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Neurotrophin-Produktion von Atemwegs-Epithelzellen

beim allergischen Asthma bronchiale:

Ein Modell für Neuro-Immunitätsinteraktionen

*“Neurotrophin Production by Respiratory Epithelial Cells in Allergic Airway
Diseases: A Model of Neuro-Immune Interactions”*

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1. Introduction

Neurotrophins are a family of polypeptide growth factors that are essential for the development of the vertebrate nervous system; they regulate the survival, death, and differentiation of neurons in the embryonic and postnatal stages, and also the neuronal maintenance later in life. In recent years, data have emerged indicating that neurotrophins could have a broader role than their name might suggest. In particular, their functions in other biological processes including, immune regulation and neuroendocrine control. Recent studies show that neurotrophins affect differentiation and function of a wide range of immune cells including T cells, B cells and granulocytes. Therefore, neurotrophins could act as an autocrine or paracrine mediators in the cellular communication between immune cells. Over that, the recent publications started to explain the possible role of neurotrophins in some immunological disorders like autoimmune diseases and allergy.

1.1. Neurotrophins and their receptors

The term neurotrophins refers to a group of proteins that have common structural features, similarity in receptor utility and physiological activities. They consist so far of four major members: nerve growth factor (NGF), brain derived growth factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). All neurotrophins have similar biochemical characteristics. They are secretory proteins that are synthesized as precursor proteins, which are then cleaved intracellularly to mature proteins of about 120 amino acids in length (20). After that, the mature proteins associate as non-covalent homodimers with a high degree of genomic, structural and biological homology among various species (approximately 50% of the amino acids are common to all neurotrophins). The biological effects of neurotrophins are mediated by binding to their receptors. There are two classes of neurotrophins cell surface receptors (Figure 1). The p75 receptor (known as the low affinity pan neurotrophins receptor) is common to all members of the neurotrophin family. The high affinity receptors (having binding constants on the order of 10^{-11}) include receptor tyrosine kinase proteins TrkA, TrkB, and TrkC. These receptors have different specificity for different members of the neurotrophins family. TrkA is the receptor for NGF, TrkB is the receptor for BDNF and NT-4, and TrkC is the receptor for the NT-3. However, NT-3 can also bind to TrkA and TrkB, but with low affinity

than to TrkC, and with lower affinity than the primary ligands for these receptors. Similarly, NT-4 also binds to TrkA but with lower affinity. In addition to these (classical) receptors, the issue is complicated by the existence of isoforms of TrkB and TrkC, which lack the cytoplasmic tyrosine kinase catalytic region (4). These receptors are found throughout the developing body as well, and it is not known if these noncatalytic forms of the receptors act as agonists or inhibitors. Binding of neurotrophins induces receptor dimerization at the cell surface followed by phosphorylation of receptor tyrosine residues. The phosphorylated tyrosine then recruits intracellular signal transduction proteins. These factors initiate functional changes such as survival, proliferation and differentiation of the corresponding target cell.

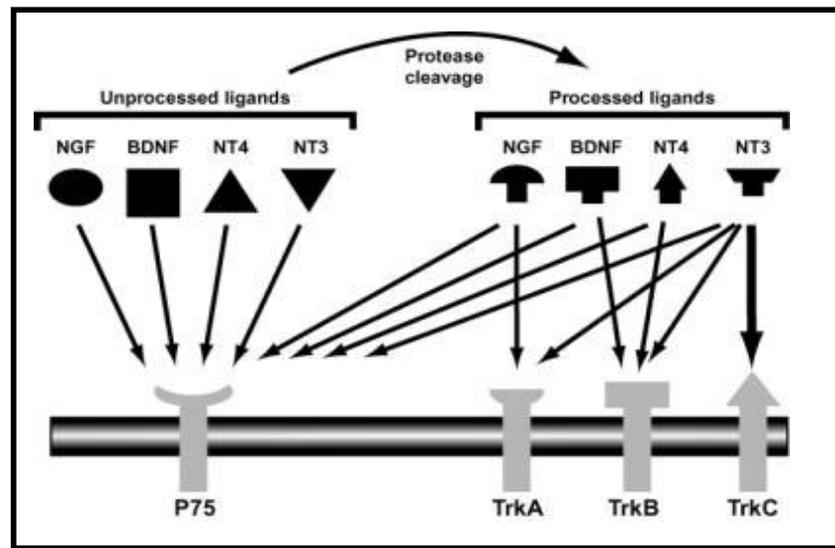


Figure 1: neurotrophins and their preferred receptors. Reprinted from Annual Review of Neuroscience 26, R. A. Segal, *Selectivity in Neurotrophin Signalling: Theme and Variations*, PP. 299-330, 2003

All neurotrophins also bind to the low affinity nerve growth factor receptor, p75. The p75 receptor belongs to the tumor necrosis factor receptors family and was the first identified neurotrophins receptor (51). This receptor binds the neurotrophins with equal affinity, but it has no cytoplasmic tyrosine kinase domain (21, 41, 86). The role of these receptors is controversial, as it may also be involved in either promoting or down regulating the response to the neurotrophins. P75 may function to increase the affinity of the Trk receptors for their respective neurotrophins, or it may bind the neurotrophins and prevent them from binding to the high affinity receptors. Although

it does not have a catalytic intracellular tyrosine kinase domain, it is capable of mediating the neurotrophin signals. The ligand binding of p75 increases the high affinity TrkA binding sites enhances TrkA autophosphorylation, and selectivity for neurotrophins ligands (53). Conversely, TrkA activation can inhibit p75 mediated signaling, but the mechanism of this inhibition is unclear (53). The TrkA neurotrophins receptor has been linked to human diseases. The TrkA gene was originally described as an oncogene in colon cancer (68) and its translocations are common in papillary carcinoma (8). Recent study has shown a possible link between the expression of the P75 neurotrophins receptor and the acute leukaemia (6).

1.2. Neuroimmune interaction

The essential role of the immune system is to protect the host from pathogens, and at the beginning, it has been thought that the immune system functioned in isolation, but now there is growing evidence that the immune system and, especially, the nervous system are functionally interconnected, also the importance of this interaction is still matter of debate. Different hypotheses aimed at explaining, at least partially, the pathogenesis of several diseases have emerged on the bases of current knowledge of the role of neurotrophins in the immune system. Depending on the widespread expression of neurotrophins in the immune organs, and immunocompetent cells, they may be the candidate molecules for regulating immune as well as neuroimmune interaction.

1.2.1. Distribution of neurotrophins and their receptors in the immune system

Neurotrophins and their receptors are widely expressed in various cell type of the adaptive and innate immune system. The traditional sources of neurotrophins under physiological conditions are primarily nerve associated cells such as glia cells, Schwann cells, fibroblast and neurons themselves (65, 67). The sources of NGF in the inflammatory process are a wide range of haematopoietic cells including mast cells (64), macrophages (10), T cells (33), and B cells (94). Whereas, BDNF synthesis has been detected in activated T cells, B cells and macrophages (11, 56); in mast cells (91), and in thrombocytes. (111) In addition to neurotrophins, their receptor P75 NTR and the specific receptor Trk were found in various immune cells including T cells, B cells, Monocytes and mast cells (12). TrkB was expressed by T cells and macrophages. One study has shown that Trk B and Trk C positive eosinophils were

described in the human bone marrow (61). Other study has described the expression of Trk A, Trk B, Trk C by eosinophils purified from human blood. (72)

1. 2.2. Function of neurotrophins in the immune system

Most of the available data about the function of neurotrophins in the immune system belong to the NGF effects over different immune cells. NGF increases the number of mast cells in neonatal rats (46), and also stimulates rapid degranulation of mast cells and basophils (7, 52), at the same time promotes differentiation of mast cells, granulocytes and macrophages (43, 69, 90, and 106). NGF also activates eosinophils (74), promotes proliferation of B- and T-cell subsets (74, 92). It has been found also that NGF enhances T_H2 cytokine production and immunoglobulin IgE synthesis (10) and induces differentiation of activated B cells in Ig-secreting plasma cells (13). In general, we could conclude from all of these data that most of NGF effects have pro-inflammatory properties, and the NGF by itself does not appear to activate the immune cells in physiologically relevant concentrations, but rather modulates their threshold to other triggering stimuli (66). There is very little data available about possible functions of other neurotrophins in the immune system.

1.3. Neurotrophins in allergic Asthma

1.3.1. Allergic Asthma

Chronic airway inflammation, development of airway hyperreactivity and recurrent reversible airway obstruction are the characteristic features of allergic bronchial asthma. The development of airway hyperreactivity is an important hallmark in the pathogenesis of allergic asthma.

1.3.1.1. Immunopathology of allergic Asthma

Based upon animal studies and limited bronchoscopic studies in adults, the immunologic processes involved in the airway inflammation of asthma are characterized by the proliferation and activation of helper T lymphocytes (CD4+) of the subtype T_H2. The T_H2 lymphocytes mediate allergic inflammation in atopic asthmatics by a cytokine profile that involves IL-4 (which directs B lymphocytes to synthesize IgE), IL-5 (which is essential for the maturation of eosinophils), and IL-13 and granulocyte-macrophage colony-stimulating factor. Eosinophils are frequently

present in the airways of asthmatics (more commonly in allergic but also in nonallergic patients), and these cells produce mediators that can exert damaging effects on the airways. Recent knockout studies and anticytokine studies suggest that lipid mediators are products of arachidonic acid metabolism. They have been implicated in the airway inflammation of asthma, and therefore have been the target of pharmacologic antagonism by a new class of agents called antileukotrienes. Prostaglandins (PGs) are generated by the cyclooxygenation of arachidonic acid, and leukotrienes are generated by the lipoxygenation of arachidonic acid. The proinflammatory prostaglandins (PGD₂, PGF₂, and TXB₂) cause bronchoconstriction, whereas other prostaglandins are considered protective and elicit bronchodilation (PGE₂ and PGI₂, or prostacyclin). Leukotrienes C₄, D₄, and E₄ compose the compound called "slow-reacting substance of anaphylaxis," a potent stimulus of smooth muscle contraction and mucus secretion. Ultimately, mediators lead to degranulation of effector/proinflammatory cells in the airways that release other mediators and oxidants, a common final pathway that leads to the chronic injury and inflammation noted in asthma.

1.3.1.2. Neurogenic inflammation

The direct potentiation of an inflammatory process by bidirectional pathways connecting the nervous and immune system has led to the concept of neurogenic inflammation (78). The Neurogenic inflammation, which due to the release of neuropeptides from sensory nerves has been demonstrated in airways of several species, particularly rodents, and may contribute to the inflammatory response in asthmatic airways. Tachykinins (substance P and neurokinin A) released from airway sensory nerves may cause bronchoconstriction, vasodilatation, plasma exudation, and mucus secretion. The activities of substance P, the most prominent member of the tachykinin peptide family, on immune cells include a broad range of functional responses from eosinophils, neutrophils, mast cells, monocytes/macrophages as well as lymphocytes. Substance P stimulates chemotaxis, superoxide production and neutrophil adherence to the endothelium and has a degranulating effect on eosinophils. Moreover substance P activates monocytes to release inflammatory cytokines, such as TNF- α or IL-1 and stimulates T_H1/T_H2 phenotype switch in T cells as well as immunoglobulin switching in B cells. Airway epithelial damage in asthma exposes sensory nerves which may become sensitized by inflammatory

products (including prostaglandins and cytokines) so that neuropeptides are released via a local reflex trigger such as bradykinin, resulting in exaggerated inflammation. The effects of tachykinins may be amplified further by loss of the major degrading enzyme, neutral endopeptidase, from epithelial cells.

The neurotrophin induced stimulation of neuropeptide synthesis from sensory lung innervations and also regulate neuropeptide synthesis in immune cells. By regulating the neuropeptide synthesis in lymphocytes and neuronal cells, NGF can influence the intensity and duration of the local immune response. At the same time, neuropeptides are able to induce neurotrophin synthesis in a positive feed back mechanism. Substance P and also the β -adrenergic agonist isoproterenol significantly increased NGF synthesis in lymphocytes (5). The enhancement of neurotrophin synthesis by neurotransmitters represents a further amplification pathway of neuroimmune interaction during neurogenic inflammation.

1.3.1.3. Neuronal plasticity

Neuronal plasticity is the ability of nerve cells to change their prosperities (by sprouting new processes, making new synapses or altering the strength of existing synapses). In allergic asthma, the qualitative and quantitative changes in the functional activity of the peripheral lung sensory neurons are an important feature. The mechanism by which airway inflammation influences neuronal activity has not been worked out in detail. In animal models it is clear that allergic inflammation directly amplifies the efferent limb of the parasympathetic ganglia, as well as, at the level of the postganglionic nerve varicosities. In addition to directly increasing the activity in parasympathetic nerves, allergic inflammation can affect afferent (sensory) nerves in the airways. And there is a growing evidence now that inflammation induced alterations in airway sensory nerve activity, and the central processing of the activity, contributes to the symptoms of asthma. Indeed, an allergen challenge, in vivo, has been found to lead to discharge in mechano-sensing afferent fibres in the rabbit (70). Inflammatory mediators associated with asthma likely lead to activation of nociceptors in the airway wall. The airway nociceptores can be stimulated by low pH, hypertonic saline and chemicals such as bradykinin and capsaicin (97). Upon stimulation of these fibres, neuropeptides are released into synapses in the nucleus of

the solitary tract in the brainstem where central sensitization may occur. In addition to inducing action potential discharge in this population of sensory nerves, airway inflammation may increase their neuropeptide content. The sensory neuropeptide content increases in guinea-pig lungs 1 day after allergen inhalation and then returns to basal levels within 3 days (35). Airway inflammation may also change the nature of the airway nerves producing neuropeptides. Sensory neuropeptides in guinea-pig trachea/bronchi are found exclusively in nociceptive C-fibres derived from small diameter cell bodies situated in the jugular ganglia (80). We have found, however, that in inflamed airways non-nociceptive nerves change their phenotype such that they begin to synthesize substance P and calcitonin gene related peptide. Thus, within 1 day of allergen challenge, or within 4 days of respiratory viral infection, low threshold mechanosensitivity trachea/bronchi neurons with large diameter cell bodies begin to synthesize these neuropeptides. This effect can be mimicked by application of nerve growth factor to the airway wall (47). The sensory neurons innervating the lung are also responsive to neurotrophins since local increase of neurotrophins in the lung could mediate similar neuronal changes in animal models as seen during human allergic inflammation. NGF was shown to contribute to inflammatory airway hyperresponsiveness by upregulation of substance P production in sensory neurons. Other groups indicate that NGF not only increases the neuropeptide expression in airway neurons but also increases the absolute number of tachykinergic neurons. Tracheal injection of NGF changed induced substance P production in mechanically sensitive Aδ-fibres that do not produce this peptide under physiological conditions. Other studies with genetically modified animals gave more evidence that neurotrophins regulate sensory nerve plasticity, one of these studies has shown that in transgenic mice overexpressing NGF in Clara-cells of the lung, a marked sensory and sympathetic hyperinnervation as well as increased neuropeptide content in projecting sensory neurons was observed (45).

