Distribution of Pulmonary Neuroendocrine Cells and Neuroendocrine Bodies among Healthy and Asthmatic Adult Lung Subjects

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1. ABSTRACT
The airways of the lungs contain an important collection of pulmonary neuroendocrine cells (PNECs). The role of these critical cells in development and in disease states is still being elucidated. The pulmonary neuroendocrine system’s known general function is cell signaling, as the cells are innervated and release a combination of peptides and amines. Gastrin-releasing peptide (GRP) is the most well-known peptide used for signaling in the human pulmonary neuroendocrine system, and it can be used as a biological marker of neuroendocrine cells via antibody. The cell organization is unique as the cells are present either as individual cells or as neuroendocrine bodies (NEBs) made of two or more cells. It has been previously suggested that asthmatic subjects have greater densities of PNECs and NEBs. Our objective of this project was to discern the impact of an asthma disease state on the overall organization of the pulmonary neuroendocrine system in terms of cell distribution on the proximal-distal axis and density. Following immunohistochemistry (IHC) staining for GRP, we measured cell density in the form of PNECs and NEBs for both normal and asthmatic subject samples. In normal subjects, the solitary pulmonary neuroendocrine cells presented with a notably higher density in the trachea than in more distal airways, whereas neuroendocrine bodies had the greatest density in distal airways, especially in terminal bronchioles. The results from IHC on normal subjects were confirmed by quantitative PCR (qPCR) performed on additional normal lung samples. The qPCR data revealed an increasing amount of GRP mRNA in the proximal-distal axis of the lungs. In asthmatic subjects, these same trends along the proximal-distal axis were present but less strong. In addition, there was a drastic decrease in NEBs in terminal airways compared to healthy subjects. Given that the two types of cell organization seen within the pulmonary neuroendocrine system are found in their highest densities in distinct lung sections, it is possible that unique roles
are maintained by these cells depending on their structure or location. In addition, the significant decrease in NEB density in distal airways of asthmatic subjects in comparison to healthy subjects may open doors for therapies and increased understanding of disease pathology.
2. INTRODUCTION

Pulmonary neuroendocrine cells (PNECs) are a specialized cell type found in the epithelium of the lungs. They were first noted in scientific literature in the 1940s when Feyrter and Fröhlich discovered clear cells, which were soon thereafter named as neuroendocrine cells. As research is still developing and solidifying in regards to the pulmonary neuroendocrine system, there have been many names and proposed roles for these cells throughout the years. During the 1990s, a fairly concrete consensus was reached among researchers, and the two major, yet distinct, functions of the pulmonary endocrine system were clarified. The first role of the proposed dual role is, during early lung development, PNECs may function to monitor and modulate the growth and differentiation of the lung via their ability to secrete peptides, amines, and neurotransmitters. Due to this supposed role of the system, it is commonly found that the densities of PNECs and NEBs are higher in fetuses and very young, developing humans. The second half of the dual role is, later in fetal life and postnatally, the cells may act as a system of chemoreceptors. There is data that suggests that PNECs sense inhaled inputs (allergen, toxins, etc.) or states such as hypoxia and coordinate lung outputs to respond to the stimuli. While the embryonal lung and neonatal lung have been large areas of investigation for researchers, the adult lung is a very active area for discovery and growth with respect to the understanding of the physiology and function of the pulmonary neuroendocrine system in both normal subjects and diseased subjects.

Pulmonary neuroendocrine cells can exist independently of one another as solitary cells or in clusters as neuroendocrine bodies. These cell types are primarily localized at airway branch points throughout the lung where they can transmit signals to other nearby cells. Neuroendocrine cells are distinctive in that they are long, extending from lumen to basement membrane with fingerlike projections that extend in many directions away from the main cell.
body. The irregular cell shape is accompanied by secretory granules that contain amines and peptides for secretion and signaling, with the capability of eliciting an immune response. There is a multitude of secretions that a neuroendocrine (NE) cell is capable of releasing. The most common human secretion is gastrin-releasing peptide (GRP), often called bombesin-like peptide. GRP is thought to be responsible in part for the production of some cytokines which work to recruit immune cells. In addition, PNECs appear to work through another of their products, GABA, to stimulate epithelial mucus production. Adding to their distinction, NEBs are typically more heavily innervated than PNECs, and it is presumed that solitary PNECs and NEBs occupy different roles within the lung epithelium.

