom small arteries, as qualitative findings of the research [5]. Both an enhanced blood vessels count and a greater percent of total vascularized area were quantified in severe persistent asthma (303.7 microvessels/mm², 9.7% area of vascularization) in comparison with moderate asthma (205 microvessels/mm², 9.1% area of vascularization) and with mild persistent asthma (185.3 microvessels/mm², 7.5% area of vascularization) [5].

The vascularization amplification in the bronchial mucosa is also a subject of study as a target of pharmacological therapeutically modulation [8], the microvessels count and individual caliber being equally enhanced in asthmatic patients in comparison with healthy subjects.

Our data concur to other research reports of increased blood vessels count in the bronchial wall of asthmatic patients, supporting the hypothesis that angiogenesis is a component of the chronic inflammatory response occurring in the bronchial wall. This complex process seems to be initiated in the intermittent and mild persistent forms of asthma, under FGF [26], TNF-α [27] and VEGF [8, 24] coordinated regulation.

Notwithstanding methodology, the importance of a better knowledge of the hypervascularization mechanism in asthma provides perspectives for its inhibition with bronchial wall thickness and edema reduction [21, 22]. Within this perspective, researches testing the impact of therapy on bronchial mucosa vascularization have been developed. A beneficial corticosteroids role in vascularization reduction has been demonstrated [9, 14]. Their effect is mediated by inhibition of neoangiogenesis growth factors [28], mainly of EGF [29]. Moreover, methylprednisolone inhibits other angiogenic factors, such as IL-8, IL-18, MMP 9 [30, 31]. Nevertheless, the accumulated research results cannot support without doubt the beneficial role of the corticosteroid inhaler treatment in reducing the number of blood vessels in the bronchial wall (using the currently prescribed dosage at least) [13, 32, 33].

Conclusions

Our data confirms the close correlation between the bronchial mucosa microvascularization count and density and the asthma severity, reflected by the FEV1 values. The increased mucosal blood vessels count initially facilitates the edema development and later on appears to be involved in the bronchial wall thickening, with concomitant distensibility reduction, as asthma characteristic features.

The bronchial mucosa microvascularization evaluation, either by qualitative and quantitative based methods or by pro- and anti-angiogenic factors quantification, opens new perspectives for advanced therapies, with beneficial effects for asthmatic patients’ quality of life.

References


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