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UPREGULATION OF BASIC FIBROBLAST GROWTH FACTOR IN SMOKERS WITH CHRONIC BRONCHITIS

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Running title: bFGF in central airways of smokers

ABSTRACT

The aim of the study was to investigate the expression of basic Fibroblast Growth Factor (bFGF) and its receptor (FGFR-1) in central airways of smokers with chronic bronchitis.

We examined lobar bronchi obtained from 17 subjects undergoing thoracotomy for solitary nodules. All had a history of cigarette smoking, nine had symptoms of chronic bronchitis and airflow limitation and eight were asymptomatic with normal lung function. Using immunohistochemical methods, we quantified bFGF and FGFR-1 expression in the total airway wall, and in the different airway compartments: bronchial glands, submucosal vessels and smooth muscle. Moreover, to investigate the role of bFGF in angiogenesis, we quantified the number of submucosal vessels.

Smokers with chronic bronchitis had an increased bFGF expression in total wall compared to asymptomatic smokers ($p=0.02$), which was mainly due to bFGF upregulation in bronchial glands ($p=0.05$). By contrast, the expression of the receptor FGFR-1 and the number of submucosal vessels were similar in the two groups of subjects examined. In conclusion, smokers with chronic bronchitis have an increased expression of bFGF in central airways which is mainly due to an increased expression in bronchial glands suggesting the involvement of this growth factor in the pathogenesis of chronic bronchitis.

Key words: Airway remodeling, cigarette smoking, COPD, FGFR-1

INTRODUCTION

Chronic bronchitis is a clinical syndrome defined by chronic sputum production. The major risk factor for the development of chronic bronchitis is cigarette smoking, but the precise pathogenetic mechanisms of the disease are largely unknown. Chronic bronchitis is characterised by an airway inflammatory process involving epithelium, subepithelium, bronchial glands and bronchial smooth muscle [1-3]. This inflammatory response is associated to a structural airway remodeling which includes hypertrophy of submucosal glands, enlargement of smooth muscle mass, fibrosis of airway wall, epithelial metaplasia and goblet cells hyperplasia [4-8]. An important component of airway remodeling is angiogenesis, which has been well documented in asthma, but poorly investigated in chronic bronchitis [5, 9].

In the last few years, several studies have highlighted the role of fibroblast growth factors (FGFs) in regulating both inflammatory and remodeling processes in the lung [10-15]. FGFs represent one of the largest families of growth and differentiation factors for cells of mesodermal and neuroectodermal origin [16]. One of the best characterised members of FGF family is the basic FGF (bFGF or FGF-2) which is a pleiotropic polypeptide often regarded as a prototypic factor of the family. Among its different functions bFGF is involved in mitotic activities, angiogenesis, tissue repair, and inflammatory processes [16]. bFGF may be produced by several cell types including fibroblasts, endothelial cells, macrophages, T-lymphocytes, mast cells [12, 13, 17, 18], and exerts its biological effects through an interaction with a high-affinity transmembrane receptor, FGFR-1 [16]. In the lung, bFGF has been shown to be involved in several inflammatory diseases. An increased expression of bFGF has been found in bronchoalveolar lavage and lung tissue of subjects with bronchial asthma and pulmonary fibrosis [10-12, 19]. Moreover, bFGF has been implicated in the

pathogenesis of sarcoidosis [10] and post transplant obliterative bronchiolitis [20]. Recently, Kranenburg and coworkers examined the expression of this growth factor in the bronchial walls of smokers with COPD [15]. The authors showed that bFGF and its receptor FGFR-1 were upregulated in bronchial epithelium and bronchial smooth muscle suggesting a role for bFGF pathway in the airway remodeling characteristic of COPD [15].

In this study we evaluated the expression of bFGF and its receptor in the whole airway wall and in its different compartments, particularly in the bronchial glands. Moreover, to investigate its angiogenetic potential, we examined whether the expression of bFGF and its receptor was related to the number of submucosal vessels.

METHODS

Subjects

The study population was composed of 17 subjects with a history of cigarette smoking, who were undergoing lung resection for a solitary lesion. Nine had symptoms of chronic bronchitis and eight were asymptomatic (control smokers).