1.3.2. Cellular sources of neurotrophins in allergic asthma

The traditional sources of neurotrophins under physiological conditions are thought to be nerve-associated cells or neurons themselves; nevertheless also immune cells are able to produce these factors. In allergic asthma, different studies have shown that neurotrophins worked as mediators of asthma in humans and animal models. The first indication of this concept came from a study of Bonini and his co-workers, in which

serum levels of NGF were measured in patients with allergic diseases (9). Serum NGF levels were found to be elevated in patients with asthma, allergic rhinitis, or urticaria. The highest levels of NGF, approximately 35 times those of healthy controls, were found in allergic asthmatics. Production of neurotrophins in the lungs of asthmatics has been examined by measuring NGF and BDNF levels in bronchoalveolar lavage fluid following segmental allergen challenge. NGF, BDNF, and NT-3 were increased in lavage fluid 18 h, but not 10 min following allergen challenge, consistent with neurotrophins release during the late-phase asthmatic response (103). The concentration of NGF in lavage fluid was >10-fold higher than the other neurotrophins. Airway epithelial cells, smooth muscle cells, and inflammatory cells appeared to be the major sources of NGF in bronchial biopsies following allergen challenge. In cultured cells and purified primary cells the production, regulation and release of NGF has intensively been investigated. Interestingly, the NGF production is often regulated by inflammatory signals. NGF production has been shown in mast cells (91, 110), and the release is triggered by activation through cross-linkage of the high affinity IgE receptors (110). Trigger factor for NGF in peripheral blood-derived monocytes/macrophages, which express this neurotrophin constitutively, is lipopolysaccharide (LPS) (18, 83), and HIV infection (39). Interestingly, peripheral monocytes obtained from patients suffering from allergic asthma show elevated NGF production (83). Synthesis of NGF in T-cells is controversially discussed. Although NGF synthesis was not detected in purified human T-cells (74), a constitutive mRNA expression encoding NGF in CD8⁺ cells (50) and CD4⁺ T-cell clones has been shown. After stimulation with mitogen, NGF synthesis in T_H2 clones was further increased, and also T_H0 cell clones started to produce NGF whereas expression in T_H1 clones were unaltered (15, 50, and 51). Additional sources for NGF are B-cells (85, 94) and eosinophils (57, 89). New synthesis of NGF in eosinophils is enhanced by Fc-receptor-mediated stimuli, such as IgA and IgG immune complexes and interleukin IL-5 (57). NGF synthesis has been also demonstrated in structural cells. Thus, it has been shown that cultured lung fibroblasts (73), alveolar epithelial cells (36), and airway smooth muscle cells (37) may contribute to the NGF production. Interestingly, NGF synthesis in these cell types is regulated by similar mechanisms: Stimulation with interleukin IL-1 β caused an upregulation in each of these cell types (36, 37, and 73). In addition, NGF synthesis in fibroblasts and alveolar epithelial cells was increased by tumour necrosis

factor TNF- α and significantly reduced by glucocorticosteroids (36, 73). Braun and his colleagues have been working to find out the cellular sources of enhanced Brain-derived neurotrophic factor production in a mouse model of allergic inflammation, and they found that in allergic airway inflammation BDNF production is upregulated and the immune cells (T. cells, activated macrophages) serve as a source for BDNF (11). Immunohistochemical in the human lung tissue reveal NGF production in the ciliated bronchial epithelium, in bronchial smooth muscle cells, neurons, satellite cells of intrapulmonary ganglia (79), blood vessels (25, 96), and fibroblasts (73). Both alveolar and interstitial macrophages express NGF protein after allergen provocation, as has been demonstrated by Braun et al. In this study, NGF was determined in bronchoalveolar lavage fluid cells as well as in lung tissue after allergen challenge in ovalbumin-sensitized and challenged mice in comparison to control animals. The dysregulation of neurotrophins production in asthma raises the possibility that these factors may contribute to disease pathology. Potential mechanisms by which this may occur include direct effects of neurotrophins on inflammatory cells, indirect effects on inflammatory cells via action of neurotrophins on nerves, and direct effects on neurons. Evidence implicating each of these potential pathways in driving pulmonary inflammation and airway hyperreactivity has been obtained in animal models.

1.4. Airway epithelial cells and Asthma

1.4.1. Histological structure of the airway and the airway epithelium

The conducting airways are lined by epithelium and their walls contain mucus-producing glands, cartilage, smooth muscle and connective tissue. A pseudo-stratified columnar epithelium composed of several different cell types. In the proximal parts of the lower airways, the basal cell, the goblet cell, and the ciliated cell are the principal cell types. In the distal airways, the Clara cell and the ciliated cell are present. The pseudo-stratified columnar epithelium lines the central airways and gradually reduces in height to form a low cuboidal lining in the distal conducting airways. A thin, viscous layer of fluid covering the epithelial surface of the conducting airways lies on top of a thicker layer of less viscous periciliary fluid. The other structural parts of the airway (Figure 2.) are the bronchial mucus-secreting glands which account for about

12% of the wall thickness in the mainstem bronchi and gradually diminish in both number and size in the peripheral conducting airways.

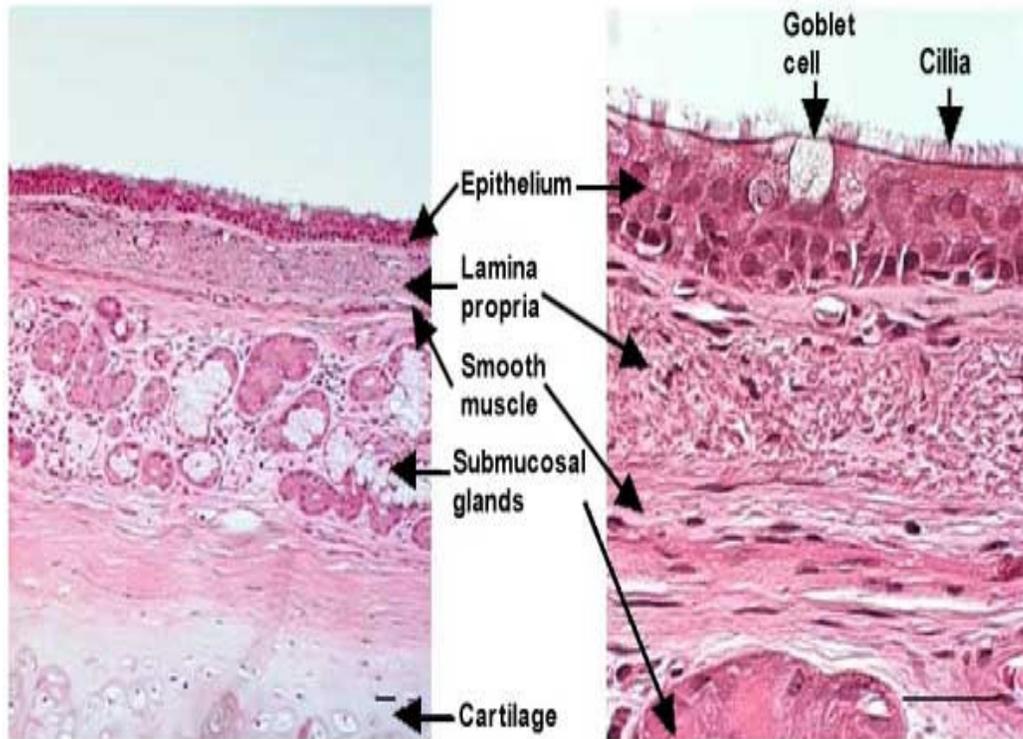


Figure 2. Histological structure of the airway showing the epithelium with its components (basal, Goblet and ciliated cells), Lamina propria, smooth muscles and the submucosal glands and finally the cartilage.

They empty their secretions via ducts onto the airway surface. Cartilage makes up about 30% of the wall thickness in the mainstem and lobar bronchi and decreases to the extent where it is no longer present in the bronchioles. The bronchioles are defined, therefore, by the absence of either cartilage or mucous glands, and represent the smallest purely conducting airways in the tracheobronchial tree. Smooth muscle makes up about 5% of the airway-wall thickness in the mainstem bronchus, increasing gradually to about 20% of the wall thickness in the bronchioles. Respiratory bronchioles are defined by the presence of alveolar openings on their luminal surface. The number of these openings increases progressively down the bronchial tree until they cover the entire airway surface in the alveolar ducts and sacs.

1.4.2. Epithelium changes in allergic Asthma

Epithelial damage is a characteristic feature of asthma. The epithelium is not merely a passive barrier but can generate a range of mediators that may play a role in the inflammatory and remodelling responses that occur in the lungs with asthma. For example, eotaxin (the principal chemokine of eosinophils), the bronchial biopsies of patients with allergic asthma have been taken 2 h after antigen provocation with inhaled house dust have demonstrated a marked upregulation of eotaxin mRNA in the epithelium. (15) In addition, there was a subsequent increase in eotaxin release in bronchoalveolar lavage (BAL) fluid at 4 h after allergen exposure. The epithelium generates a large number of other proteins that may be pro-inflammatory. These not only recruit eosinophils, but in the case of GM-CSF and IL-5, also prolong their survival within the tissue. Many of the cytokines that seem to be generated in asthma are anti-apoptotic, i.e. they will rescue eosinophils from programmed cell death. Park et al. (75) took BAL fluid from symptomatic patients with asthma and studied the survival of eosinophils in the absence or presence of various cytokines. They found that monoclonal antibodies to interleukin IL-5 and IL-13 only had a small effect in accelerating programmed cell death, whereas an antibody against granulocyte macrophage colony-stimulating factor (GM-CSF) led to very rapid eosinophil death. This suggests that GM-CSF is the main cytokine enhancing the survival of eosinophils in the lumen of asthmatic airways. The bronchial epithelium is the principal source of GM-CSF in the asthmatic airway, although other cells such as macrophages, T cells, mast cells and myofibroblasts can generate it as well (75, 24). Deposition of collagen in the epithelial subbasement membrane is a characteristic feature of the remodelling response in asthma. This may be due to abnormal associations between myofibroblasts and epithelium, both of which are involved in early lung development (epithelial–mesenchymal trophic unit). It is likely that bronchial epithelial cells initially affected by bronchial injury may be able to initiate repair of an injured area by producing chemotactic factors for epithelial cells. Fibronectin is a large glycoprotein present in the extracellular matrix with a large number of binding sites for both cells and for other molecules. It is involved in epithelial cell adhesion and spreading and has been shown to increase epithelial cell regeneration suggesting an important role for fibronectin in the repair mechanisms of epithelial cell injury. Studies using bovine bronchi have shown that these epithelial

cells can release a chemotactic factor for airways epithelial cells and that factor is most likely to be fibronectin. (87) Campbell and his colleagues have shown that epithelial cells of asthmatics obtained by brushing release more fibronectin than those of normal subjects (16) suggesting that bronchial epithelial cells, especially the cells that are affected by bronchial injury in asthma, are able to initiate repair of an injured area by producing a chemotactic factor for intact bronchial epithelial cells. Epidermal growth factor (EGF) appears to be a key factor in bronchial epithelial repair; it stimulates epithelial cell proliferation and migration. The 3v isoform of the adhesion molecule CD44 is overexpressed in damaged epithelium and seems to regulate the repair response by presenting EGF more efficiently to its receptor. Although EGF receptor expression is increased in asthma, it does not lead to an appropriate proliferative response and restitution of normal epithelium. Other factors such as transforming growth factor (TGF) β which are generated by inflammatory cells and epithelium are also upregulated in asthma. The activation of the airway epithelial cells in asthma can be through different mechanisms. It has been recently observed those epithelial cells of asthmatics but not those of normal subjects bear the Fc ϵ RI and Fc ϵ RII receptors (17) and that could be directly activated by anti-IgE. It is therefore possible that cells can be directly triggered by allergens. Pollutants including NO₂ and ozone can activate bronchial epithelial cells (28, 31). Viruses can directly damage epithelial cells (88, 95). Histamine (102), platelet activating factor (84) or cytokines can activate bronchial epithelial cells. Other mediators such as cysteinyl leukotrienes have been shown to enhance growth of bronchial epithelial cells in vitro (63).

1.4.3. Neurotrophins and their receptors in the airway epithelial cells

The cellular sources of the neurotrophins in the airways have not been defined; many cells more likely contribute to the synthesizing and releasing neurotrophins, but there is now growing evidence that epithelial cells are a major source of neurotrophins in the lung. Actually there were many works trying to find out the possible role of the airway epithelial cells in the expression of the Neurotrophins and their receptors but most of it has been done over the mouse and few of them was over the human.

One of those studies was the work of Armin Braun and his colleagues in which they have developed a mouse model of chronic and severe ovalbumin (OVA)-induced airway inflammation for this purpose. And they found that the airway epithelium was

thickened and displayed a goblet-cell hyperplasia with a marked mucus production. And also BDNF was detected by using *in situ* hybridization and enzyme-linked immunosorbent assay. And there was a constitutive expression of BDNF messenger RNA (mRNA), it was observed in the respiratory epithelium of sensitized and nonsensitized mouse lungs, these data indicate that in allergic airway inflammation BDNF production is unregulated and immune cells serve as a source of BDNF (11). At the same time Lommatzsch and his colleagues found that the respiratory epithelium was strongly BDNF mRNA positive from trachea up to the bronchioli. In a human study, bronchial biopsies of asthmatic patients revealed strong NGF expression in epithelial cells. However, epithelial NGF expression was observed in either patients were provoked with antigen or not and thus the NGF expression is not likely a result from the acute inflammatory process but rather from the underlying chronic allergic inflammation or simply constitutive in nature. Unfortunately in this study neither biopsies from nonasthmatic controls were included nor was NGF expression quantitatively investigated in biopsies with and without allergen provocation. In a review from the same group, NGF immunolabeling of bronchial epithelium in biopsies from control patients has been described, but so far no other original work supported these findings.

