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SUMMARY

Immunohistochemical profile of epidermal growth factor EGF and its receptors in the pathogenesis of chronic bronchitis

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INTRODUCTION

Chronic bronchitis represents a significant problem in the public health domain, with a powerful negative impact over the quality of patient life. Worldwide absolute figure is estimated about 210 million cases of COPD, its prevalence rate is estimated at 1% in the general population of all ages, and 8-10% and even higher among people over 40 years.

According to global data from 2002, obstructive chronic bronchitis represented the fifth cause of death and it is anticipated to become the fourth until 2030.

The objectives of the study were to extend knowledge about clinic factors, immunohistochemical factors, histopatologic factors and morphometric factors that are involved in the pathogenesis of chronic bronchitis, for a better understanding of its mechanisms.

Key words: chronic bronchitis, immunohistochemistry, EGF, EGFR1, TGFb-1, TGFbR1, outcome.

STATE OF KNOWLEDGE

Chapter I – “Epidemiologic data” – covers recent data from specialty literature about the incidence, prevalence, mortality and morbidity of chronic bronchitis in various geographic areas.

Chapter II – “Risk factors involved in etiopathogeny of chronic bronchitis” – investigates the main risk factors involved in triggering the disease.

Chapter III – “Pathogenic mechanisms for inflammation in chronic bronchitis” – describes the action mechanisms of the main cellular populations involved in the inflammatory process.

Chapter IV – “Clinical stage of diagnose” – analyzes pacient symptomatology with chronic bronchitis and imagistic paraclinic investigation or laboratory investigations usefull for the diagnostic.

STUDY OBJECTIVES

The study propose a full and thorough evaluation of the pathogenesis process of chronic bronchitis using classic investigation methods, and modern techniques like immunohistochemistry and morphometry. When identified, the complex mechanisms which develops at molecular level and the interactions between them provides us with valuable information regarding the occurrence, the development and the outcome of chronic bronchitis.

Main specific objectives of the study include:
1. Expanding knowledge about clinical factors, histopathological, immunohistochemical and morphometric involved in the pathogenesis of chronic bronchitis, in order to consolidate its mechanisms.
2. Filling morphological diagnosis of chronic bronchitis assessment by highlighting potential molecular targets;
3. Identifying mechanisms and markers involved in the pathogenesis of chronic bronchitis;
4. Identification of specific markers of prognosis in chronic bronchial lesions;

CHAPTER VI - „Material and Methods”

The material investigated was human material originating from patients hospitalized in the Clinic of Pneumology Clinical Hospital of Infectious Diseases and TB ` Dr. Victor Babes ` Craiova and was represented by a total of 1667 patients diagnosed with chronic obstructive bronchitis in the period 2009-2011.

Histopathological material came from casework Laboratory of Pathology of the same hospital and was the archived paraffin blocks made from bronchial biopsy specimens collected during the exploration of bronchoscopy.

The pieces were processed by the classical inclusion in paraffin and Hemalaum-Eosin staining, achieving also special stains such as Masson trichrome, which allow a good classification of lesions according to histological appearance.

The histopathological study included a total of 25 cases of chronic bronchitis. As control group were enrolled in the study five non smoking healthy subjects.
The material for immunohistochemical study was selected from histopathological casuistic presented above, respectively the same subjects, performing during histopathological processing additional number of sections that were further processed immunohistochemically.

Immunohistochemical study was a of type with enzyme detection using the LSAB technique working method (labeled Streptavidin-Biotin2 System) development with DAB (diaminobenzidine).

Interpretation of immunohistochemical reactions aimed the expression of markers of bronchial biopsy specimens. Semiquantitative assessment was based on the pattern of distribution of these growth factors and respectively their receptors correspondents in bronchial epithelial thickness, made to a microscope objective magnification x200 in accordance with criteria established by Merrick et al.[1] .

**Immunohistochemical diagnosis algorithm:**

Antibodies used in this study were addressed to:

► Specific identification of different cell subpopulations in inflammatory and non-inflammatory bronchial biopsy specimens (CD3 - T lymphocytes, CD20 - B lymphocytes, CD68-macrophages, mastocytes tryptase, CK 5/6- bronchial basal cells);

► visualization of cells proliferation (Ki-67);

► to identify the sources of growth factors and their receptors through reaction double which in the first part of reactions are specifically identified those cells, and the 2nd part are specifically identified growth factors and their receptors correspondents.

**Statistical analysis** used average values, standard deviations and confidence intervals, and comparison tests (Student t, unifactorial ANOVA, chi square, Pearson) for the formes groups, made with SPSS10 soft.

**CHAPTER VII – „Results” and CHAPTER VIII- „Discussions”** - render the study results, these being related to recent data from the literature.

