INTRODUCTION

Asthma is recognized as a chronic inflammatory disease of the airways, characterized by reversible airflow limitation and airway hyper responsiveness. Its prevalence is continuously increasing and it becomes one of the most important socio-medical problems.\(^1\)

The inflammatory process in asthma is dominated by Th2 type inflammation, characterized by T cells that make interleukin IL-4, -5, and -13 along with eosinophils, mast cells, basophiles, and macrophages.\(^2\)

Although asthma has been considered as a condition of reversible airflow obstruction, many asthmatic patients, children and adults, have evidence of residual airway obstruction that may even be detected in asymptomatic patients. This clinically demonstrable irreversible component of airways obstruction also is reflected by the presence of structural changes of the bronchi that contributes to geometric changes of the airways and to a various degree of functional impairment.\(^3\)

Asthmatic airway inflammation is generally believed to cause tissue injury and subsequent structural changes, so-called airway remodeling.
An increase in airway smooth muscle mass is the most prominent feature of airway remodeling in asthma. Smooth muscle proliferation consists of hypertrophy (an increase in size of airway smooth muscle) and hyperplasia (an increase in the number of airway smooth muscle). The airway remodeling is an important feature of asthma, as it is an early and consistent component of childhood asthma. Some biopsy studies described collagen deposition in the lamina reticularis and underlying fibroblasts proliferation and other tissue restructuring up to 4 years before the onset of symptoms in young children. This indicates that this process begins early in the development of asthma and may occur in parallel with inflammation.  

A variety of mediators have been described in airways of asthmatics that could theoretically be relevant to airway remodeling, but it is not entirely known how various stimuli or mediators of the inflammatory response are linked to the processes of airway remodeling. The lung is exposed continuously to inhaled antigen, yet inflammatory lesions in the lung are relatively mild to protect the gas-exchange surface to overt damage. Dendritic cells (DCs) and alveolar macrophages play a central role in this regulatory process. While macrophages generally serve to dampen the pulmonary immune response, DCs are clearly specialized to initiate immune responses. Airway DCs capture inhaled antigen and migrate to the draining nodes of the lung where they specifically react with naive specific T cells. When DCs reach the draining lymph node T cell area, they select only those T cells with a receptor specific for the antigens carried from the periphery. DCs that have migrated from the periphery of the lung become mature and express co stimulatory molecules, which interact with their ligands on T cells to form the immunological synapse. After a number of divisions (>4), primed T cells return to the lung as effectors and secret cytokines like IL-4, IL-13 and IL-5.

Not all inflammatory responses to allergen are closely associated with Th2 pathways. There are studies that measured increases in the acute inflammatory response element, thrombin, in the airways of subjects with asthma after segmental allergen challenge. This was accompanied by increases in fibronectin and TGF-β. TGF-β is increased in the lungs of individuals with asthma and may modulate airway inflammation and remodeling. In asthmatics, some irritants, such as house dust mites, degrade the tight junction of the epithelium, and from damaged tissue is released TGF-β, which interacts with the receptors from fibroblasts. TGF-β is a family of multi-functional cytokines, including five known isoforms, three of them being expressed in mammalian cells (TGF-β₁,₂,₃). These three isoforms have different expression genes, and their promoter structures and regulatory elements are different.

Based on these data, results that one of the most important cells in pathogenesis of asthma are the fibroblasts. It has been shown that after tissue injury, they migrate into the damaged tissue and synthesize extracellular matrix components stimulated by cytokines released from inflammatory and resident cells. In case of mechanical challenge, fibroblasts develop contractile fibers composed of cytoplasmic actins, converting into “progenyfibroblast”. These are stable phenotype in culture, representing an intermediate step toward the “differentiated myofibroblasts” that are characterized by expression of α-SMA, the most important molecular marker.

In this study we evaluate the effect of four important cytokines that are present in inflammatory process from asthma – IL-4, IL-13, TGF-β₁ and TGF-β₂ – on pulmonary fibroblasts. We hypothesized that these cytokines and growth factors increase α-SMA expression in fibroblasts, inducing the airway remodeling. Therefore, we established primary fibroblasts cultures from pulmonary biopsies and compared the ability of IL-4, IL-13, TGF-β₁ and TGF-β₂ to promote myofibroblasts transformation with that of two pulmonary fibroblasts lines.

**MATERIALS AND METHODS**

**Cell sources and culture**

Two fibroblasts lines were used, obtained from Promo Cell - Human Pulmonary Fibroblasts (HPF, Cat. No. C-12360) and Lonza - Normal Human Lung Fibroblasts (NHLF, Cat No CC-2512). The NHLF line was a generous gift from Blutspendedienst, Frankfurt, with the kindness of Prof. Dr. Torsten Tonn. The cells were cultured at 37°C, 5% CO₂ in DMEM, supplemented with 20% FCS, 2 mM L-glutamine, 150 U/I ml penicillin, 100 µg/ml streptomycin. Medium was changed two times/ week. The cells were used between passage 5 and 7.