Fox and Pons (36, 77) have been focused over the actual role of the airway epithelial cells in the production of Neurotrophins. Fox has investigated the production of NGF from a human airway epithelial cell line (A549), Under basal conditions, and after treated them with interleukin-1beta (IL-1beta) or tumour necrosis factor-alpha (TNF-alpha) and he found that basal release was significantly augmented in a concentration-dependent manner in cells treated with interleukin-1b (IL-1b) or tumour necrosis factor-a(TNF-a) and inhibited by dexamethasone. And he suggested that NGF released from structural cells may be an important target for the anti-inflammatory effects of steroids in asthma therapy. At the same time, Pons and his colleagues and by doing almost the same work found that the human airway A549 epithelial cells are an important source of NGF. The pro-inflammatory and asthma-associated cytokine interleukin-1b enhances this NGF secretion, whereas the anti-inflammatory glucocorticoid dexamethasone reduces the NGF production triggered by interleukin-1b. And they assumed that this result will add some part in the mechanism of the anti-inflammatory action of glucocorticoids, suppressing NGF oversecretion by airway epithelial cells. These divergences indicate that further studies are necessary to clarify

the epithelial NGF expression in vitro. Also it has to be indicated that A549 cells are a human pulmonary epithelial cancer cell line derived from lung alveolar adenocarcinoma. Whereas these cells are representative of airway epithelial cells in some properties they are nevertheless tumour cells and it is well known that tumours have a modified expression pattern of growth and survival factors. Therefore, it will be necessary to reinvestigate neurotrophin expression in primary cultures of lung epithelia.

2. Objective

Allergic bronchial asthma (BA) is a model of neuro-immune diseases, and is characterized by chronic airway inflammation, development of airway hyperactivity and recurrent reversible airway obstruction. Neurotrophins (NGF, BDNF, NT-3, and NT-4) are important mediators in the immune system and they might be the candidate molecules for orchestrating neuro-immune interaction during chronic airway inflammation.

Animal studies have shown that in allergic airway inflammation neurotrophin production is upregulated, but the cellular sources of the neurotrophins in the airways have not been defined; and many cells could contribute to the synthesizes and release of neurotrophins.

There is now growing evidence that epithelial cells are a major source of neurotrophins in the lung. Actually there have been many attempts and reports to find out the possible role of the airway epithelial cells in the expression of the neurotrophins and their receptors but most of it was done by using human or mouse tumour cells. Two of these studies have shown that human pulmonary epithelial cancer cell line (A549) can produce NGF.

From this point, the aim of the present work was to find out the actual role of the primary cultured murine tracheal epithelial cells in the regulation of neurotrophin production, and as a prerequisite to establish a protocol for isolation and culturing of mouse tracheal epithelial cells. Next, the basal production of neurotrophins by those cells was measured over time. The effect of various pro-inflammatory cytokines (IL-1 β , IL-13, and IL-4) on neurotrophin production by mouse tracheal epithelial cells was investigated.

3. Material and Methods

3.1. Isolation and culturing the mouse tracheal epithelial cells

A special technique was used for isolating and culturing mouse tracheal epithelial cells, which is a modified one of the co-existing protocols, established by Davidson and his colleagues (26, 27), Kumar and his colleagues (59), and also You and his co-workers (112).

3.1.1. Medium preparation

3.1.2. Coating protocol of the insert with Rat Tail Collagen, Type I

3.1.3. Isolation and culturing Protocol

3.1.1. Medium preparation

The following preparation method was used for the composition of medium for collecting, dissociating and culturing the cells.

Collection medium:

- DMEM. F-12 medium. (20 ml)
- Penicillin (100 IU/ml) +streptomycin (100 µg/ml). (0,2 ml)

Dissociation medium:

- Minimum essential medium (MEM) consisted of calcium- and magnesium-free (23 ml)
- Penicillin (60 IU/ml) + streptomycin (60 IU/ml). (0,15 ml)
- 1.4 mg/ml of Pronase. (1,75 ml)
- 0, 1 mg/ml of DNase. (0,25 ml)

Culture medium:

- DMEM. F-12 medium. (23 ml)
- Penicillin (100 IU/ml) +streptomycin (100 µg/ml). (0,25 ml)
- 5% fetal calf serum. (1,75 ml)
- 120 IU/l of insulin. (3 IU)

Culture medium plus:

- Airway epithelial Cell Basal Medium 500ml
- Airway Epithelial Cell Growth Medium:
 - BPE-26 Bovine Pituitary Extract, 100µl (13mg/ml)
 - hEGF-0.25 EGF, human, recombinant, 250µl (0.25 µg / 500 µl)
 - Insulin-2.5 Insulin, bovine, 25 µl (2.5 mg / 500 µl)
 - HC-250 Hydrocortisone, 25 µl (250 µg / 500 µl)
 - Epinephrine-250 Epinephrine, 25 µl (250 µg / 500 µl)
 - T3-3.35 Triiodthyronine, 25 µl (3.35 ng / 500 µl)
 - Transferrin-5 Transferrin, 25 µl (5 mg / 500 µl)
 - RA-50 Retinoic Acid, 25 µl (50 ng / 500 µl)

Reagents were kept in dark.

Ultraser G (USG) Medium:

- DMEM. F-12 Medium. (97 ml)
- Penicillin (100 IU/ml) +streptomycin (100 µg/ml). (1 ml)
- 2% USG serum substitute. (2 ml)

3.1.2. Coating protocol of the insert with Rat Tail Collagen, Type I**Material**

- Rat tail Collagen. Type I (4,74 mg/ml)
- 0,02N Acetic acid (sterile)

For preparing 100 ml stock solution of 0,02N Acetic acid: 2 ml 1N Acetic acid was added to 98 distilled water.

For preparing 1 ml of rat tail collagen type I working solution (200µg/ml solution of Human Placental Collagen, Type I) : 10 µl collagen stock solution (4,74 mg/ml) was taken +990 µl acetic acid 0,2%.

Method

For the Cell culture insert:

The tissue culture insert semi-permeable support membranes were placed in a 24 well plate, and then the insert was coated with 300 µl of collagen working solution in each insert (10 µg /cm). Inserts were then dried in a bio safety cabinet over 24 hours; then washed twice with phosphate-buffered saline (PBS) before use.

For the 24 well culture plate:

The wells were coated with 1 ml of collagen working solution in each insert (10 µg /cm), and twice washed with phosphate-buffered saline (PBS) before use.

3.1.3. Isolation and culturing protocol

Using the cell culture insert:

For the study, BALB C / wild type mice of both sexes were used, from Harlan Winkelmann GmbH, and followed the precautions of the German law for protection and usage of animals for a scientific research.

The experiment was started by sacrificing 10 mice for every experiment and then briefly immersed those dead mice in 70% ethanol. Then the tracheas were isolated from the larynx to the bronchial main branches (in a sterile technique). Every five tracheas were collected in 50 ml plastic tube containing ice cold-PBS medium in a tissue culture hood, the muscles and vascular tissues from tracheas in PBS cold medium were dissected, and then the tracheas were washed with PBS medium, opened them longitudinally and washed in PBS, after that these opened tracheas were transferred to the collection medium pre-warmed at 37°C for 10 minutes. Then the pieces of five tracheas to 20 ml of pre-warmed dissociation medium containing Pronase and DNase were transferred and incubated at 37°C for 120 minutes, after that 5 ml of sterile fetal calf serum were added to stop the enzymatic digestion.

In order to dissociate epithelial cells, the trachea were gently agitated (the tube containing the tracheae was carefully inverted 12 times, without shaking). The tracheal debris's were removed from the suspension, placed in 10 ml of culture medium, and gently agitated 10 times as before to release more epithelial cells. The resultant cell suspensions were pooled and centrifuged at 1,000 rpm (120 g) for 5 min at room temperature (21-23°C). Then supernatants were removed, the pellets were resuspended and washed in 10 ml of culture medium then centrifuged at 1,000 rpm for 5 min, and resuspended in 5 ml of culture medium. The suspension was incubated

at 37°C for 4 h in a 100-mm culture dish to remove contaminating nonepithelial cells. The medium was removed with a fine-tip flexible pipette to collect the unattached cells centrifuged the collected medium at 1,000 rpm for 5 minutes, resuspended the resultant sediments cells in culture medium plus (200 µl per trachea) and counted. The dissociated cells from two tracheae ($\sim 4 - 5 \times 10^5$ cells) were seeded onto one tissue culture insert semi permeable support membrane in 300 µl of culture medium plus, with 600µl outside the insert. Then those cultured cells were incubated at 37°C in 5% CO₂ in a humidified incubator for 4 – 5 days.

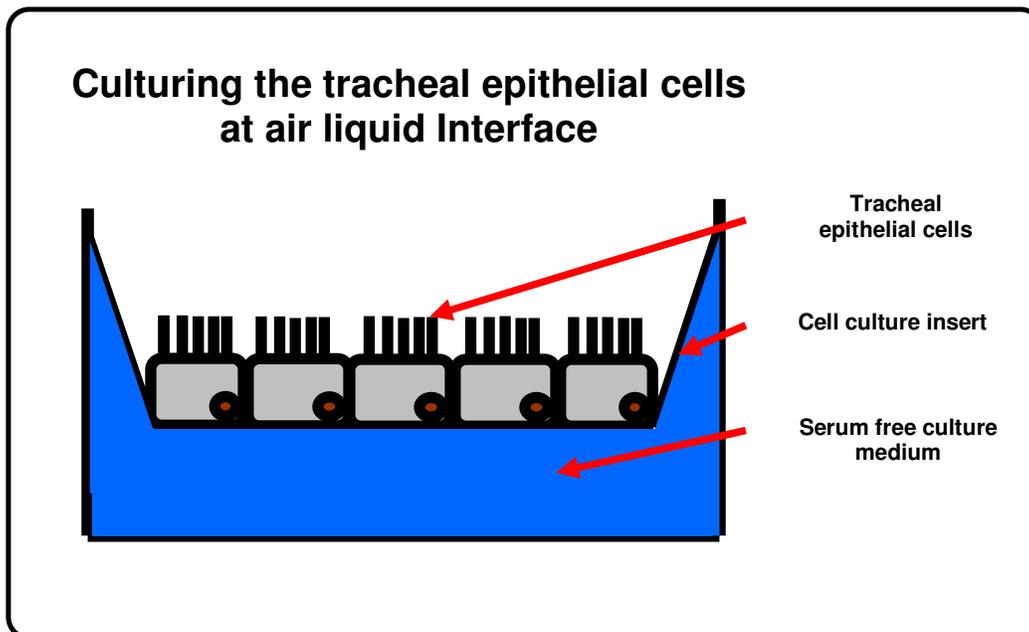


Figure 3: The basic principle of the air-liquid interface: The porous bottom of the insert provides independent access to both sides of a cell monolayer giving researchers a versatile tool to study cell transport and other metabolic activities in vitro

And after 4 – 5 days the medium were removed on the apical surface of the cultured cells along with any nonadherent cells and debris offered an air-liquid interface as seen in figure (1). Then the medium on the outside of the insert (bathing the basolateral surface) is replaced with 600 µl of Ultrosor G (USG) medium and once the cells had reached confluence, the apical surface of the insert appeared dry; this normally occurred from day 5 onward. After that the medium bathing the basolateral surface is replaced twice weekly. And the primary cultures grown at an air-liquid interface remained viable for up to 40 days.

Using the 24 well normal culture plates:

The same isolation and preparation procedures were performed as before, until the step of culturing cells over the pre-coated well plate, from then onward the following procedures were used:

The dissociated cells from four tracheas ($\sim 1 \times 10^6$ cells) (or the LA4 cells) were seeded onto one well of the 24 well culture plate along with 1 ml of the culture medium plus , and those cultured cells were incubated at 37°C in 5% CO₂ in a humidified incubator for 4 – 5 days.

After the cells had reached confluence (from day 5 onward), the medium was changed with a new serum free medium (USG) in order to feed the cells, and this step was repeated every two days as long as we needed the cells.

3.2. Identification of cultured cells.

Protocol for staining Tracheal Epithelial Cells by immunohistochemistry:

The primary cells were stained (Mouse tracheal epithelial cells) and also LA4 (airway epithelial tumour cell line) were used as positive control.

3.2.1. Preparation of working solutions:

- Biotinylated Antibody:

Three (3) drops (150 µl) of normal blocking serum stock (yellow label) were added to 10 ml buffer in mixing bottle and then added one (1) drop (50 µl) of biotinylated antibody stock (blue label).

- Vectastain® ABC Reagent:

Exactly two (2) drops of reagent A (gray label) were added to 5 ml of buffer in the ABC Reagent large mixing bottle. Then exactly two (2) drops of REAGENT B (gray label) were added to the same mixing bottle, mixed immediately, and let Vectastain® ABC Reagent to stand for about 30 minutes before use.

- Blocking Serum (Normal Serum):

Three (3) drops (150 µl) of stock (yellow label) were added to 10 ml of buffer in mixing bottle (yellow label). The preferred serum for blocking was prepared from the same species in which the biotinylated secondary antibody was made

3.2.2. Staining Procedures:

- The frozen slides were removed from the freezer and allowed it to come to the room temperature in order to dry the slides.
- After that the fixation step were made by leaving the slides 10 minutes in -20 c acetone, and then dried those slides.
- All slides were labelled by using secure line pen, and then rinsed the slides 2-3 times in PBS for 2-5 min.
- Then the excess buffer was wiped from around the specimen.

From here onward all steps in humid chamber were performed.