Much research is currently being conducted to determine if PNECs may be useful for therapies for various lung conditions and diseases including chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and asthma. Prior research has indicated that there are notably more PNECs in lung samples of asthmatics. Pengfei Sui, et al. conducted a research project to determine the role of PNECs during an allergen challenges in asthmatics. They determined that the secretions of the pulmonary neuroendocrine system affected goblet cell hyperplasia and interleukin 5 (IL5) production, both of which have notable roles within an allergen response (mucus production and immune response). These results coupled with the increased density of PNECs suggest causality for the increase in allergen responses in subjects with asthma. Knock-out mice for this cell system showed decreased allergen response efficiency and effectiveness. While the density of PNECs relative to other cell types within the lung is minimal, they seem to play an important and essential role in amplifying and perpetuating the allergen response signal. While the pulmonary neuroendocrine system has been relevant in scientific research for over 60 years, there are still many questions regarding the true role the cells play in the lung environment.
as a whole throughout development and into adulthood. Understanding the way these cells distribute themselves throughout the lung based on their two known structures could provide a basis for research into physiology and purpose. A distinct or opposing distribution pattern of PNECs and NEBs in healthy or diseased subjects could uncover nuances of the pulmonary neuroendocrine system that the community is still unfamiliar with. In an attempt to map the relative distributions of PNECs and neuroendocrine bodies (NEBs) in lungs of healthy adult subjects, I performed immunohistochemistry in various lung regions of twelve subjects (six healthy and six asthmatic). The immunohistochemistry results were confirmed through qPCR analysis of nine healthy subjects. In this study, we were able to examine the relationship between cell type and location in order to advance the understanding of the roles of pulmonary neuroendocrine bodies and solitary neuroendocrine cells. In addition, through the comparison of samples from healthy subjects and asthmatic subjects, this project allowed us to explore potential differences in the proliferation pattern of the pulmonary neuroendocrine system in diseased systems.
3. METHODS

3.1. Lung Sample Donor Demographics

Lung tissue was taken from 16 donors. The demographics of the donors are provided in Table 1, along with information related to the types of experiments in which they were used.

Table 1: Demographics of Subjects and Experimental Use

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Disease</th>
<th>Smoking History</th>
<th>IHC</th>
<th>qPCR</th>
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<tr>
<td>H10260</td>
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<td>43</td>
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<td>Never smoker</td>
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<tr>
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<tr>
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<td>Never smoker</td>
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<tr>
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<tr>
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<td>Never smoker</td>
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<td>H10700</td>
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<tr>
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<tr>
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<tr>
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3.2. Tissue Preparation for Immunohistochemistry

Lung tissue samples from 16 human subjects were used for immunohistochemistry. All lung tissue samples were acquired from the University of North Carolina Tissue and Cell Culture Procurement Core. Human lung tissue was procured under the UNC Office of Research Ethics Biomedical Institutional Review Board-approved protocol No. 03-1396. Tissues were fixed in
10% neutral buffered formalin for 24-36 hours, embedded in paraffin, cut into 5µm thick sections, and placed on slides. Prior to beginning the steps of immunohistochemistry, the slides were baked in an oven at ~48°C oven for 6-12 hours. The tissue samples were then deparaffinized through a series of changes in enough xylene and ethanol to cover the sections. The first step of deparaffinization was three changes in xylene for ten minutes each. This was followed by two changes in 100% ethanol for five minutes each, then one change in 95% and 70% ethanol both for five minutes each. To rehydrate the slides, they soaked in phosphate buffered saline (PBS) pH 7.2 for five minutes in glass Coplin jars. To block endogenous peroxidase activity, the slides were placed in Coplin jars containing a solution of 60mL methanol and 1mL hydrogen peroxide for 15 minutes. The slides were transferred to a Coplin jar containing PBS for and gently shaken for five minutes.

3.3. Immunolabeling of Gastrin Releasing Peptide

A solution of 4% donkey serum was prepared by combining a ratio of 1mL normal donkey serum (Jackson Immunoresearch Labs Inc.) to 24 mL of phosphate buffered saline with Tween (PBST). The solution was transferred to plastic Coplin jars (large enough for five slides) and the slides soaked for two hours at room temperature. After the slides blocked with donkey serum, the slides were placed in a slide tray with a very small pool of water to maintain humidity (not touching the slides).