In the clinical and epidemiological study we found that the incidence of chronic bronchitis was slightly decreased from 608 cases in the 2009 to 511 cases in 2011.
According to WHO, the prevalence of COPD was estimated to a total value of 63.6 per 1 million inhabitants. The prevalence of chronic bronchitis was estimated at 34 cases per 1000 persons [2]. In Europe, the prevalence of chronic bronchitis cases reported was 6.2% [3].

Our study showed an increased frequency of male patients with chronic bronchitis 79.90% (with a ratio 4:1 - men: women aged 50-70 years (91%), environment of origin being urban (65.81%). Our study data correspond with most international studies, which indicated chronic obstructive bronchitis as a disease of people aged 45-65 years (60%) [4].

**Analysis of data concerning risk factors involved in the pathogenesis of chronic bronchitis**

In our study, the main risk factor incriminated in the pathogenesis of chronic bronchitis was smoking present at 90.22% of patients, the rest, of 9.78%, being represented by: ex-smokers (7.5%) and nonsmokers (2.28%). Most were smoking more than a pack of cigarettes / day (69%) compared with 31% who smoked less than one pack / day.

The lot of smokers aged 50-60 years were the most numerous (68.42%), followed by those aged 60-70 years (23.07%), explained by increasing average lifespan, more especially in industrialized countries and intensifying pollution [5].

Studies conducted between 1990-2004 [6] who added a Japanese study [7] showed that the prevalence of chronic obstructive bronchitis was higher among active smokers and ex-smokers, over 40 years. Other studies have suggested that women seem to be more susceptible to the effect of cigarette smoke than men [8].

Other risk factors involved in the etiopathogenesis of chronic bronchitis outbursts in our study were represented by: bacterial infections (63%), viral (23.76%), pollutants and irritants (13.02%).

**Analysis of clinical data of patients with chronic bronchitis**

Our study showed that 80% of patients were hospitalized for increased cough with mucopurulent sputum increased amount relatively constant during the 3 years, only 20% were admitted for increasing dyspnea.

**Analysis of data from sputum examination in the diagnosis of chronic bronchitis**

The analysis of data obtained in our study, after microbiological examination of sputum concerning the types of bacteria involved in the production of bacterial infectious syndrome was
found that pneumococcus is the main causative agent (40.51%), followed by H. influenzae (16.70 %) and Klebsiella (10.82%).

**Analysis of data from respiratory functional explorations used in the diagnosis of chronic bronchitis.**

In our study, the most numerous patients (66.89%) were detected in stage III disease (FEV30-50%) and 18.78% patients in stage IV. Stage III disease prevailed in all decades of age, and stage II was more common in older than 70 years.

**Analysis of imagistic explorations data**

In 158 patients with chronic bronchitis, smokers with all over a pack cigarettes / day, clinical symptoms (occurrence of hemorrhagic sputum and intensifying dyspnea) suggested the risk of developing proliferative process. These patients underwent chest CT and only 85 patients could perform fibrobronchoscopy. In 17 cases (1%) was confirmed lung cancer and we were able to sample conclusive material from 25 patients for histopathological and immunohistochemical study.

**Morphological study of the lesions in chronic bronchitis**

Our investigations have shown that patients with chronic bronchitis have an intact bronchial epithelium with caliciform cell hyperplasia and squamous metaplasia. Bronchial epithelial basement membrane in these patients appears to be thickened compared with control subjects. Other researchers have shown that in patients with chronic bronchitis, bronchial epithelial basement membrane thickness is within normal [9].

Our study revealed an increased number of caliciform cells in the bronchial epithelium in patients with chronic bronchitis smokers and non-smokers. Most of these cells were positive for Alcian blue staining, showing the prevalence of acidic mucins in the secreted mucus structure. However, Davies et al. showed that respiratory mucins from patients with chronic bronchitis have a structure similar to the mucus from healthy individuals, only that they would be less acidic [10].

Similar with other results, we observed a hypertrophy of submucosal glands, especially the mucous type, which were more frequently positive for PAS staining, showing abundant neutral mucin. Glynn and Michael have shown in patients with chronic bronchitis a disproportionate increase of mucous acini and decrease in serous acini [11].
In our study we showed that smokers with symptoms of chronic bronchitis had a higher number of inflammatory cells in the bronchial structural compartments compared with asymptomatic non smoking subjects. In terms of cells bronchial inflammatory process was dominated by CD3 + lymphocytes, macrophages and mastocytes.

Most studies conducted on bronchial biopsy samples taken from patients with chronic bronchitis have revealed that morphological changes produced are the result of inflammation, and especially bronchial wall infiltration with mononuclear cells [12] and also the presence of a large numbers of neutrophils in the lower airway lumen [13].