- 1 drop of the hemogen peroxidas blocking solution were added for 10 min.
- After that the slides were rinsed 2-3 times in PBS, and blocked with 5%normal serum (goat) (in PBS+3%BSA) for 60 min.
- Then enough diluted primary antibody (monoclonal anti-pan cytokeratin) 1:100 (diluted with PBS+3%BSA) was applied, covered tissue sections on slide and incubated over night at 4 °C.
- The slides were rinsed 2-3 times in PBS for 2-5 minutes, and then incubated all sections for 30 minutes with diluted biotinylated secondary antibody solution.
- The slides were washed for 5 minutes in buffer.
- Then the sections were incubated in peroxidase substrate solution until the desired stain intensity developed. The substrate was prepared immediately before use, and the preparation of the substrate solution was as follows.
- 2 drops of Buffer Stock Solution was added to 5.0 ml of distilled water and mixed very well. Then 4 drops of DAB Stock Solution was added and mixed well. After , 2 drops of the Hydrogen Peroxide Solution was added and also mixed very well. After finishing the preparation of the substrate solution the tissue sections were incubated with this substrate solution at room temperature until a suitable staining developed. Actually the development time was determined by us but generally 2-10 minutes was enough time for providing a good staining intensity. Then all sections were washed for 5 minutes in water.
- The last step in our staining protocol was the counter stain step by using the Vector® Hematoxylin QS kit and it was as follow:
 - First of all the slides were rinsed in tap water.

- And then the slides were immersed in Vector® Hematoxylin QS counter stain or the counter stain was applied directly to the slide. The tissue sections were covered completely.
- Then all sections were incubated with Vector® Hematoxylin QS for 5-45 seconds. Later rinsed with the running tap water until the rinse water was colourless or put under water for approximately 10 seconds.
- The slides were transferred into the different upgraded dilution ethanol bath starting with 70% up to 100% and xylol as in normal stain. All sections were mounted in aqueous or permanent mounting media as per standard protocols.

3.3. Detection of tracheal epithelial cells NGF and BDNF by PCR

3.3.1. Isolation of RNA from the primary cultured cells

3.3.2. DNA-Digestion.

3.3.3. First strand cDNA synthesis.

3.3.4. PCR.

3.3.1. Isolation RNA from the primary cultured cells

Using materials:

RNeasy® Mini Kit (QIAGEN Cat. No.74103).

Medium preparations:

RBE Puffer + 4 Volumes Ethanol (96 – 100%): (RBE Puffer working solution)

1000µl RLT + 10 µl Beta-Mercaptoethanol (RLT/Mercapto working solution)

Procedures:

Cell pellet in 350 µl of RLT/Mercapto ($\leq 5 \times 10^6$ cells) or 600 µl ($\leq 1 \times 10^6$ cells) were used. And then one volume of 70% Ethanol were added to the cell suspension and mixed. Then up to 700 µl of the sample to RNeasy mini column was applied placed in a 2 ml collection tubes. The tub gently closed and centrifuge for 15 S. at 10000 rpm, discarded the flowthrough. Then 700 µl Buffer RW1 were added to the RNeasy column, closed the tub gently and centrifuge for 15 S. at 10000 rpm. The flowthrough was discarded, and the RNeasy column was transferred into a new 2 ml collection tube. Then 500 µl Buffer RPE was added to the RNeasy column, and

closed the tub gently and centrifuged for 2 min. at 10000 rpm. The flowthrough was discarded, and then the tub is centrifuged for 1 min. at 13000 rpm. After that the column was placed in a new 2 ml collection tube and discarded the old collection tube with the flowthrough. The tub was centrifuged in a micro centrifuge at full speed for one minute. Then 30 – 50 RNase – Free water was added directly onto the RNeasy silica – gel membrane. The tub was closed gently and centrifuged for 1 min. at 10000 rpm, and then stored the resulting sample at -80 °C.

3.3.2. DNA-Digestion.

Using material:

DNA-free from Ambion (Cat. No.1906)

Procedure:

Sample preparation: the following materials were mixed gently together

- 25 µl RNA
- 2,5 µl 10x DNase I puffer
- 1 µl rDnase I

After that the mixture was incubated for 20 min. at 37°C., added 5 µl DNase inactivation reagents, and incubated for 2 minutes at room temperature with light mixing. After that the mixture was centrifuged for 2 minutes at 10000 g at 4 °C. Then the flowthrough was transferred to a new eppendorf tube.

3.3.3. First strand cDNA synthesis:

Using materials:

- SuperscriptTMII RT (Invitrogen, Cat. No. 18064-014)
- RNaseOUTTM (Invitrogen, Cat. No. 10777-019)
- 10 mM dNTP Mix, PCR Grade (Invitrogen Cat. No. 18427-013)
- Random Primers (Invitrogen Cat. No. 48190-011)

Procedure:

The 0,5 ml Nuclease –free micro centrifuge tube was used in my procedure and the following components were added to the tub:

- 0,5 µl Random Primers (Diluted 1:30)
- 0,5 µl 10 mM dNTP Mix
- RNA
- Sterile distilled water to reach total volume 6 µl.

The mixture were heated to 65°C for 5 min. and then quick chilled on ice followed by brief centrifugation. After that the following materials were added:

- 2 µl 5X puffer
- 1 µl 0,1M DTT
- 0,5 µl RNaseOUT

The content of the tube was mixed gently, and then 1 µl of Superscript™II RT was added in every tube but not the –RT and mixed gently by pipetting up and down. Then the mixture was incubated for 10 minutes at 25°C., and after that for another 50 minutes incubation time at 42°C. At the end the reaction was activated by heating at 72°C for 15 minutes. The resultant DNA can be used as a template for amplification in PCR.

3.3.4. PCR:**Material:**

- REDTaq™ ReadyMix™ PCR Reaction Mix With Mgcl2
- SIGMA – ALDRICH (Cat. No. R 2523)

Procedure:

the following reagents were added to 0,5 ml PCR tube:

- 25 µl 2X REDTag ReadyMix
- 1µl Forward primer
- 1 µl Reverse primer
- X µl Template DNA
- Sterile distilled water to reach total volume 50 µl

The mixture was mixed gently and briefly centrifuged. After that 50 µl mineral oil was added to the top of each tube. Then the amplification of 20 -30 cycles was made by thermocycler in this order:

- 1 min. at 94°C
- 2 min. at 55°C
- 3 min. at 65°C

At the end the amplification DNA was added to the Agarose gel.

3.4. Measuring the Neurotrophin basal production by the primary cultured mouse tracheal epithelial cells.

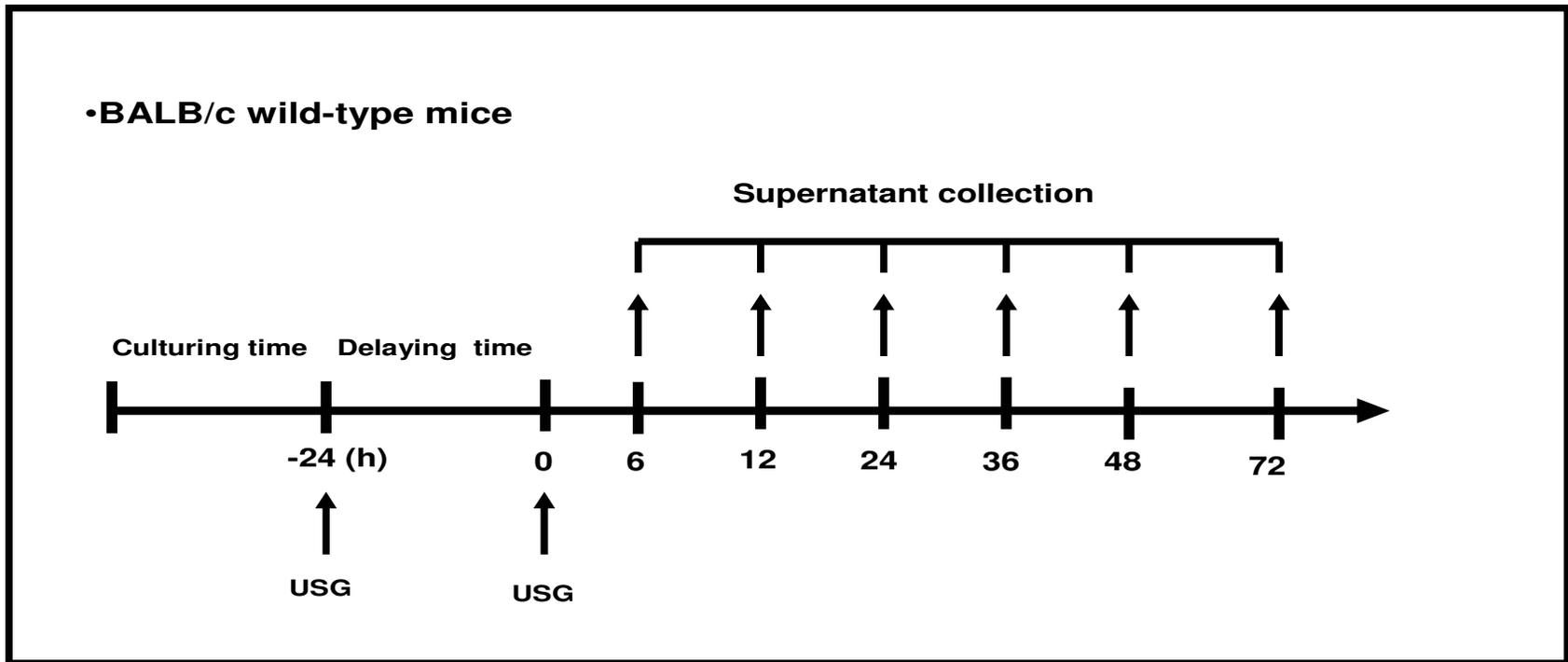
3.4.1. Collecting Protocol

3.4.2. ELISA measurement procedures

3.4.1. Collecting Protocol

After culturing the tracheal epithelial cells for four to five days, the medium on the apical surface of the cultured cells along with any nonadherent cells and debris were removed, and also the medium on the outside of the insert (bathing the basolateral surface) were replaced with 600 µl of Ultrosor G (USG) medium and once the cells had reached a confluent state, the apical surface of the insert appeared dry; this normally occurred from day 5 onward. After that the medium on the outside of the insert (bathing the basolateral surface) were removed with a new 600 µl of Ultrosor G (USG) medium and left for 24 hours. (Delaying time for 24 hours). Then the medium was changed with new serum free medium. (USG), and the mediums were collected after 6h, 12h, 18h, 24h, 48h, and 72h, as shown in the protocol for this step (tab.1) . Normal USG medium were used as a blank.

The same collecting protocol were applied but by using LA 4 cell line and primary tracheal epithelial cells which have been cultured onto normal 24 well culture plates pre-coated with rat tail collagen type I, for comparison reasons.



Tab.1. Collecting Protocol of the supernatant medium after 6h, 12h, 18h, 24h, 48h, and 72h . USG is the Ultraser G (serum). .)free medium)

3.4.2. ELISA measurement procedures:

All measurements were performed in duplicates and under the same circumstances. They were performed for three times and by using new primary epithelial cells at every time. The ELISA Kits used for measuring the NGF, BDNF, NT-3, and NT-4 were from Promega, and the ELISA reader was from Tecan (Tecan Sunrise).

Medium preparation:

1N hydrochloric acid

- 82.7ml concentrated hydrochloric acid
- 917.3ml deionized water.
- Carbonate coating buffer
- 0.025M sodium bicarbonate
- 0.025M sodium carbonate
- The pH adjusted to 9.7 (± 0.1) by using 1N HCl or 1N NaOH.

Lysis buffer

- 137mM NaCl
- 20mM Tris-HCl (pH 8.0)
- 1% NP40
- 10% glycerol
- 1mM PMSF
- 10 μ g/ml aprotinin
- 1 μ g/ml leupeptin
- 0.5mM sodium vanadate

DPBS (per liter)

- 0.2g KCl
- 8.0g NaCl
- 0.2g KH₂PO₄
- 1.15g Na₂HPO₄
- 100mg MgCl₂ • 6H₂O
- 133mg CaCl₂ • 2H₂O

Deionized water was added at room temperature to KCl, NaCl, KH₂PO₄, and Na₂HPO₄ to a final volume of one litre. The PH was adjusted to 7.35 by using 1N

HCl or 1N NaOH. Then the $MgCl_2 \cdot 6H_2O$ were added; and mixed thoroughly. At the end the $CaCl_2 \cdot 2H_2O$ was added and mixed thoroughly.

ELISA procedure:

The NGF, BDNF, NT-3, Nt-4 ELISA kits from Promega in our measurements was used and in principle they have almost the same procedures with some differences.

○ **Plate Coating**

- Exactly 2 μ l of the Anti-NGF pAb was added to 12.5ml of carbonate coating buffer which is enough for 96-well plate (NGF ELISA measurements).
- Exactly 10 μ l of the Anti-BDNF mAb was added to 10 ml of carbonate coating buffer which is enough for 96-well plate (BDNF ELISA measurements)
- Exactly 20 μ l of the Anti-Human NT-3 pAb was added to 10 ml of carbonate coating buffer which is enough for 96-well plate (NT-3 ELISA measurements)
- Exactly 40 μ l of the Anti-Human NT-4 pAb were added to 9.96 ml of carbonate coating buffer which is enough for 96-well plate (NT-4 ELISA measurements) .
- Then thoroughly mixed, and avoiding creating excess bubbles.
- After that 100 μ l were added to each well of a polystyrene ELISA plate.
- The wells were sealed with a plate sealer and incubated the whole plate overnight at 4°C.

○ **Preparing Block & Sample 1X Buffer**

For NGF, NT-3, NT-4 measurements:

43ml of Block & Sample 1X buffer for 96-well plate were prepared to be used on the second day. And to prepare Block & Sample 1X Buffer, 34.4ml of deionized water was added to 8.6ml of Block & Sample 5X Buffer, and then gently mixed and completely by inversion prior to use.

For BDNF measurements:

53ml of Block & Sample 1X Buffer for 96-well plate were prepared to be used on the second day. And to prepare Block & Sample 1X Buffer, I 42.4 ml of deionized water was added to 10.6 ml of Block & Sample 5X Buffer, and then gently mixed and completely by inversion prior to use.

- **Blocking the Plate**
 - The coated plate was removed from the refrigerator.
 - Then the contents of the wells were flicked out and slapped the plate upside down three times on a paper towel.
 - All wells were vigorously washed with TBST wash buffer.
 - Then 200µl of Block & Sample 1X Buffer were added to each well, after that the plate was incubated at room temperature for one hour without shaking.