Chronic bronchitis was defined as cough and sputum production occurring on most days of the month for at least 3 months a year during the 2 years prior to the study. Subjects with chronic bronchitis had no exacerbations, which were defined as increased dyspnea associated with a change in the quality and quantity of sputum that led the subject to seek medical attention, during the month preceding the study [21]. All subjects in both groups had been free of acute upper-respiratory-tract infections and none had received glucocorticoids or antibiotics within the month preceding surgery, or bronchodilators within the previous 48 h.

The subjects were nonatopic (i.e., they had negative skin tests for common allergen extracts) and had no past history of asthma or allergic rhinitis.

The study conformed with the Declaration of Helsinki, and informed written consent was obtained for each subject undergoing surgery. Each subject underwent an interview, chest radiography, electrocardiography, routine blood tests, skin tests with common allergen extracts, and pulmonary function tests in the week before surgery.

Pulmonary function tests were performed according to the previously reported methodology [21]. Briefly, pulmonary function tests included measurements of FEV_1 and FVC in all the subjects examined. In subjects with a baseline FEV_1>80% predicted, inhalation challenge with methacholine was performed and the results were expressed as the provocative dose that elicited a 20% decline in FEV_1 (PD20 FEV_1) (mg methacholine). In order to assess the reversibility of airway obstruction in subjects with a baseline FEV_1< 80% predicted, the FEV_1 measurement was repeated 15 min after the inhalation of 200 μg of salbutamol.

Histology

One ring from each subject was taken from the lobar or segmental bronchus of the lobe obtained at surgery, away from the tumor site, fixed in 4% formaldehyde and embedded in paraffin as previously described [2].

The expression of bFGF and its receptor FGFR-1 as well as the number of submucosal vessels were detected by immunohistochemical ABC peroxidase method. The following antibodies were used: polyclonal anti-bFGF antibody (Sigma BioScience, St. Louis, MO), monoclonal anti-FGFR-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and monoclonal anti-CD34 antibody (Dakopatts, Copenhagen, DK) for identification of vessels. As positive control we used non-small cell lung cancer sections, known to be

immunoreactive for bFGF and FGFR-I and as negative control we omitted the specific primary monoclonal antibody.

Quantitative assessment of bFGF and FGFR-1 expression was performed using a stereological method as previously described [13, 22]. In each subject, 150 microscope fields (total wall) were evaluated: 50 in the bronchial glands, 50 in the bronchial smooth muscle and 50 in the submucosal vessels. Point counting was performed in each field by ligth microscopy under a magnification x1000 using a 30-point Weibel grid. The percent of points falling on positive staining was determined in the total wall and in the separate compartments (bronchial glands, smooth muscle, submucosal vessels) as follows: Pi/Pt x 100, where Pi = the number of points that fell on positive staining and Pt = the total number of points counted. The results were expressed as percent volume density, as previously described [13, 22].

A quantitative assessment of bFGF and FGFR-1 was also performed in the bronchial epithelium of the subjects with intact epithelium. The number of bFGF and FGFR-1 positive cells within the airway epithelium were counted and the results were expressed as number of positive cells per millimiter of basement membrane.

Submucosal vessels was assessed recording the highest value of vessel count in the three highest vascularized microscopic fields as previously described [23] and expressed as number of vessels/square millimetre of tissue examined.

Reid's index was calculated as ratio between the maximum thickness of each bronchial gland and the bronchial-wall thickness as measured from basement membrane to inner perichondrium along a single axis. Smooth muscle proportion was calculated as ratio between thickness of smooth muscle and bronchial-wall thickness.

To avoid observer bias, the cases were coded and measurements were made without knowledge of clinical data.

Statistical analysis

Differences between groups were analysed using the analysis of variance for clinical data and the Mann-Whitney U test Group data for histologic data. Correlation coefficients were calculated using Spearman's rank test. Wilcoxon Signed Rank test was used to compare bFGF and FGFR1 expression in the different compartment. Probability values of 0.05 or less were accepted as significant. Group data were expressed as means and SEM or as medians and range when appropriate. Statistical analysis was done by using the Stat View 4.5 software package (Abacus Concepts Inc., Berkley, CA).