Statistical analysis of the data of our study revealed the existence of significant differences in the density of inflammatory cells for the the study groups and the control group for both bronchial epithelium and the submucosal region. Secondly we found a linear correlation for each class of inflammatory cells and between classes at both epithelial and submucosal region.

We also found a significant correlation between the density of different classes of inflammatory cells (excluding mast cells from the bronchial epithelium) and FEV\textsubscript{1} values in both bronchial epithelium and submucosal region for all study and control groups investigated.

**Immunohistochemical study of EGF growth factor and its receptor EGFR1 and EGFR2 in chronic bronchitis**

In our study reactivity for the EGF showed significant differences according to smoking status and FEV\textsubscript{1} values. Thus, we have shown that smokers with chronic bronchitis in patients with the highest scores FEV\textsubscript{1} was noted the highest reactivity for the EGF. These results are in contradiction with the literature which showed that there were no significant differences in the expression of EGF and smoking status [14;15;16].

Comparing the damaged epithelium expression of EGF in ex-smokers with and without COPD, the authors found that expression was higher in ex-smokers with COPD. It has been suggested that cigarette smoking may play a role in inhibition of EGF expression in COPD.

When comparison was made with normal subjects, the authors found that EGF expression was higher in smokers with chronic bronchitis or patients with moderate or mild COPD compared with non smoking control patients [14;15;16].

We identified a nuclear reactivity for the EGF, present especially at specimens with much thickened bronchial epithelium.
In addition, this was more evident in smoking patients with chronic bronchitis who presented hyperplasia and metaplasia in the bronchial epithelium.

In previous studies such a nuclear reactivity for the EGF was observed in normal and pathological conditions [17;18], suggesting that when EGF is secreted it can be internalized in the cytoplasm and then incorporated into the nucleus, where it exerts different functions such as regulating gene transcription.

We also reported reactivity for the EGF also in submucosal glands (especially serous acinar cells) in vascular endothelium in inflammatory cells (macrophages, eosinophils and lymphocytes), and smooth muscular fibers. In a similar manner we investigated responsiveness in EGF, we also noted that the EGFR1 highest scores of immunoreactivity were present in samples taken from smokers chronic bronchitis patients.

Other authors found no significant differences in terms of ErbB receptor expression level in patients with chronic bronchitis asthma according to smoking status, but they were found when compared to normal subjects [14;15;19].

Our study also highlighted that while EGF expression was restricted to basal cells and ciliated cells in both normal and pathological conditions, however responsiveness for EGFR has been reported also in caliciform cells. Takeyama et al. claimed that the expression and activation of EGFR induced caliciform cell metaplasia in bronchial epithelium, but without changing the total number of cells, explained mucous differentiation of Clara cells [20].

Similarly to other papers, our study showed the same cellular localization of EGF and its receptor expression, suggesting that this growth factor is a ligand bound to these receptors.

**Immunohistochemical study of TGFβ1 growth factor and its receptor TGFβR1 in chronic bronchitis**

Our study was a qualitative one as immunoreactivity in TGF-β1 was highly heterogeneous both in bronchial biopsy samples taken from patients with chronic bronchitis and non-smokers and control subjects.

Reactivity was higher in the study groups compared with normal subjects. Bronchial epithelium reactivity for TGF-β1 was evident in ciliated columnar cells, namely the brush border and in basal cell cytoplasm, the latter being particularly evident in areas of hyperplasia.

In specimens sampled from patients with chronic bronchitis smoking reactivity for TGF-β1 was much higher mainly due to the presence of squamous metaplasia areas.
We noticed a weak reactivity even in the areas of caliciform cell hyperplasia. In the submucosal region, immunoreactivity for TGF-β1 was present at lymphoplasmocytic infiltrate level in the smooth muscular fibers, subepithelial bronchial fibroblasts, vascular endothelial cells and the mucous acini in the structure of hyperplasied and hypertrophied bronchial gland.

In our study at the level of submucosal region reactivity for TGF-β1 in patients with chronic bronchitis was present in the inflammatory infiltrate, smooth muscular fibers, mucous glands, vascular endothelial cells as well as secreted at the extracellular matrix and basement membrane level.

Such a phenotype expression of TGF-β1 in the bronchi in pathological conditions might suggest that this growth factor could play a major role in the pathogenesis of subepithelial fibrosis associated with chronic inflammation of the lower airways [16]. In this regard the authors have shown that TGF-β1 immunoreactivity correlated significantly with the number of fibroblasts and basement membrane thickness.

The ability of TGF-b1 to regulate the turnover of extracellular matrix components and activate stromal cells, especially fibroblasts was shown previously of this study by Kovacs and DiPietro [21].