- **Preparing the standard curve**

For NGF, BDNF:

The standard which was provided with this system will generate a linear standard curve from 7.8–500pg/ml. values only within the linear range were used to determine the NGF, BDNF concentration of the test samples. The NGF, BDNF standard were supplied at a concentration of 1µg/ml.

- Accurately the supplied NGF, BDNF Standard 1:2,000 in Block & Sample 1X Buffer were diluted to achieve a concentration of 500pg/ml.
- Following plate blocking, the contents of the wells were flicked out over a sink. And then the plate three times upside down slapped on a paper towel.
- Then washed once with TBST wash buffer.
- Two columns of wells (16 wells) for the standard curve designated.
- Then 100µl/well of the Block & Sample 1X Buffer was added to wells in rows B through H in the two columns designated for the standard curve.
- 200µl of the diluted NGF, BDNF Standard (500pg/ml) were added to the first well in each column designated for the standard curve.
- Then serial 1:2 dilutions (100µl/well) down the plate in the columns designated for the standard curve were immediately performed. In the last set of wells for the standard curve, no NGF, BDNF (control) were added. The final concentrations (in duplicate) within the plate will be 0–500pg/ml

For NT- 3, NT- 4:

The standard which was provided with this system will generate a linear standard curve from 4,7 –300pg/ml. Use only values that are within the linear range to

determine the NT- 3, NT- 4 concentration of the test samples. The NT- 3, NT- 4 standard were supplied at a concentration of 0, 6 µg/ml.

- Accurately the supplied NT- 3, NT- 4 Standard 1:2,000 in Block & Sample 1X Buffer were diluted to achieve a concentration of 300pg/ml.
- Following plate blocking, the contents of the wells were flicked out over a sink. And then I slapped the plate three times upside down on a paper towel.
- Then washed once with TBST wash buffer.
- Two columns of wells (16 wells) for the standard curve were designated.
- Then 100µl/well of the Block & Sample 1X Buffer was added to wells in rows B through H in the two columns designated for the standard curve.
- 200µl of the diluted NT- 3, NT- 4 Standard (300pg/ml) were added to the first well in each column designated for the standard curve.
- Then serial 1:2 dilutions (100µl/well) down the plate in the columns designated for the standard curve were immediately performed. In the last set of wells for the standard curve, no NT- 3; NT- 4 (control) were added. The final concentrations (in duplicate) within the plate will be 0–300pg/ml.

○ **Addition of Sample**

After preparing the NGF, BDNF, NT- 3, NT- 4 Standard curves, 100µl of the samples were added to each of the remaining wells. Then sealed the wells with a 96-well plate sealer and incubated the plate for six hours at room temperature with shaking (500 ± 100rpm). All wells were washed five times with TBST wash buffer.

○ **Addition of the anti – neurotrophines Ab**

For the NGF measurements:

2.5µl of the Anti-NGF mAb were added to 10ml of Block & Sample 1X Buffer (1:4,000 dilutions) to prepare enough reagents for a full 96-well plate.

For BDNF measurements:

20 µl of the Anti-Human BDNF pAb were added to 10 ml of Block & Sample 1X Buffer (1:5,000 dilutions) to prepare enough reagents for a full 96-well plate.

For NT – 3 measurements:

2.5 µl of the Anti-NT-3 mAb were added to 10 ml of Block & Sample 1X Buffer (1:4,000 dilutions) to prepare enough reagents for a full 96-well plate.

For NT – 4 measurements:

2 µl of the Anti-NT-4 mAb were added to 10 ml of Block & Sample 1X Buffer (1:5,000 dilutions) to prepare enough reagents for a full 96-well plate. And then those following steps were done:

Thoroughly mixing. And 100µl of the diluted Anti-neurotrophines Ab were added, to each well. Then the wells were sealed with a plate sealer and incubate overnight at 4°C without shaking. The next day, all wells were washed five times with TBST wash buffer without shaking.

○ **Addition of Anti-Rat IgG, HRP Conjugate**

Fresh 10ml working solution of Block & Sample 1X Buffer were prepared by combining 8ml of deionized water and 2ml of Block & Sample 5X Buffer. Then gently and completely mixed by inversion prior to use. 100µl of the stock Anti-Rat IgG, HRP Conjugate were added to 9.9ml of Block & Sample 1X Buffer (1:100 dilution) , and mixed thoroughly ,Then 100µl of the diluted Antibody Conjugate were added to each well. Then the mixture was incubated for 2.5 hours at room temperature with shaking (500 ± 100 rpm). All wells were washed five times with TBST wash buffer.

○ **Colour Development**

100µl of TMB One Solution were added to each well (at the room temperature). Then the mixture was incubated at room temperature with shaking for 10 minutes. After that the reaction was stopped by adding 100µl of 1N hydrochloric acid to the wells in the same order in which TMB One Solution was added in Step 1 above. The blue colour will change to yellow upon acidification. At the end the absorbance at 450nm on a plate reader within 30 minutes of stopping the reaction were recorded.

3.5. Measuring the Neurotrophin production after stimulating the primary cells by different types of cytokines.

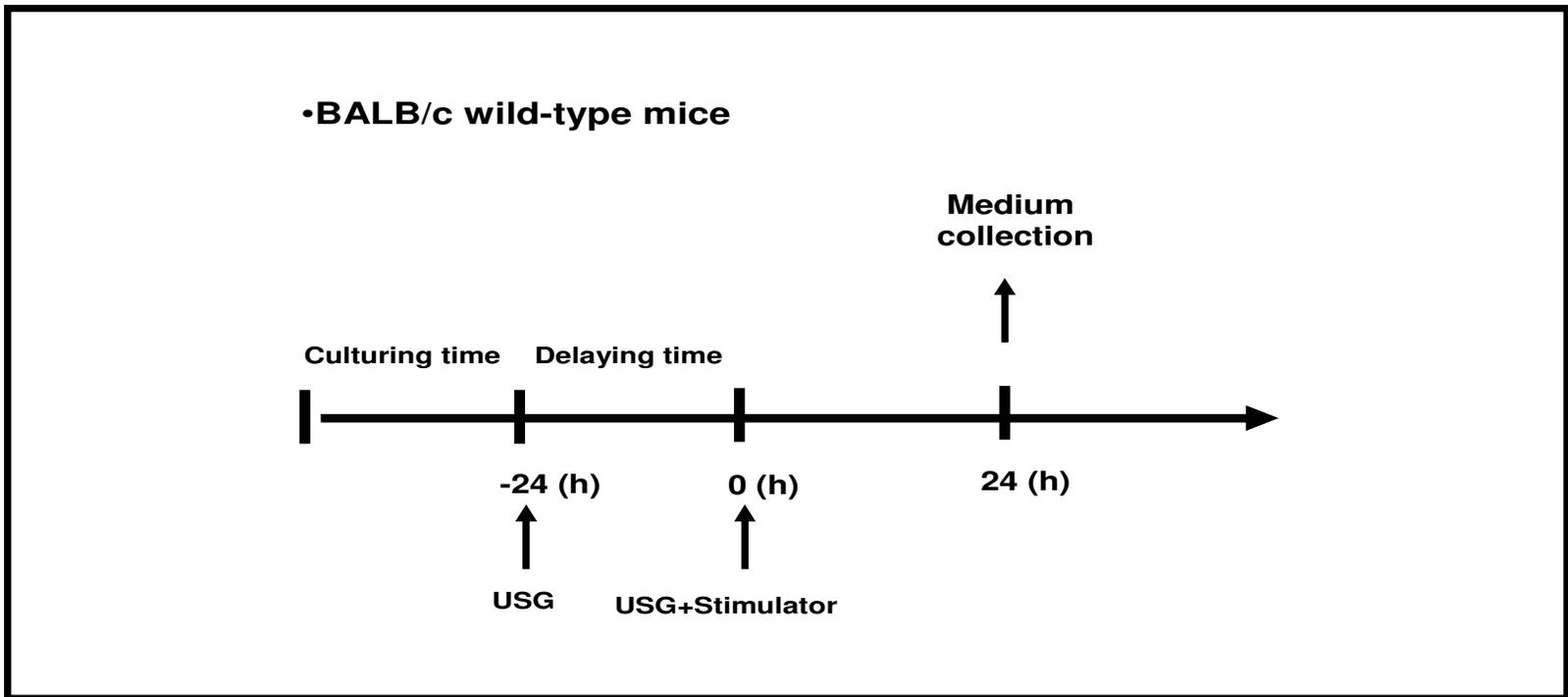
3.5.1. Collecting Protocol:

After culturing the tracheal epithelial cells for four to five days, the medium on the apical surface of the cultured cells along with any nonadherent cells and debris were removed, and also the medium on the outside of the insert (bathing the basolateral surface) was replaced with 600 µl of Ultrosor G (USG) medium and once the cells

had reached confluence state, the apical surface of the insert appeared dry; this normally occurred from day 5 onward. And then the medium on the outside of the insert (bathing the basolateral surface) with a new 600 μ l of Ultrosor G (USG) medium were removed and for 24 hours (starvation time for 24 hours) was left. Then the medium with new serum free medium + different concentrations of different cytokines were changed in this order:

- IL1 β : 0.1, 1, 10 ng/ml
- IL4: 0.1, 1, 10 ng/ml
- IL13: 0.1, 1, 10 ng/ml

After 24h the medium was collected, as shown in the protocol for this step (Tab.2). Normal USG medium was used as a blank. 24 hours incubated USG medium was used as an indicator for basal production. The same collecting protocol was applied but by using LA 4 cell line and primary tracheal epithelial cells which have been cultured onto normal 24 well culture plate pre-coated with rat tail collagen type I, to be sure that our stimulators are working and also for comparing reasons.



Tab.2.Collecting Protocol of the supernatant medium 24h, incubation time. USG is the Ultraser G (serum free medium)

3.5. 2. ELISA measurement procedures:

All the measurement was done by the same way as used for the basal production and the USG medium as a blank was used, and also the USG which was collected after 24 hours incubation time was used as a standard to find out the behaviour of the neurotrophin production by the primary cultured tracheal epithelial cells after stimulating those cells by different types of cytokines.

3.6. Measuring the Neurotrophin production by LA 4 cell line after stimulation with different types of cytokines

The same collecting protocol and measuring procedures but the LA 4 cell line was used as a cultured and test cells

3.7 The list of using materials

- DMEM. F-12 Medium

SIGMA – ALDRICH (Saint Louis, Missouri, USA)

Cat. No. D6434.

- Minimum Essential Medium(MEM)

SIGMA – ALDRICH (Saint Louis, Missouri, USA)

Cat. No. M8167.

- Pronase

Roche Diagnostics GmbH (Roche Applied Science, Mannheim, Germany)

Cat. No. 165 921 (1g).

- DNase I

Roche Diagnostics GmbH (Roche Applied Science, Mannheim, Germany)

Cat. No. 1 284 932

- Rat tail Collagen, Type I

BD Biosciences (two Oak Park, Bedford, MA 01730)

Cat. No. 354236

- Supplement Pack / Airway Epithelial Cell Growth Medium

PromoCell GmbH (PromoCell GmbH, Heidelberg, Germany)

Cat. No. C-39160

Contains:

- Bovine Pituitary Extract, 100µl (13mg/ml)

- EGF, human, recombinant, 250µl (0.25 µg / 500 µl)
- Insulin, bovine, 25 µl (2.5 mg / 500 µl)
- Hydrocortisone, 25 µl (250 µg / 500 µl)
- Epinephrine, 25 µl (250 µg / 500 µl)
- Triiodthyronine, 25 µl (3.35 ng / 500 µl)
- Transferrin, 25 µl(5 mg / 500 µl)
- Retinoic Acid, 25 µl (50 ng / 500 µl)
- Airway epithelial Cell Basal Medium

PromoCell GmbH (PromoCell GmbH, Heidelberg, Germany)

Cat. No. C-21260

- Ultrosor G (USG)

Ciphergen Biosystem, INC. (BioSeprra S.A.Cergy-Christophe, France)

Cat. No. 292040

- Recombinant Human Epidermal Growth factor (hEGF)

BD Biosciences (two Oak Park, Bedford, MA 01730)

Cat. No. 354052

- Monoclonal Anti-pan Cytokeratin (Mixture)

SIGMA – ALDRICH (Saint Louis, Missouri, USA)

Cat. No. C2562

- Vectastain ABC Kit

Victor Laboratories (Vector Laboratories, INC. Burlingame, CA, USA)

Cat. No. PK- 6100

- Hematoxylin QS Nuclear Counter Stain

Vector Laboratories (Vector Laboratories, INC. Burlingame, CA, USA)

Cat. No. H- 3404

- Product Avidin / Biotin Blocking Kit

Vector Laboratories (Vector Laboratories, INC. Burlingame, CA, USA)

Cat. No. SP- 2001

- DAB Substrate Kit For Peroxidase

Vector Laboratories (Vector Laboratories, INC. Burlingame, CA, USA)

Cat. No. SK- 4100

- Biotinylated Anti-Mouse IgG (H+L)

Vector Laboratoires (Vector Laboratoires, INC. Burlingame, CA, USA)

Cat. No. BA- 9200

- DB Falcon™ Cell Culture Insert

BD Biosciences (two Oak Park, Bedford, MA 01730)

Cat. No. 353095

- BD Falcon™ Cell Culture Insert Companion Plates

BD Biosciences (two Oak Park, Bedford, MA 01730)

Cat. No. 353504

- Recombinant Mouse IL-1 β

Cell Concepts GmbH (Cell Concepts GmbH, Umkirch, Germany)

Cat. No. C- 2111-BSS

- Recombinant Mouse IL-4

Cell Concepts GmbH (Cell Concepts GmbH, Umkirch, Germany)

Cat. No. C- 21414-SS

- Recombinant Mouse IL-13

Cell Concepts GmbH (Cell Concepts GmbH, Umkirch, Germany)

Cat. No. C- 21013---SS

- NGF Emax® Immunoassay System

Promega Corporation (Madison, WI, USA)

Cat. No. G7630

- BDNF Emax® Immunoassay System

Promega Corporation (Madison, WI, USA)

Cat. No. G7610

- NT-3 Emax® Immunoassay System

Promega Corporation (Madison, WI, USA)

Cat. No. G7640

- NT-4 Emax® Immunoassay System

Promega Corporation (Madison, WI, USA)

Cat. No. G7650

- REDTaq™ ReadyMix™ PCR Reaction Mix With Mgcl₂

SIGMA – ALDRICH (Saint Louis, Missouri, USA)

Cat. No. R 2523

- RNeasy® Mini Kit

GIAGEN (QIAGEN GmbH, Germany)

Cat. No. 74103

- Superscript™ II Reverse Transcriptase
Invitrogen (Invitrogen Corporation, USA)

Cat. No.18064-014

- RNaseOUT™

Invitrogen (Invitrogen Corporation, USA)

Cat. No. 10777-019

- 10m M dNTP Mix, PCR Grade

Invitrogen (Invitrogen Corporation, USA)

Cat. No. 18427-013

- Random Primers

Invitrogen (Invitrogen Corporation, USA)

Cat. No. 48190-011

- DNA – Free™

Ambion (Ambion, Inc. USA)

Cat. No. 1906

- Cell Line Designation: LA 4

ATCC (American Type Culture Collection, VA, USA)

Cat. No.CCL-196

- BALB C / wild type mice.