The primary antibody used in this study was the mouse monoclonal antibody GRP E-11: sc-271045 kappa light chain (Santa Cruz Biotechnology Inc.). The primary antibody was combined with 4% donkey serum (reused from step before) at a ratio of 1:100. For primary antibody incubation, 500 µL of the solution was applied to all experimental slides, and they were subsequently covered with plastic cover slips. For negative (isotype) control slides, mouse IgG
antibody (Santa Cruz Biotechnology Inc.) was used in place of the primary anti-GRP antibody. The incubating slides were kept at 4°C overnight. The next day, the slides were rinsed with PBST three times for ten minutes each. The secondary antibody used in this study was biotinylated donkey anti-mouse IgG (Jackson ImmunoResearch Labs Inc.). The secondary antibody was prepared at a 1:200 dilution with 4% donkey serum in PBST. The same application technique used for the primary antibody (500 µL per slide with plastic cover slip) was applied to the secondary antibody.

The incubating slides were kept at room temperature for one hour. After the hour, the slides were rinsed in glass Coplin jars with PBST three times for ten minutes each. During these rinses, the Vector ABC (avidin/biotin complex) reagent was prepared using the Vectastain Elite ABC-Horseradish Peroxidase kit (Vector Laboratories). This solution was prepared by combining 10 mL of PBST, two drops of A (avidin), and two drops of B (biotinylated HRP). The ABC reagent was allowed to incubate on the shaker for 30 minutes. After the slides finished rinsing in PBST, the ABC reagent was applied using the same technique that was used for both the primary and the secondary antibody. The slides incubated for 30 minutes. After the 30 minutes, the slides were once again rinsed three times with PBST for ten minutes each. During these rinses, the diaminobenzidine (DAB) reagent was prepared according to the following steps. In a separate beaker, 200 mL of acetate buffer, 1.6 g of sodium chloride, and 2.0 g nickel sulfate were combined and stirred until completely dissolved. After complete dissolution, 0.095 g of 3′3 diaminobenzidine was added to the solution, and the solution was stirred for ten minutes. The completed DAB reagent was then filtered into a plastic staining dish. To the filtered DAB solution, 25 µL of hydrogen peroxide was added.
Once the slides were finished incubating with Vector ABC, the slides were then stained in the following manner. To begin, the slides were soaked in 0.1M acetate buffer for one minute. Then the slides were transferred to the DAB solution (with hydrogen peroxide) for five minutes. The slides then soaked in 0.1M Tris saline for one minute and then Tris cobalt for four minutes. Afterwards, the slides were washed in DI water briefly and then transferred to fast red to soak for four minutes. Finally, the slides were washed with multiple rounds of DI water to remove excess fast red. To dehydrate and clear the slides, they were dipped 20 times in 70% ethanol, 95% ethanol, 100% ethanol and two changes of xylene. With the experimental steps complete, the slides were finished and covered using glass cover slips and mounting buffer.

3.4. Slide and Data Analysis
The prepared slides were scanned and digitized using an Olympus VS120 light microscope with a 40X objective lens. Using ImageJ software, the basement membranes of all airways on each slide were measured. The number of PNECs and NEBs for each region of measured basement membrane was counted and recorded. These numbers were then converted to number of cells/bodies per millimeter of basement membrane in order to determine a density. The average density was calculated for each airway section (trachea, middle, and distal) for each subject. The collection of airways that makes up the middle classification are primary and secondary bronchi. Distal airways are tertiary bronchi and bronchioles.

3.5. Quantitative PCR of GRP
We also performed quantitative PCR using samples from nine normal subjects as denoted in Table 1. The relative amounts of GRP mRNA found in the various airway regions were determined via qPCR in an effort to confirm findings from the prior IHC analysis. The qPCR
protocol used in this project was based on the protocol performed by Kenichi Okuda, et al\textsuperscript{7}. All qPCR was performed using the Applied Biosystems 7500 Real Time PCR Systems.
4. RESULTS
In order to identify and quantify the cells of interest in the lung samples, we performed IHC specific for GRP, the human marker protein for the pulmonary neuroendocrine system. Once stained and identifiable, the cells were counted and normalized to the length of the basement membrane in order to determine densities. In this quantification, PNECs and NEBs were considered distinct entities and their numbers were kept separate. Densities were then compared from different lung regions in order to develop a sense of distribution throughout the lung in both healthy and asthmatic subjects. Following immunohistochemistry, quantitative PCR was done to validate and verify the findings for normal subjects.
Figure 1: Representative Microscope Images of PNECs and NEBs in the Trachea and Distal Airways of Normal Subject

Microscope images showing isotope control sections and sections using the anti-GRP antibody after IHC. The presence of neuroendocrine cells (of any form) is only seen in experimental sections, as seen from the dark, specific staining.