RESULTS

Clinical Findings

The characteristics of chronic bronchitics and control smokers are reported in Table 1. All subjects with chronic bronchitis had a FEV_1 < 80% predicted, ranging from 56 to 79% predicted, with an average response to bronchodilator of 5% (range: 0 to 13%). All control smokers had normal lung function, with a FEV_1 ranging from 86 to 116% predicted, and reactivity to methacholine within the normal range (PD_{20} FEV_1 > 1.4 μg methacholine). Subjects with chronic bronchitis had a significantly lower value of FEV_1 (% predicted) and FEV_1 /FVC ratio (%) than did control smokers. The two groups of subjects were similar with regard to age, sex, smoking history, PaO_2 and $PaCO_2$ values.

Data on underlying cancer were collected in 6 over 9 patients with chronic bronchitis and in 6 over 8 control smokers. All subjects of both groups had squamous cell carcinoma except three (one smoker with chronic bronchitis and two control smokers) who were

referred to lobectomy for a suspected carcinoma which revealed to be a benign tumour at the pathological examination. As for localization, in 3 control smokers and in 4 smokers with chronic bronchitis the lesion was localized in the lung parenchyma, while in the remaining cases it was localized in a lobar or segmental bronchus. All subjects were staged as T1 or T2, the relative proportion of each being similar in smokers with chronic bronchitis and control smokers.

Histological findings

The quantification of the expression of bFGF and of its receptor (FGFR-1) was satisfactory in all subjects except in one subject with chronic bronchitis, in which bronchial glands could not be examined because of the lack of gland tissue.

The expression of bFGF in the total wall of central airways was increased in subjects with chronic bronchitis as compared to control smokers (Figure 1). When we examined the different wall compartments, e.g. bronchial glands, smooth muscle and submucosal vessels (figure 2 A,B,C), the increased expression of bFGF remained significant only in the bronchial glands (figure 3). By contrast, the expression of the receptor FGFR-1 was similar in the two groups of subjects both in the total wall and in each of the compartments examined (figures 1, 4).

Among the three compartments examined, bronchial glands showed the highest reactivity for bFGF and FGFR-1. Indeed, bFGF expression in bronchial glands was higher than in bronchial smooth muscle or submucosal vessels both in subjects with chronic bronchitis ($p=0.03$ and $p=0.02$ respectively) and control smokers ($p=0.02$ and $p=0.08$).

Similarly, FGFR-1 expression in bronchial glands was higher than in bronchial smooth muscle or submucosal vessels both in subjects with chronic bronchitis (p=0.01 and p=0.01) and control smokers (p=0.05 and p=0.01).

Analysis of bronchial epithelium could not be performed in 4 smokers with chronic bronchitis and 1 control smoker because of epithelial denudation. The epithelial expression of bFGF was not significantly different in subjects with chronic bronchitis as compared to control smokers (median (range): 56 (30-145) vs 57 (18-102), cells/mm). Similarly, the expression of the receptor FGFR-1 was not significantly different in the two groups of subjects examined (54 (40-70) vs 73 (29-132), cells/mm).

When we examined the structural changes, we found that the number of submucosal vessels, the Reid's index and the proportion of smooth muscle were not significantly different in subjects with chronic bronchitis and control smokers (table 2).

When all the subjects were grouped together, the total wall expression of bFGF showed a significant positive correlation with the number of vessels (r= 0.47, p=0.05) (figure 5) as did the expression of bFGF in bronchial glands (r=0.49, p=0.05). This latter correlation remained significant when only smokers with chronic bronchitis were considered (r=0.78, p=0.03). Moreover, bFGF expression in smooth muscle showed a positive correlation with Reid's index in all the subjects of the study (r=0.55, p=0.03). This correlation remained significant when only smokers with chronic bronchitis were considered (r=0.73, p=0.05). Finally, a positive correlation was observed in bronchial glands between the expression of bFGF and its receptor FGFR-1 (r=0.52, p=0.04). This correlation remained significant when only smokers with chronic bronchitis were considered (r=0.73, p=0.05). No significant correlations were found between histologic parameters and functional data.

DISCUSSION

This study shows that smokers with chronic bronchitis have an increased expression of bFGF in central airways, which is mainly due to an enhanced expression in the gland compartment.