Harlan Winkelmann GmbH, Borchon, Germany

4. Results

4.1. Isolation and culture of Tracheal epithelial cells using air liquid interface

4.1.1. Isolation step.

This step was made for several times and under different conditions, and the latest protocol was found to be very good for isolating the tracheal epithelial cells; especially after the steps in the field of determination the purity of the cultured cell. Several identification steps were made by staining my cells at different stages:

- directly after isolating those cells from the trachea
- After incubation those cells in a betray dish for 4 hours.

In the first occasion the percentage of the Pan-Cytokeratin positive cells was between 45-50% of the total cells whereas the percentage was over 60% to 75% after 4 hours incubation time in a betray dish.(Fig.4). And this result was due to the attachment of large numbers of the fibroblasts and other non epithelial cells to the surface of the betray dish whereas the tracheal epithelial cells will not attach to it.

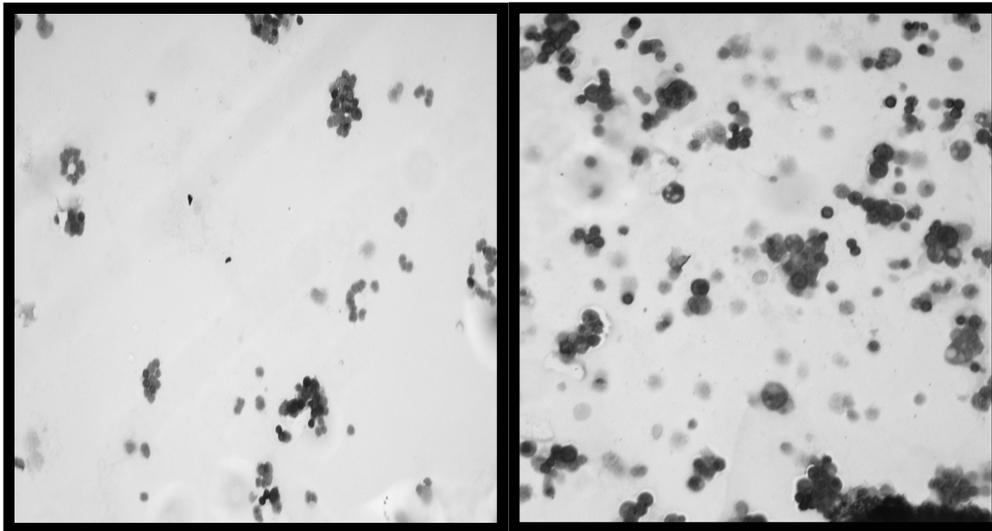


Figure 4: isolated mouse tracheal epithelial cells at different stages stained by Pan-Cytokeratin and viewed by light Olympus Microscope at 10X magnification. Left side: Directly after isolation. Right side: after 4 hour's incubation time

4.1.2. Culturing step.

The cell suspension was added directly to the culture insert and after four to five days incubation time, most of those cells were attached to the insert semi-permeable collagen coated membrane and the remaining cells stayed in the supernatant along with non-adherent cells and debris at the top of the insert. Then the medium was replaced from the outer part of the insert with Serum free (USG) medium. At day 6 all cells reached the confluence state as seen in the picture which was captured directly from the insert Fig. (5).

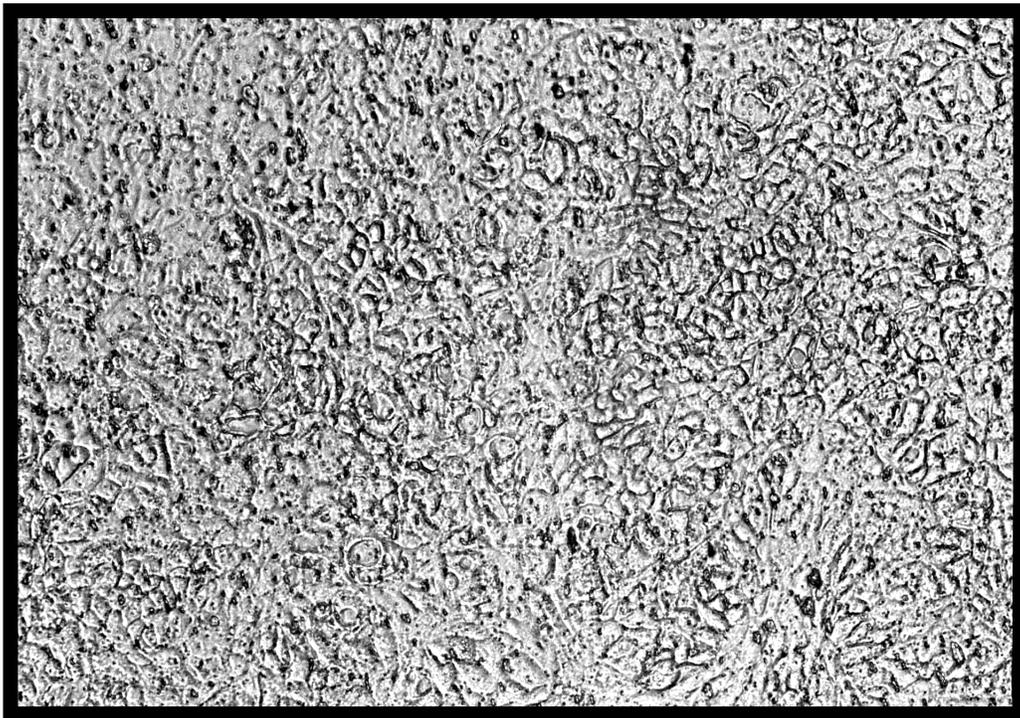


Figure 5, Cultured Tracheal epithelial cells at the air liquid interface (20X magnification by Olympus inverted Microscope)

For determination of neurotrophines production by the tracheal epithelial cells, cells from the second passage were cultured in order to increase the purity of our cultured cells. And that by comparing the percentage of the Pan- Cytokeratin positive cell among the cultured cells at 0 passage and 2nd passage were confirmed, and there was an increase in the purity percentage for more than 15% (at the 0 passage the percentage was between 80 and 85%, whereas at the 2nd passage the percentage was more than 95%).

4.2. Identification of cultured cells

Immuno-histochemical staining was performed with Monoclonal Anti-pan Cytokeratin for those cells which were isolated directly from the insert after reaching the confluent state in two ways:

- by using primary antibody (normal staining protocol)
- Without using the primary antibody (staining control)
- Staining the LA4 cells (positive control).

The result was as following:

- By using the normal staining protocol, more than 95 % of the primary cell were found to be Pancytokeratin positive as shown in Fig.(6) .

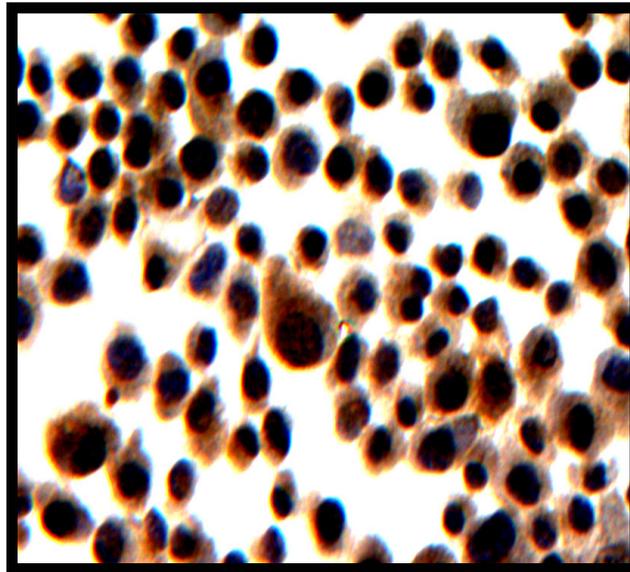


Figure 6: Pancytokeratine positive primary cultured tracheal epithelial cells at 40X magnification

- By using the staining protocol without primary antibody (control for the validation of my staining), all the primary cells were found negative as shown in Fig. (7)

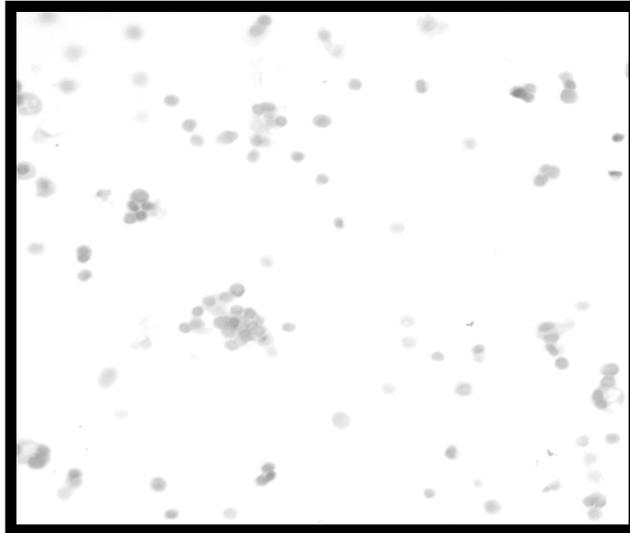


Figure 7: Negative staining of the primary cultured tracheal epithelial cells without using primary antibody (10X Magnification)

- By using the normal staining protocol, more than 95% of the LA 4 cells were found to be Pancytokeratin positive (positive control) as shown in Fig.(8) .

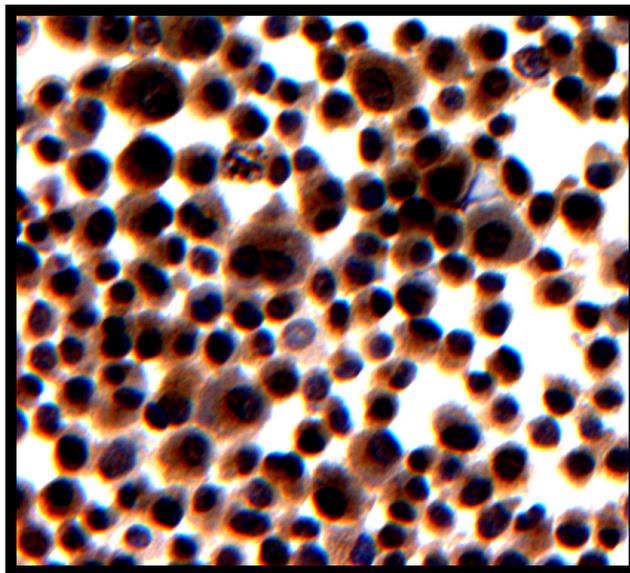


Figure 8: Positive stained LA 4 cells (positive control) (40X magnification)

4.3. RT-PCR detection of tracheal epithelial cells NGF and BDNF

Specific bands after cDNA synthesis from its complementary mRNA encoding for NGF, BDNF were detectable in all samples after reverse transcription as appeared in Fig. 9 and Fig.10. And during the PCR amplification, control reactions were negative for amplification products, demonstrating that the PCR method and reagents which was used yielded specific amplified products only when a cDNA source was included.

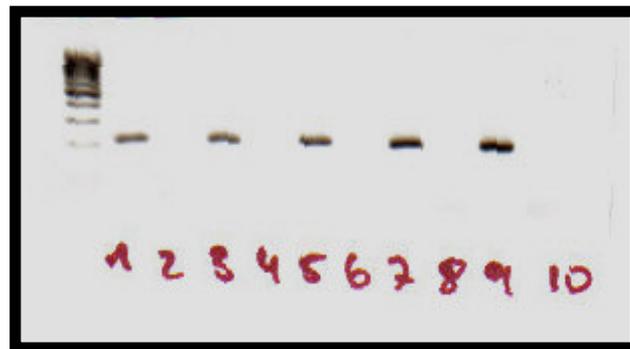


Figure 9: RT-PCR amplicates of the Neurotrophins NGF. Lane 1: positive control. Lane 2: water. Lane 4, 6, 8, 10: (-RT). Lane 3, 5, 7, 9: samples from the primary culture.

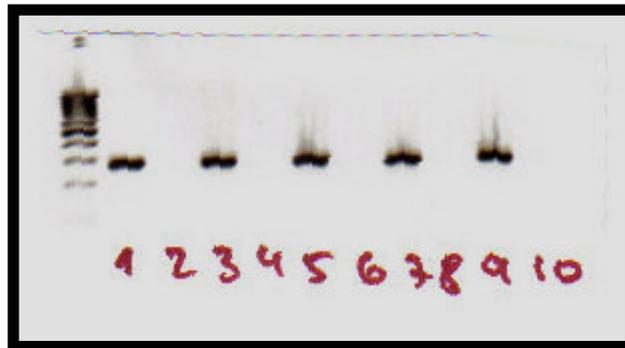


Fig 10: RT-PCR amplicates of the Neurotrophins BDNF. Lane 1: positive control. Lane 2: water. Lane 4, 6, 8, 10: (-RT). Lane 3, 5, 7, 9: samples from the primary culture

4.4. Neurotrophin basal production by the primary cultured mouse tracheal epithelial cells.

All our measurements were made in duplicate and by using Tecan's Sunrise absorbance micro plate reader.