Primary fibroblasts (PF) were established from human biopsies tissue, derived from normal areas of surgically excised lung specimens undergoing resective surgery for lung cancer; 5 patients, 4 male, 1 female, aged 54±6.16.8 (mean ± SEM). All biopsies were taken after the patients had given their informed consent. Cells were isolated and separated in unicellular suspension by interjecting the tissue through 70 µm cell strainer, after a preliminary digestion with collagenase (200
U/ml), 30 minutes at 37°C. Cells were then collected by centrifugation, washed twice and resuspended in high-glucose medium supplemented with 20% FCS (Gibco). All cells were plated in T25 cm² culture flasks in growth medium with a medium change twice a week until cells reached confluence. The cells were used between passage 2 and 3. For the assay, cells were seeded in 6 well plates and at specified time points cells were harvested to extract RNA and seeded on slides for immunohistochemistry.

Effect of cytokines and transforming growth factor β on smooth muscle α-actin expression in cultured pulmonary fibroblasts

The study totals a number of 6 experiments, two for each type of cells: HPF, NHLF and PF.

The following experiment was designed to assay the effect of pro-inflammatory factors on the phenotypic change of fibroblasts. The cells were plated in 6 well plates (4000 cells/cm²). This was referred as day 2. Twenty-four hours later (day 1) the FCS concentration was reduced to 4% to decrease the proliferating rate and maintained for the rest of the experiment. Twenty-four hours later the medium was replaced by medium containing IL-4 (R&D, 204-IL-050, 25 ng/ml), IL-13 (R&D, 213-IL-025, 25 ng/ml), TGF-β1 (Sigma Aldrich, T7039, 1.5 ng/ml), TGF-β2 (Sigma Aldrich, T2815, 1.5 ng/ml) and one medium which contained all factors in the same concentrations. One well was maintained with diluent only (0 ng/ml factor). This was referred for day 0. Each experiment was proceeded in duplicate – one sample for RT-PCR and the other for immunohistochemistry. The cells were pulsed for 14 days, when the medium was discarded, cells were rinsed with PBS.

Analysis of smooth muscle α-actin by reverse transcription polymerase chain reaction

Specific sense (forward) and antisense (reverse) primers were designed for α-SMA, using the published sequence (NM001613). The product size was 611 bp. Total RNA was extracted from fibroblasts cultured with or without factors (Sigma, RTN70). Reverse transcription and cDNA amplification were proceeded in one-step reaction (Qiagen, One step RT-PCR kit, 210212). The final reaction volume was 50 μL, containing specific sense and antisense primers (3 μL each), 10 μL 5x Buffer, 10 μL 5x Q Solution, 2 μL dNTP, 2 μL Enzyme Mix, RNA 10 μL (100 ng/reaction) and free RNA-ease water up to 50 μL. PCR was performed for 30 cycles, each cycle including denaturation (94°C, 1 min), annealing (60°C, 2 min) and extension (72°C, 3 min) and a final incubation (72°C, 10 min). One blind sample was used for negative control, which contained distilled water (DW) instead of RNA. PCR products were electrophoresed onto 2% agarose gels, containing ethidium bromide and visualized and imaged on a UV transilluminator equipped with a camera. For comparing the obtained value of the bands the TrackIt 50 bp DNA Ladder (Invitrogen) was used.

Characterization of fibroblasts/myofibroblasts by immunohistochemistry

After 14 days of cell culture with or without factors cells were harvested for immunohistochemistry. Cells were washed two times with sterile PBS, trypsinized using 0.25% trypsin EDTA solution (Sigma, T4049), centrifuged 5 min at room temperature and resuspended in 1 mL PBS. The suspension was layout on slides (Thermo Scientific, A78710003) using Cytospin (Thermo Scientific, A78300002). Slides were rinsed twice with PBS 10 min at room temperature and to enhance permeability and fix the cells, they were washed with 0.02% formalin and then with 95% ethanol. After air-drying, immunohistochemistry was carried-out in an automatic mode. The cells were assessed for α-SMA (Dako Cytomation, N1584) and vimentin (Dako Cytomation, N1521).

Morphometric assessment of smooth muscle α-actin immunoreactivity

The slides were counted for the express of α-SMA. The cells that did not show any staining at all were considered negative. The cells that showed a minimal positive immunoreactivity were considered positive. Five randomly selected fields were examined on each slide and were counted the positive cells, as well as the total number of cells in the same area at 40x magnification. The percentage of positive cells was calculated for each area and then the average of the five areas was considered the percentage of α-SMA positive cells.

RESULTS

Effect of cytokines and transforming growth factor β on smooth muscle α-actin expression in cultured airway fibroblasts tested by immunohistochemistry

The assay of fibroblasts pulsed with factors showed a positive reaction for expressing α-SMA, but in different ways, depending on the factor that was used. The percentage of positive cells was higher in fibroblasts pulsed with all factors together (p=0.00801), and tended to be lower in fibroblasts pulsed with TGF-β1 or TGF-β2 (p=0.008532 or p=0.017819). (Fig. 1) The cells were all positive close to 100% for vimentin, the fibroblasts cultured with factors, as well as the fibroblasts cultured in normal medium. (Fig. 2)
Figure 1. Representative immunohistochemical staining of α-SMA in fibroblasts pulsed with TGF-β, IL-4, IL-13 (a), all factors at once (e) and no stimulated fibroblasts (f) (trypsinized cells).