The precise source of bFGF in central airways is unknown, but previous reports have shown that several cell types may produce this growth factor and, among them, inflammatory cells. In particular, it has been shown that T-lymphocytes and macrophages, which are increased in the central airways of smokers with chronic bronchitis [1], can secrete bFGF [17, 18]. It is therefore plausible that these inflammatory cells may contribute to the upregulation of bFGF observed in our study.

Among its potential effects, bFGF may induce the migration and proliferation of endothelial cells, leading to angiogenesis [16]. New vessel formation is an important component of airway remodeling, which has been well documented in bronchial asthma [5, 9] but scantily investigated in COPD [5]. Our finding of a similar number of submucosal vessels in smokers with chronic bronchitis and asymptomatic smokers is in agreement with the results by Kuwano and coworkers who reported a similar number of vessels in peripheral airways of subjects with COPD and controls [5]. Interestingly, despite the lack of difference between the two groups of subjects, we found that the number of submucosal vessels was positively correlated with the expression of bFGF, suggesting an angiogenetic activity for this growth factor in smokers. A possible explanation for the lack of difference in the number of vessels in our study is that all subjects in the control group were heavy smokers and it is known that smoking itself is able to induce an airway inflammatory process, which may potentially promote angiogenesis [24]. It must be highlighted, however, that, without a proper control group of non-smoking subjects, a firm conclusion can not be drawn on the

presence of angiogenesis in smokers. Unfortunately, our study lacks such a control group, since the study population consisted of patients undergoing thoracotomy for lung cancer, which is less frequent among nonsmokers than it is in smokers.

When we quantified bFGF expression in the different compartments of central airways, we found an upregulation of this growth factor in bronchial glands of smokers with chronic bronchitis. Chronic bronchitis is usually associated with hypertrophy of bronchial glands, a pathological feature traditionally considered to account for the increase sputum production [25, 26]. It is somewhat surprising that in our study the enhanced bFGF expression in smokers with symptoms of chronic bronchitis was not associated with gland hypertrophy as measured by Reid's index. However, this finding is in agreement with previous reports showing a lack of correlation between sputum production and gland hypertrophy in smokers [27]. In this context, it is interesting to note that the inflammatory response involving the bronchial glands has shown a better concordance with mucus hypersecretion than gland size per se [26, 28]. In the bronchial glands, this inflammatory response is characterised by neutrophils, mast cells, macrophages and CD8 T lymphocytes [2, 28], all of which can potentially produce bFGF [12, 17, 18, 30]. It is therefore conceivable that the increased expression of bFGF that we observed in bronchial glands of smokers with chronic bronchitis may be due to the inflammatory cells infiltrating the glandular compartment. Neutrophils are an important component of this inflammatory response, and it has been shown that bFGF may regulate the neutrophil recruitment by modulating the expression of adhesion molecules on these cells [31]. Interestingly, neutrophil elastase is able to release, and therefore activate, the bFGF bound within the extracellular matrix [32], suggesting that the interaction between this growth factor and neutrophils may create a self maintaining loop.

In our study, the up-regulation of bFGF in bronchial glands of subjects with chronic bronchitis was not paralleled by the increased expression of its receptor FGF-R1. Although previous observations suggest that bFGF is able to up-regulate the expression of its receptor FGFR-1 via autocrine signals [33], this is not the only mechanism. Indeed, the regulation of FGF-R1 expression is a complex mechanism, not completely understood, that can be influenced by many different factors such as hypoxemia, cell phenotype or changes in the extracellular matrix environment [34-36]. These confounding factors may explain why, in our study, the increased expression of bFGF was not paralleled by an increased of its receptor FGF-R1.