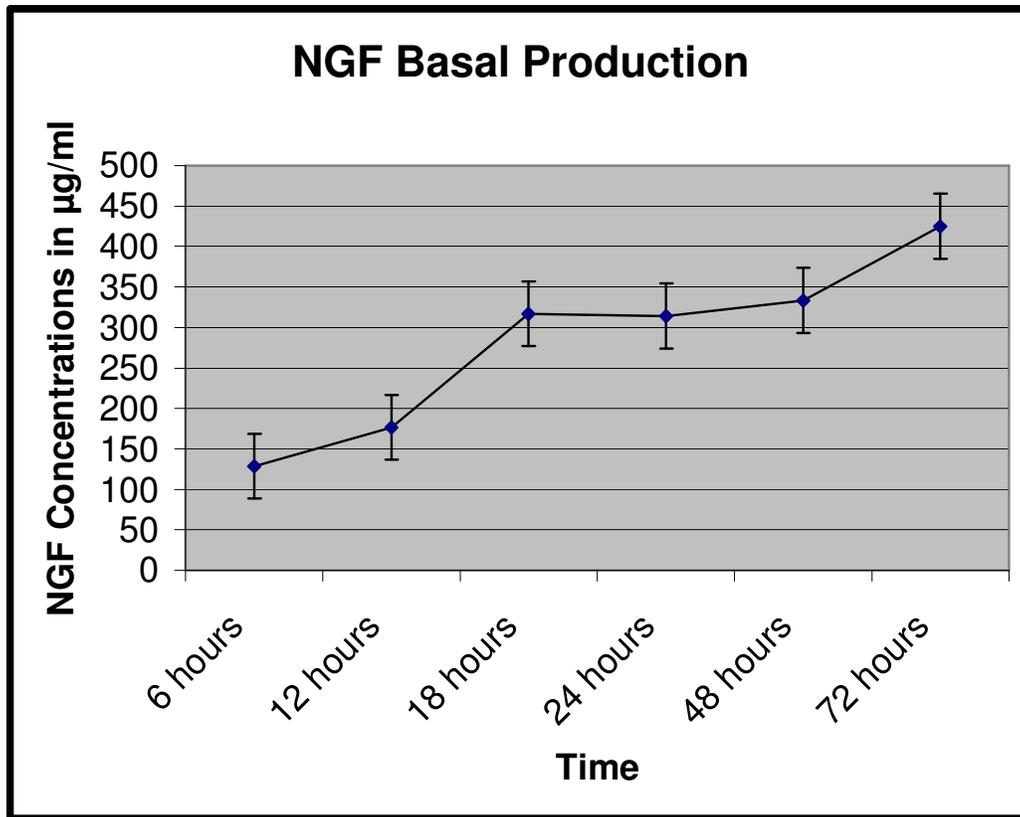
4.4.1. NGF basal production

4.4.2. BDNF basal production

4.4.3. NT3, NT4 basal production

4.4.1. NGF basal production

The measurements of NGF basal production were done several times and the USG medium (serum free medium) as a culture medium was used. The USG medium was tested whether it contains neurotrophines, and at all times were found that there was no presence of any types of neurotrophines by PCR and ELISA. After 6, 12, 18, 24, 48, 72 hours incubation time all culture mediums were collected and ELISA measurements were done in duplicate. And from the values of five different experiments, the basal production was found to be increased over the time starting by 128, 64 ng/ml after 6 hours incubation time to reach 424, 95 ng/ml after 72 hours. And from that result, the NGF production was concluded to be up regulated in a time dependent manner as seen here in Tab.3.

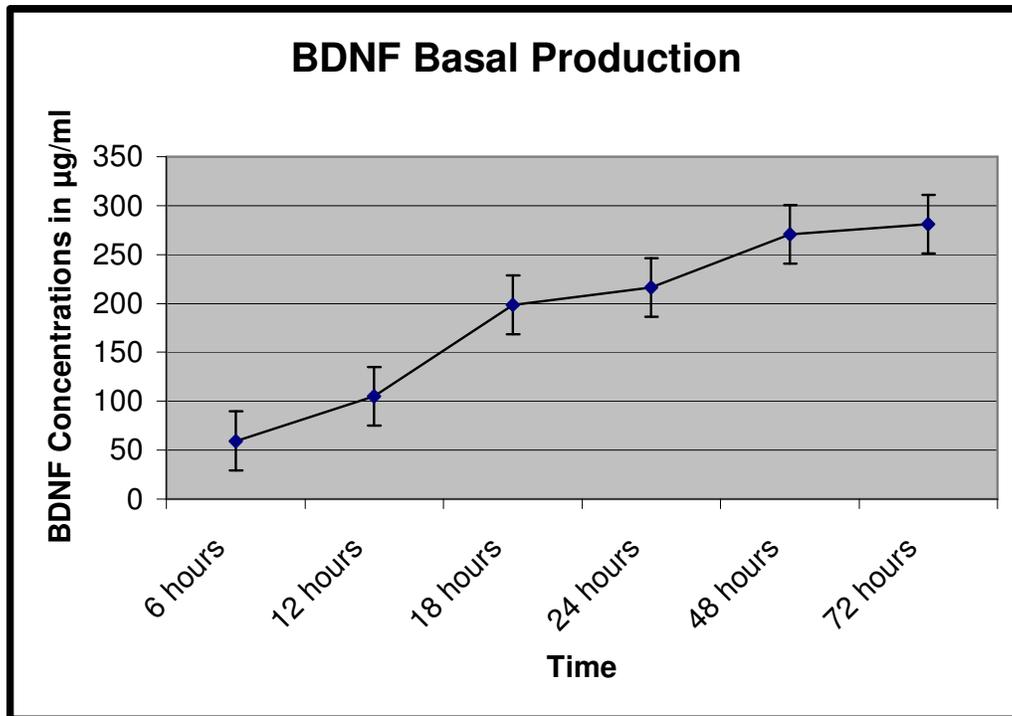


Tab.3: The time-dependent release of nerve growth factor NGF from the primary cultured tracheal epithelial cells. Results are shown as the mean" S.E.M. of five different determinations

4.4.2. BDNF basal production

The same procedures were repeated for BDNF. Basal production after 6, 12, 18, 24, 48, 72 hours incubation time all culture mediums were collected and ELISA measurement were done in duplicate.

And from the values of five different experiments, the basal production was found to be increased over the time starting by 59, 52 ng/ml after 6 hours incubation time to reach 280, 89 ng/ml after 72 hours. And from that result, the BDNF production was concluded to be up regulated in time dependent manner as seen here in Tab.4.



Tab.4: The time-dependent release of Brain derived growth factor BDNF from the primary cultured tracheal epithelial cells. Results are shown

4.4.3. NT3, NT4 basal production

By using the same procedures just to find out if there were any productions of the NT3 or NT4, no productions of NT3, NT4 by the primary cultured tracheal epithelial cells were found.

4.5. Neurotrophin production after stimulating the primary cells by different Types of cytokines

4.5.1. NGF production after stimulation of the epithelial cells by IL1 β , IL4, and IL13 at different concentrations

4.5.2. BDNF production after stimulation of the epithelial cells by IL1 β , IL4, and IL13 at different concentrations

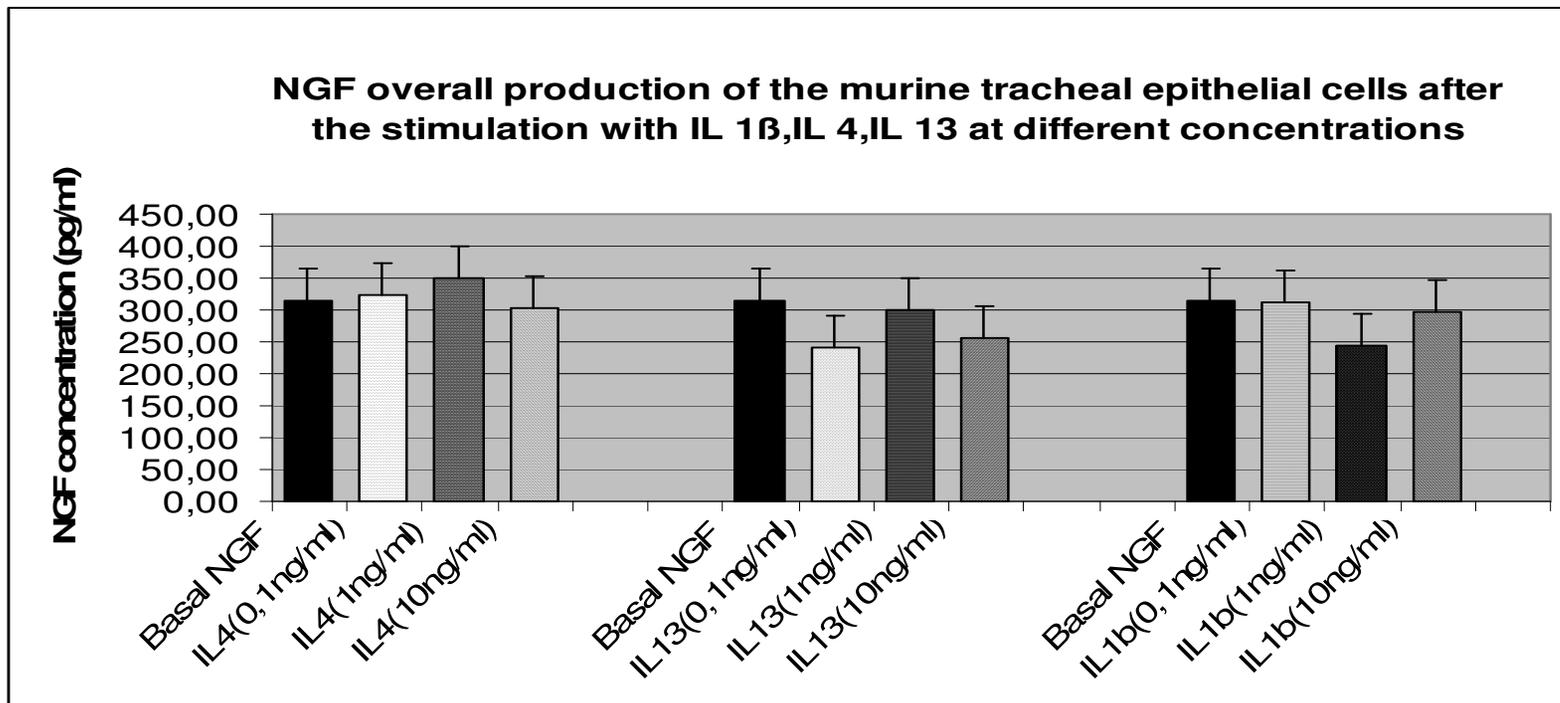
4.5.1. NGF production after stimulation of the epithelial cells by IL-1 β , IL-4, and IL-13 at different concentrations

After replacing the basolateral culture medium, with serum free medium (USG), it was left for 24 hours (Delaying time for 24 hours). The medium with a new serum free medium (USG) + IL-1 β , or IL-4, or IL-13 in different concentrations (0.1, 1, 10 ng/ml) were changed. After 24 hours incubation time the medium again were collected. Then, the NGF measurements were done by using the ELISA techniques.

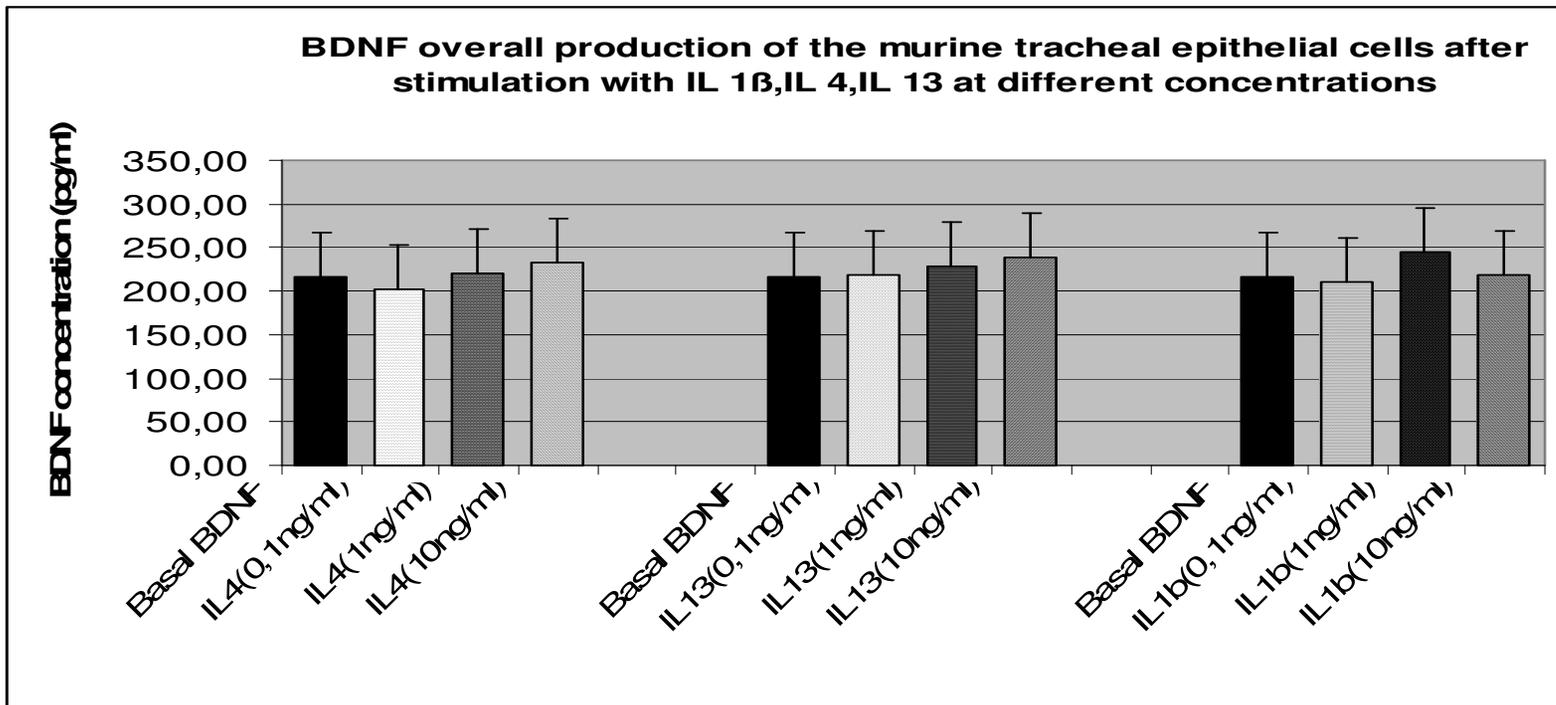
No differences in the production of the NGF by the primary cultured tracheal epithelial cells after 24 hours stimulation with different concentration of IL-1 β , IL-4, and IL-13 were found as seen here in Tab. 5.