4.1. Normal/Healthy Adult Subjects
Initially, we performed IHC specific for GRP on the samples from normal subjects. We measured the density of both solitary neuroendocrine cells and neuroendocrine bodies from three distinct lung sections (trachea, middle airway, and distal airway) in order to decipher any significant distribution patterns. On average, for each subject 2-4 middle and distal airways were studied. In normal lung samples, we found a statistically significant increase in PNECs in the
trachea versus the distal airways (Welch’s t-test, \( p \leq 0.05 \)) and a statistically significant increase in NEBs in distal airways versus in the trachea (Welch’s t-test, \( p \leq 0.01 \)) (Figure 2).

**Figure 2:** Density and Distribution of PNECs and NEBs in the Lungs of Normal Healthy Adult Subjects.

Based on the high density of pulmonary neuroendocrine bodies found in distal airways (shown in Figure 2 right panel), we decided to conduct specific distribution studies in only the distal airways. There is evidence to suggests that other cell types in the airway epithelium have significant differences in genes and in the proteins they involve with when comparing non-terminal and terminal bronchioles\(^9\). As a next step, we separated the distal airway classification into terminal bronchioles and non-terminal bronchioles to quantify the difference in distribution within these sub-classifications (Figure 3). We found a statistically significant increase in the number of NEBs in terminal bronchioles versus non-terminal bronchioles (Welch’s t-test, \( p \leq 0.05 \)).
Figure 3: NEB Density in the Distal Airway of Normal Healthy Subjects

Column graph comparing NEB density in normal subjects, organized by sub-classification of the distal airway. There is a significant difference between the two sub-classes (Welch’s t-test, p ≤ 0.01).

To enhance the results we obtained from IHC, we performed qPCR on freshly excised airway tissue sections from 9 normal adult subjects listed in Table 1. We used qPCR to determine the relative amounts of GRP mRNA located in the various lung regions (Figure 4). Although there was a trend towards more GRP mRNA in the distal airways, this was not statistically significant (Welch’s t-test, p=0.1306).
Figure 4: mRNA Expression of GRP in Normal Subjects

Column graph demonstrating relative amounts of GRP mRNA expression from 9 normal subjects, organized by lung section. There are no significant findings in regards to trachea/middle airways versus distal (Welch’s t-test, p=0.1306).

4.2. Asthmatic Adult Subjects

To expand the scope of our study, we conducted IHC on tissue samples from 6 asthmatic subjects. We calculated the density of both solitary PNECs and NEBs in the trachea, middle, and distal sections from the six asthmatic subjects (Figure 5). Unlike the normal subjects, the IHC results from the asthmatic subjects did not show significant region-specificity in PNECs and NEBs densities, although there was an average increase in cell density in the trachea for solitary PNECs and in the distal airways for NEBs, which was a similar trend seen in the normal subjects (Welch’s t-test, p=0.0596 and p=0.1095).
Figure 5: Densit and Distribution of PNECs and NEBs in the Lungs of Asthmatic Subjects.

Column graph depicting densities of PNECs and NEBs in asthmatic lung tissue, organized by lung section. No significant difference in cell density was found between any of the sections (Welch’s t-test, p=0.0596 and p=0.1095).

As with the normal subjects, we did more specific IHC analysis of the NEB densities within distal airways. Separating the distal classification into non-terminal and terminal bronchioles allowed us to break down the distribution of NEBs further for comparison (Figure 6). There was a significant difference in distribution of NEBs between non-terminal and terminal bronchioles, with a noted increased in terminal bronchioles (Welch’s t-test, p≤0.01).
Figure 6: NEBs in the Distal Airway of Asthmatic Subjects

Column graph demonstrating the respective densities of NEBs in asthmatic lung samples, organized by sub-class of the distal airway. There is a significant increase in NEB density as airways move from non-terminal to terminal (Welch’s t-test, $p \leq 0.01$).

Given that an objective of this study was to elucidate potential differences in lung physiology between diseased and normal subjects, we chose to compare the relative densities (from IHC) of NEBs in terminal airways. Both normal and asthmatic subjects produced lung samples with a statistically significant increase in NEB densities along the proximal-distal axis of the lung airways, so we chose to explore this trend for significant differences (Figure 7). There was a significant difference in NEB densities of the terminal airways between asthmatic and normal samples, with a noted decrease in the asthmatic subjects (Welch’s t-test $p \leq 0.05$).
Figure 7: Density of NEBs in Terminal Bronchioles Normal and Asthmatic Subjects.