Surprisingly, we did not find any difference in the expression of bFGF and its receptor in bronchial smooth muscle of smokers with chronic bronchitis and control smokers. This finding appears to contrast with the results of a recent report by Kranemburg and coworkers showing that both bFGF and its receptor FGFR-1 were upregulated in bronchial smooth muscle of subjects with COPD [15]. However, there are important clinical and methodological differences between the two studies that may have influenced the results. Indeed, in our study, the subjects were selected on the basis of chronic bronchitis symptoms, while in that study they were selected on the basis of airflow limitation, regardless of the presence of chronic bronchitis. Although the two conditions can share some pathogenetic traits, there can be some discrepancies as well. Moreover, we used different antibodies and a different quantitative analysis to detect FGF and FGFR-1 expression, thus making a direct comparison between the two studies difficult. Despite this, our main finding of an increase bFGF expression in central airways of smokers with chronic bronchitis confirms the observation by Kranenburg and coworkers and is consistent with their hypothesis that bFGF could be implied in the airway remodelling that characterizes COPD. In a previous report, the same authors showed that both bFGF and its receptor FGFR-1 were up-regulated even in

pulmonary arteries of subjects with COPD, suggesting a role for the bFGF pathway in the vascular remodelling observed in the disease [14]. In this context, the recent observations by Shute and coworkers are of interest [19]. These investigators demonstrated an increased expression of bFGF in bronchial biopsies of asthmatic subjects indicating that in asthma, as in COPD, an up-regulation of bFGF is present and may contribute to airway remodelling. However, the precise role of bFGF in the development of the structural changes characteristic of the two diseases still remains to be investigated. In particular it is still unknown whether the increased bFGF expression we observed in bronchial glands of smokers with COPD is present even in bronchial glands of asthmatic subjects and is associated with mucus production in these subjects.

Although in our study, subjects with chronic bronchitis were selected only on the basis of the presence of symptoms, they all had fixed airflow limitation. Therefore, whether the observed upregulation of bFGF is related to chronic bronchitis or to airflow limitation still remains to be investigated. It should be underlined, however, that this distinction may be problematic, since the role of symptoms of chronic bronchitis in the development of chronic airflow limitation is still controversial. Traditionally, mucus hypersecretion has been considered irrelevant for the development of airflow limitation [37], and more recently it has been shown that chronic sputum production in smokers with normal lung function (GOLD stage 0) does not predict a subsequent establishment of airflow limitation [38]. Conversely, when COPD is established, as it is in the subjects of the present study, chronic sputum production has been found to be associated with both an excess of FEV_1 decline and an increased risk of subsequent hospitalisation [38-39] suggesting a role for mucus hypersecretion in the progression of the disease.

In any study on surgically resected specimens of patients with lung cancer the presence of cancer itself may influence the results. However, as compared with bronchial

biopsy, which sample only a small portion of the bronchial wall, surgical specimens allow for a better examination of the whole central airways. Moreover, as a result of our examining only tissue away from the tumour site, and having included subjects with lung cancer in the control group, we feel rather confident that our finding of an increased bFGF expression in the central airways of subjects with chronic bronchitis is valid.

When considering the relationship between lung cancer and chronic obstructive pulmonary disease, it is noteworthy that COPD, which is characterized by chronic lung inflammation, is associated with an increased risk of lung cancer [40] thus suggesting a pathogenetic link between tumor and inflammation. Interestingly, bFGF has the potential to stimulate tumour proliferation by promoting angiogenesis [16]. It is therefore conceivable that this growth factor could have a key role in the association observed between lung cancer and COPD.

In conclusion, smokers with chronic bronchitis and airflow limitation have an increased expression of bFGF in central airways which is mainly due to an enhanced expression in the bronchial gland compartment. These results suggest that bFGF may have a role promoting mucus hypersecretion in smokers. However, further studies are required to clarify the multiple roles played by bFGF in the pathogenesis in chronic bronchitis and COPD.

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Table 1: characteristics of the subjects

	Chronic bronchitics	**Control smokers**
Subjects examined (number sex)	9M	8M
Age (years)	68±3	69±2
Smoking history (pack years**)**	57±8	45±5
FEV_1 (% of predicted**)**	68±3*	98±4
FEV_1/FVC (%)	67±2*	78±3
PaO2 (mm Hg)	85±4	89±3
PaCO2 (mm Hg)	41±2	36±3

Values are expressed as means ± SEM

* significantly different from control smokers ($p < 0.01$)

Table II: morphometric results

	Chronic bronchitics	Control smokers
Vessels (number/mm^2)	80 (58-112)	80 (53-138)
Reid's index (%)	47 (32-64)	36 (17- 50)
Smooth muscle thickness (%)	17 (13 – 52)	22 (15 – 74)

Values are expressed as medians with ranges shown in parentheses

FIGURE LEGENDS

Figure 1- Individual measurements of total bFGF expression in smokers with chronic bronchitis and control smokers. Horizontal bars represent median values.