TGF-β1 has been proven to be essential to reduce the secretion of mucin after Haemophilus influenzae infection, frequent pathogen in COPD [22].

In our study reactivity for TGFβR1 was obviously lower in the bronchial specimens collected from healthy subjects. This was limited epithelial in bronchial columnar cells and was present in the smooth muscle cells and rare inflammatory cells of the subepithelial region.

In pathological specimens, TGFβR1 was present mainly in bronchial epithelium superficial caliciform cells and ciliated columnar cells. We highlighted this receptor in the secretion of mucus from the surface of epithelium with hyperplasia. Reactivity in the areas of basal cell hyperplasia was an reduced, superficial cells showing weak cytoplasmic reactivity. However, in areas of mucosal epithelial cell hyperplasia we observed an intense cytoplasmic reaction for TGFβR1 within the superficial layers and the rest predominantly membranous.

The most intense bronchial epithelial reactivity for this receiver in areas of squamous metaplasia. Subepithelial reactivity for TGFβR1 was present in the lymphoplasmocytic inflammatory infiltrate and also in fibroblasts.
Overall reactivity for the two receptors in COPD was recorded in the fibroblasts [23], endothelial cells and muscle fibers of arteries and arterioles [24;25], bronchial epithelium [26;25], bronchial submucosal glands [26] and bronchial inflammatory infiltrate.

Pons et al. recorded a decrease in TGF-β1 expression in alveolar macrophages of patients with COPD compared with control subjects smokers and nonsmokers, suggesting that these patients would produce a low grade anti-inflammatory response mediated by this factor growth [27].

CHAPTER VIII "Conclusions":

❖ Epidemiological study of 1667 cases with chronic obstructive bronchitis, hospitalized in Craiova Pneumology "Victor Babeş" Hospital between 2009-2011 revealed a higher frequency in males (79.90%), with a sex ratio 4:1 - men: women with predominance in the age group 50-70 years (91%), being urban environment of origin (65.81%), the main risk factor found in 90.22% cases was smoking with over a pack cigarettes/day.

❖ The clinical symptomatology on admission was dominated by mucopurulent expectoration cough (79%) and dyspnea (21%) with ascending thermal curve.

❖ Analysis of data from sputum examination of patients with chronic bronchitis showed the presence of the following pathogens: pneumococcus (40.51%), H. Influenzae (16.7%), Klebsiella (10.82%), followed by Staphilococcus.

❖ Exploration of respiratory function in patients with chronic bronchitis, determined the severity of the disease in study group and found increased frequency of stage III disease (66%), predominantly in smokers in the age group 50-60 years.

❖ Analysis of data from pulmonary radiological imagistic explorations supported the diagnostic of chronic bronchitis, on the investigated casuistics(1509). Chest CT examination proved very useful to 158 cases. In 85 cases who received fibrobronchoscopy, in 17 cases was diagnosed lung cancer.

❖ Histopathological investigation revealed heterogeneity of lesions with involvement of all cellular and extracellular compartments in bronchial biopsy specimens sampled from the patients with COPD.
Regardless of smoking status, we revealed the presence of microscopic constant: focal squamous metaplasia changes in the bronchial epithelium, caliciform cell hyperplasia, hypertrophy and hyperplasia of bronchial submucosal glands and abundant inflammatory infiltrate composed predominantly of mononuclear cells.

Morphometric analysis of bronchial inflammatory cells showed that regardless of cell type and regardless of smoking status, their density was correlated with FEV₁ values both in the epithelium as well as in bronchial submucosa, the abundance of inflammatory cells being a sign of severity of bronchial damage.

The immuno-reactivity for EGF and receptors (EGFR1 and cerB2) was higher in COPD patients compared with control subjects, significant differences existing in the reactivity according to smoking status and FEV₁ values. Thus the highest reactivity we mentioned in smokers COPD patients which spirometric tests had the highest values of FEV₁.

Data analysis of immunoreactivity for TGFβ-1 and its receptor showed that TGFβR1 implication was particularly pathogenic in inducing lesions such as hyperplasia and metaplasia of bronchial epithelium and in modulating inflammatory response, acting mainly on lymphocyte population.

Distribution and cellular immune responses pattern for all these markers are suggestive of autocrine and paracrine action mechanisms by which these growth factors via their receptors correspondents intervene in generating lesions at bronchial level in patients with COPD.

Such immunophenotype proves first the complexity of the molecular mechanisms governing the cellular interrelations at bronchial level in both physiological and pathological conditions, namely in COPD, and on the other hand highlights the potential benefits that therapy based on targeting these molecules in patients with COPD can bring.
REFERENCES