Column graph showing the densities of NEBs in terminal bronchioles, organized by subject demographic (Normal versus Asthmatic). The average decrease in NEB density in asthmatic subjects in comparison to normal physiology is statistically significant (Welch’s t-test, p ≤ 0.05).

To go along with the data in Figure 7, we chose to compare density of PNECs between normal and asthmatic subjects in both the trachea and in the middle airways using the data from IHC (Figure 8). In both regions (trachea and middle airways), the sections from the asthmatic subjects had lower PNEC densities than the normal subjects. The difference is not statistically significant, though the trend may still be of value (Welch’s t-test, p=0.4832 and p=0.1637).
Figure 8: Density of PNECs in Trachea and Middle Airway Sections in Normal and Asthmatic Subjects

Column graph showing the densities of PNECs in normal and asthmatic subjects, organized by section (trachea or middle airway). The average decrease in PNEC density in trachea and middle airway sections from asthmatic subjects compared to normal subjects is clear, but not statistically significant (Welch’s t-test, $p=0.4832$ and $p=0.1637$).
5. DISCUSSION AND CONCLUSIONS

Throughout the course of this project, we were able to discern a few key details regarding the organization and distribution of the cells within the pulmonary neuroendocrine system that have yet to be stated in existing literature. The first of these was that in tissue samples from normal subjects, the density of PNECs in proximal airways is greater than in more distal airways. The trend is reversed for NEBs in normal subjects, with their highest densities in distal airways, especially terminal bronchioles. For asthmatic subjects, the findings are similar, but less convincing given the weaker statistics. Potentially the most interesting finding is the significant decrease in the density of NEBs found in terminal bronchioles in asthmatic subjects when compared to healthy subjects. Taking all of these findings together, there are some apparent differences in distribution depending on cell structure and on subject pathology.

The opposing distribution pattern of PNECs and NEBs suggests distinct roles for the two cell forms. The regions in which these two cell forms are primarily located are distinct and have different roles in the respiratory system. While PNECs are located in the largest conducting airway of the respiratory system, NEBs are located most heavily in the smallest airways that are more involved with chemo-sensing and detoxification\(^5\). Terminal bronchioles represent the junction of the conduction zone and respiration zone of the lung, and there may be a correlation between this unique location and the nuanced role of NEBs. Previous research has provided much evidence to support the claim that NEBs are key players in chemo-sensing and oxygen-sensing in the lungs, and the findings from our project coincide with the previous literature regarding this topic\(^8\).

The distinct decrease in NEB density in terminal airways of asthmatic subjects in comparison to healthy subjects should be explored for its potential for therapeutic implication. These findings
are corroborated by the decrease in PNEC density in asthmatic subjects in both the trachea and the middle airways. Although the findings were not statistically significant, the data shows an overall decrease in neuroendocrine cells (of any form) in subjects with asthma. The presence of a difference between normal and asthmatic pathology yields many possibilities for exploration in regards to disease prevention, treatment, and therapy.

Given the nature of the study, which utilized human lung samples, there was little room for standardization beyond assessing smoking history and general lung health. The tissue samples used in the study were taken from lungs rejected for transplant due to having been on the ventilator for an extended period of time. Human subjects leave room for a vast quantity of confounding factors and inconsistencies in data, which cell culture can mostly eliminate. There is some chance that variability among the subjects yielded unexpected and unknown data discrepancies. In addition, the sample sizes are each relatively small (<10), and conclusions drawn from the data could become stronger and more supported with an increase in sample size.

In order to confirm the findings from asthmatic subjects, qPCR should be done in the same fashion that it was done for the normal subjects. Finally, a large aspect of this study revolves around the comparison of different disease states, and extrapolating the study to include additional diseases (including chronic obstructive pulmonary disease or cystic fibrosis) would continue to progress the goal of the study.
6. ACKNOWLEDGEMENTS
I would like to thank the Wanda O’Neal, PhD, Kenichi Okuda, MD, and Richard Boucher, MD for their continued support of my work during my time at the University of North Carolina at Chapel Hill. I would like to send my appreciation to the UNC CF Center Tissue Procurement and Cell Culture Core for providing human lung tissues. In addition, I want to thank the Cystic Fibrosis Foundation, Cystic Fibrosis Research Incorporated, and the National Institutes of Health for their sponsorship of this project. Finally, I would like to thank my mother, Dana Minnick, PhD, for her love and support throughout all stages of my growth in scientific writing.
7. REFERENCES