Figure 2- bFGF expression (positive staining in brown) in bronchial glands (panel a), smooth muscle (panel b) and submucosal vessels (panel c) of a subject with chronic bronchitis.

Figure 3-Individual measurements of bFGF expression in bronchial glands, smooth muscle and submucosal vessels in smokers with chronic bronchitis and control smokers. Horizontal bars represent median values.

Figure 4-Individual measurements of FGFR-1 expression in bronchial glands, smooth muscle and submucosal vessels in smokers with chronic bronchitis and control smokers. Horizontal bars represent median values.

Figure 5-Relationship between bFGF expression in the bronchial wall and the number of submucosal vessels (Spearman's rank correlation)

Figure 1

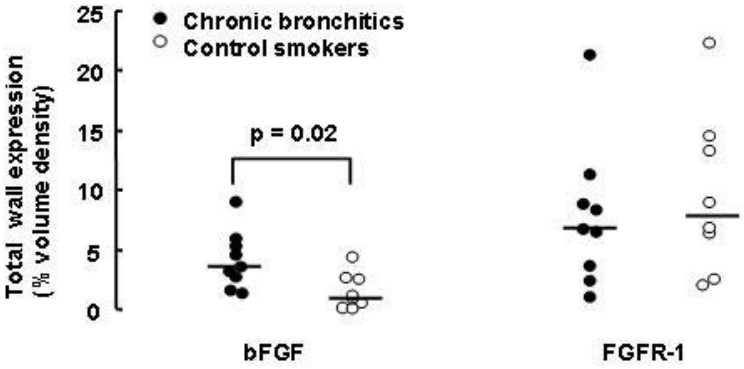
Total wall expression
(% volume density)
25
20
15
10
5
0
Chronic bronchitics
Control smokers
p = 0.02
bFGF
FGFR-1