Figure 2. Representative immunohistochemical staining of vimentin in unstimulated fibroblasts (a) and fibroblasts pulsed with TGF-β, IL-4, IL-13, all factors at once (f) (trypsinized cells).

Figure 3. Representative immunohistochemical staining of α-SMA in fibroblasts pulsed with IL-4 (a) and all factors (b) - large shaped cells.

The morphology of cells cultured with factors was also modified, they were larger shaped cells. (Fig. 3)

A very important issue of the experiment is that there were no significant differences between the two lines and primary fibroblasts, so that IL-4 seemed to be the most pro-fibrogenic factor irrespectively of the cell type, comparing to negative control (p=0.000181). (Fig. 4) The only difference observed is that primary fibroblasts had a higher transformation rate for each factor. (Fig. 5)

Effect of cytokines and transforming growth factor β on smooth muscle α-actin expression in cultured airway fibroblasts tested by polymerase chain reaction

Unstimulated lung fibroblasts expressed a weak band for α-SMA. Expression of α-SMA was enhanced in lung fibroblasts upon incubation with proinflammatory factors, and the most important effect was seen in fibroblasts stimulated with all factors. (Fig. 6)

DISCUSSION AND CONCLUSIONS

Once considered a simple allergic disease dominated by Th2 type inflammation, asthma is now
considered a very heterogeneous disease, concerning implications from immunopathology, clinical phenotypes, response to therapies and the natural history. Because in literature there already are many factors incriminated to participate in pathophysiology of asthma, the present study was designed to investigate a possible difference between the Th2 type cytokines (IL4, IL13) and the factors that are secreted from environmental cells, like TGF-β. Boxall et al. evidenced TGF-β, to be the main factor implicated in fibrosis. The results of this study also evidenced the role of TGF-β in remodeling by increasing the expression of α-SMA in fibroblasts after 14 days of exposure, but a more relevant result was obtained after fibroblasts stimulation with Th2 type cytokine, IL-4. Because Batra et al. found in asthmatic’s bronchoalveolar fluid (BAL) increased concentrations of all four factors significantly one day after segmental allergen challenge (SAC), it shows a particular role of these cytokines in significant one day after segmental allergen challenge (BAL) increased concentrations of all four factors stimulation with Th2 type cytokine, IL-4. Because Batra et al. found in asthmatic’s bronchoalveolar fluid (BAL) increased concentrations of all four factors significantly one day after segmental allergen challenge (SAC), it shows a particular role of these cytokines in remodeling. The authors related TGF-β1, TGF-β2 and IL-13 concentrations returned to baseline by one week after SAC, but BAL fluid IL-4 concentration remained elevated for two weeks at least.

Audrey Richter et al suggested that neither IL4 nor IL13 was able to promote fibroblasts differentiation by secreting SMA and have a direct role in remodeling, but both cytokines were found to stimulate release of TGF-β2 from bronchial epithelial cells. It remains to be determined if Th2 type cytokines act on secretor function of airway fibroblasts and participate through other factors in asthma remodeling.

Leigh and coworkers reported that, whereas IL-13 appeared to be important in airway hyper responsiveness in mice who underwent acute antigen sensitization and challenge, it appeared to be unimportant in the increase in airway wall remodeling (increase in goblet cells, sub epithelial collagen deposition, and α-SMA) in mice that had chronic (4-week) antigen exposure. This report important differences in acute versus chronic animal models of antigen exposure and “asthma.” Our study revealed also a week answer from fibroblasts in stimulation with IL-13, with no differences between the two lines and the PF. In severe asthma, angiogenesis and microvascular remodeling can also be seen. These changes are mediated by multiple factors, especially vascular endothelial growth factor (VEGF). VEGF is localized to airway environmental cells, mononuclear cells, and T lymphocytes. Previous clinical studies of Hashino et al. related that VEGF levels are increased in asthmatic’s sputum and BALF, and the levels correlate directly with the disease activity.

An additional mediator that is reported to promote myofibroblast activation is endothelin. This important soluble mediator is capable of rapidly transdifferentiating the stellate morphology of myofibroblasts to the activated phenotype within 30 min of addition to cell culture media.

In conclusion, the hypothesis that Th2 cytokines and the factors secreted from environmental cells, like TGF-β1, have a role in remodeling, by determining airway fibroblasts to express actin, the main marker of smooth muscle cells, was confirmed of our results and from these, IL-4 distinguished as the most profibrogenic factor. For further study of the remodeling, myofibroblasts response to allergic inflammation inductors stimulation will be assessed to observe if TGF-β1, TGF-β2, IL-4 and IL-13 stimulation will affect different the secretor capacity of myofibroblasts, through immunoenzymatic analysis of endothelin-1 and VEGF from myofibroblasts culture supernatant.

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REFERENCES