Figure 2, panel A

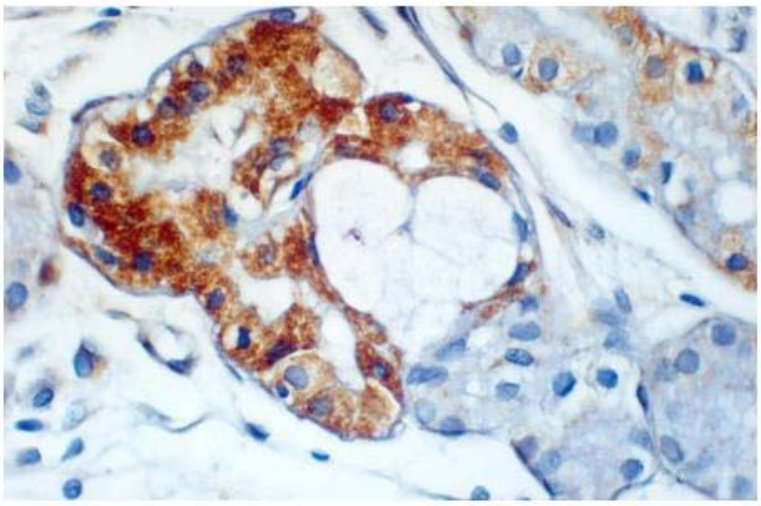

Figure 2, panel B

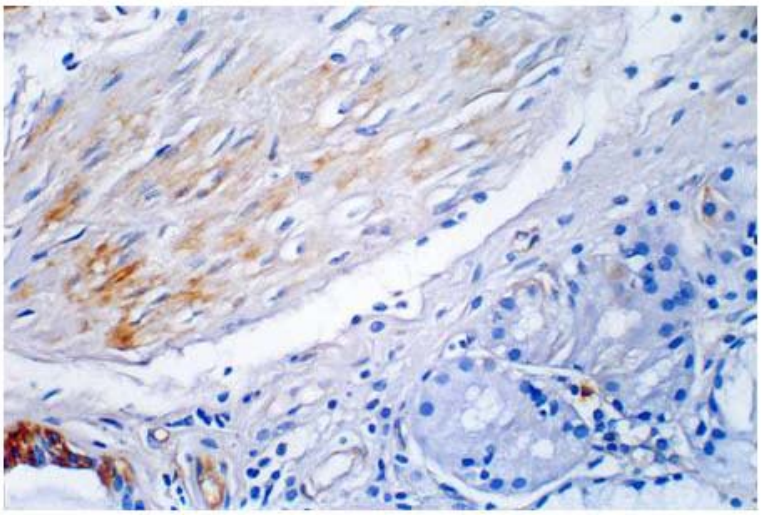

Figure 2, panel C

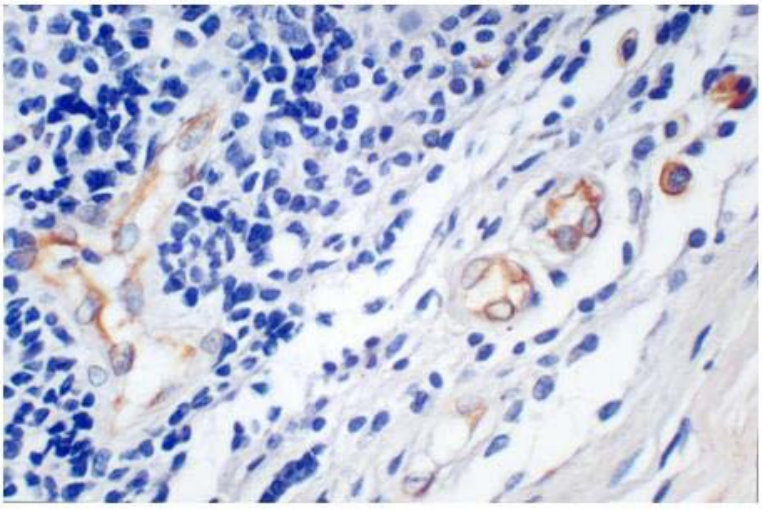

Figure 3

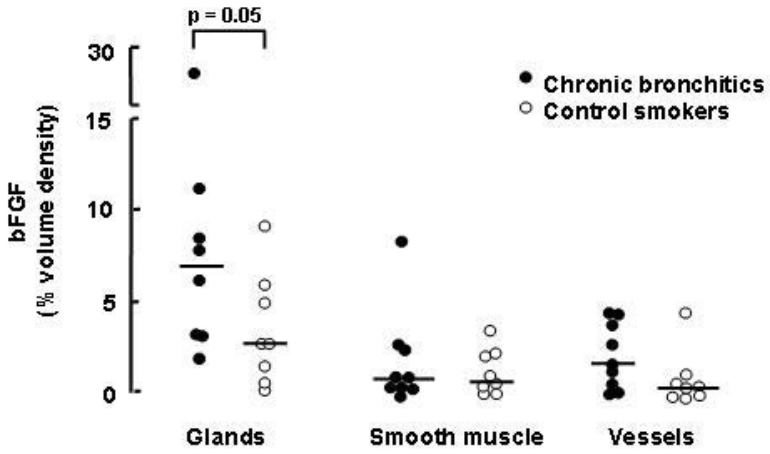

Figure 4

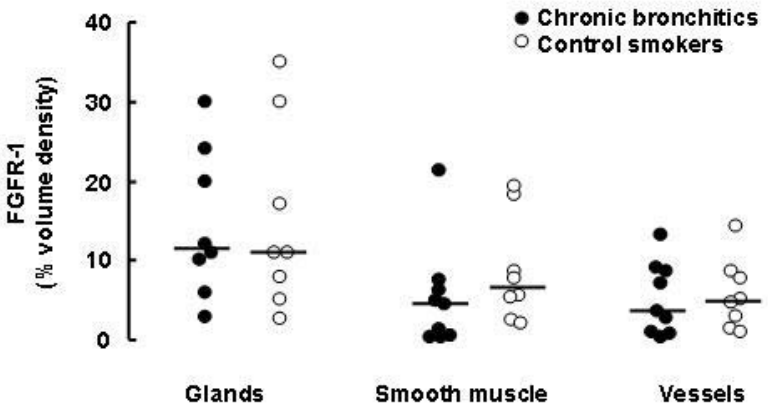

Figure 5