4.5.2. BDNF production after stimulation of the epithelial cells by IL-1 β , IL-4, and IL-13 at different concentrations

The same procedures to measure the production of the BDNF by the primary cultured tracheal epithelial cells were performed, and no differences in the production of the BDNF by the primary cultured tracheal epithelial cells were found, after 24 hours stimulation with IL-1 β , IL-4, IL-13 in different concentrations (0.1, 1, 10 ng /ml), as seen here in Tab.6.



Tab. 5: the overall production of NGF by the primary cultured tracheal epithelial cells after stimulating those cells with IL1 β , IL4, and IL13 At different concentrations.



Tab. 6: the overall production of BDNF by the primary cultured tracheal epithelial cells after stimulating those cells with IL 1 β , IL 4, and IL13 at Different concentrations

4.6. Neurotrophin production by LA 4 cell line after stimulating by different types of cytokines

4.6.1. NGF production after stimulation the LA 4 cell line by IL1 β , IL4, and IL13 at different concentrations

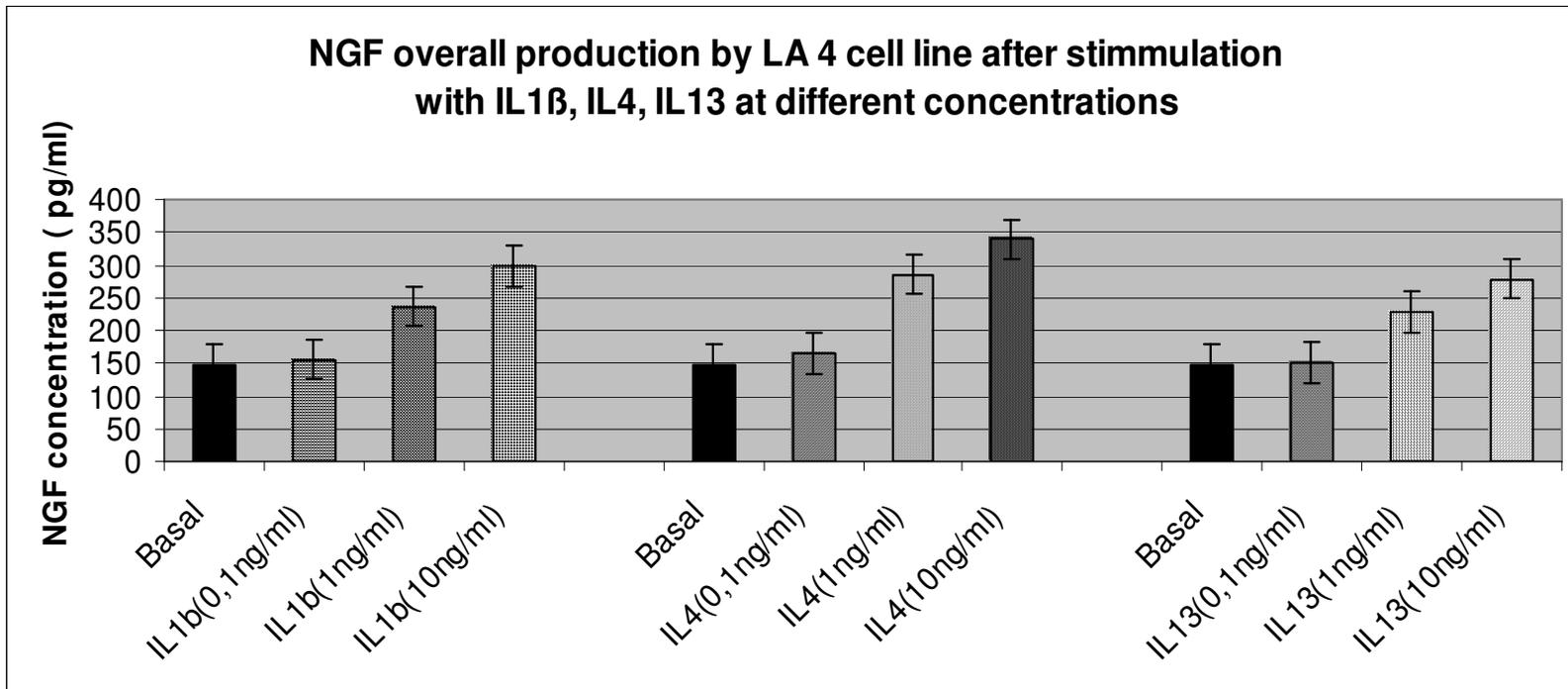
4.6.2. BDNF production after stimulation the LA 4 cell line by IL1 β , IL4, and IL13 at different concentrations

4.6.1. NGF production after stimulation of the LA 4 cell line by IL1 β , IL4, and IL13 at different concentrations

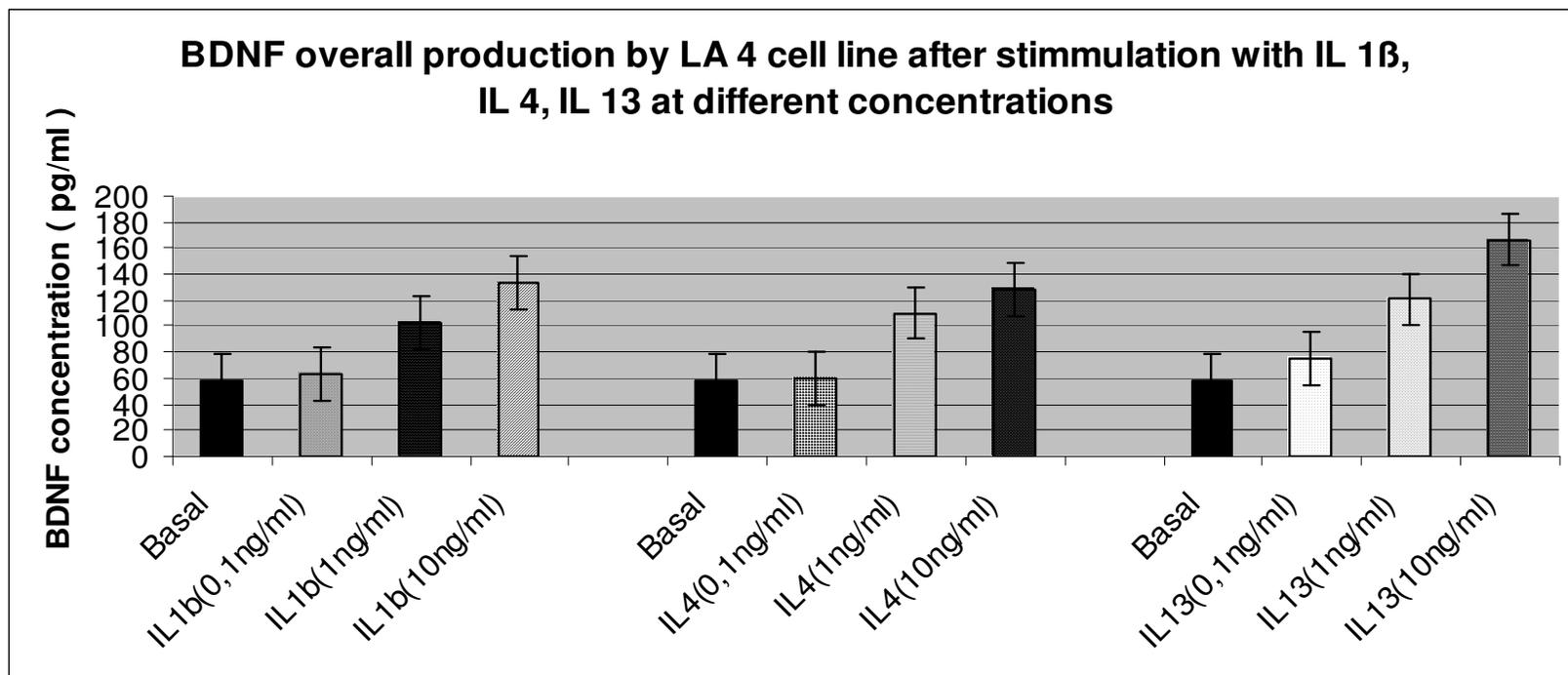
The same procedures as before to measure the overall production of the NGF by the LA 4 cell line cultured epithelial cells were performed, and a dose dependent up-regulation in the production of the NGF by the LA 4 cell line cultured epithelial cells after 24 hours stimulation with IL1 β , IL4, IL13 in different concentrations (0.1, 1, 10 ng/ml) were found as seen here in Tab. 7. There were increases in the concentration of the NGF up to 2 – 3 times compare to the NGF concentration of the LA 4 cell line basal production.

4.6.2. BDNF production after stimulation the LA 4 cell line by IL1 β , IL4, and IL13 at different concentrations

Also the same procedures to measure the overall production of the BDNF by the LA 4 cell line cultured epithelial cells were performed, and also up-regulation in the production of the BDNF by those cultured epithelial cells after 24 hours stimulation with IL1 β , IL4, IL13 in different concentrations (0.1, 1, 10 ng/ml) were found as seen here in Tab. 8. So, there were increases in the concentration of the BDNF up to 2 – 3 times compared to the BDNF concentration of the LA 4 cell line basal production.



Tab. 7: the overall production of NGF by the LA 4 cell line after stimulating those cells with IL 1 β ,IL 4,IL13 at different concentrations



Tab. 8: the overall production of BDNF by the LA 4 cell line after stimulating those cells with IL 1 β ,IL 4,IL13 at different concentrations

5. Discussion

Airway epithelium is an organized system of interacting populations of highly differentiated cells. In the last decades, there has been substantial progress in understanding the biology of normal tracheal epithelium largely as a result of the development of methods for primary culture of protease-dissociated airway epithelial cells. With this approach, useful numbers of cells can be isolated from the trachea and /or bronchi of several species ,including rats (44), guinea pigs and hamsters (98), Rabbits (55), humans (32). Improved culture techniques, including the use of appropriately supplemented serum-free media (50, 99), appropriate substrata (50, 109), and system of culture at an air-liquid interface (2, 29, 58); have permitted maintenance of a high degree of differentiation in vitro. This makes it possible to examine the molecular mechanisms of disease processes involving airway epithelial cells in parallel studies utilizing both in vivo experiments in animal models and in vitro experiments in cell culture studies.

Current studies in our laboratory focus on the role of tracheal epithelial cells in the regulation of neurotrophines expression in allergic airway diseases , and for that reason there was an increased need to develop a suitable methods for culture of adequate numbers of mouse tracheal epithelial cells .

A technique for the primary culture of murine tracheal epithelial cells was described. This method facilitates the establishment of differentiated, air-liquid interface cultures from murine tracheal epithelial cells. The medium employed in these studies utilized defined growth factor, unlike most previous reports, which have usually relied on hypothalamic tissue extract as a source of mitogenic activity. The expansion of tracheal epithelial cells which was established by a combination of mitogenes, including Epidermal growth factor (EGF) and others, was much more effective than supplementation with a single growth factor. As has long been recognized (19), cells growing on collagen film have a squamous morphology and exhibit a few if any differentiated characteristics, and the subculture cells growing on collagen film was of this phenotype. A number of maneuvers have been reported to facilitate redifferentiation, including reduction of the calcium content of the culture medium (23), supplementation with retinoids and hormones (22, 50, 99), culture on collagen gel substratum, and also culturing the tracheal epithelial cells at an air-liquid interface. All of these approaches were investigated for induction the differentiation

of the mouse tracheal epithelial cells at second passage. The most important factor was to culture on the insert semi-permeable membrane coated with rat tail collagen type I. By culturing the isolated cells in a betray dish for 4 hours directly after the isolation step, will allow for some fibroblast and some other cells but not the epithelial cell to attach to the bottom of the betray dish. That thing was confirmed by staining those cells by pan-cytokeratin, there were increasing in the percentage of the epithelial cells from 60% to 75% from the total ready to culture cells. At the other hand, using rat tail collagen type I as a coating material will form a selective culturing medium due to the ability of the epithelial cells to attach in a very good affinity to the culture insert membrane only in the presence of the collagen. The reason for culturing the murine tracheal epithelial cells from the second passage was also to improve the purity of the cultured cells, while isolating, culturing and re-isolating and then re-culturing will reduce the amount of the non epithelial cells, and that was confirmed by staining those cells by pan cytokeratin, and also there were increasing in the percentage of the epithelial cells from 75 – 80 % to more than 95 % from the total ready to culture cells. the protocol ,which used for staining the culture cells was evaluated in two different measures: the first was by using the staining protocol without primary antibody (just to find out the validation of the staining), all primary cells were found negative, and the second was to use the normal staining protocol to stain the LA 4 cells (a well known Pancytokeratin positive cells) as a positive control, and the positive result supported the implantation of the staining protocol to identify the culturing cells.

In conclusion, culturing the murine tracheal epithelial cells from the second passage over the semi-permeable membrane pre-coated with rat tail collagen type I, in the presence of some growth factors at air-liquid interface, those conditions are offering the optimal situation for those cells to grow and expand to reach the confluent state after four to five days and form a tight junction between each others. At the same time, and for the evaluation and comparison reasons, arise the need to culture the LA4 cell line, and also to use the normal culturing techniques for culturing the isolated mouse tracheal epithelial cells over a pre-coated 24 well culture plate which offered the possibility to have more sub-cultured cells and fast method to measure the neurotrophins production by the primary cells and the LA4 cell line. As a result there was an ability to duplicate the number of the tracheal epithelial cells in less than four days by using the normal culture technique.

The role of neurotrophins in the survival and function of distinct neuronal subjects is well known but there is now growing evidence that neurotrophins have profound activities on various immune cells and even the immune cells themselves could produce neurotrophins under certain conditions. There is also a possible interaction between the neurosystem and the immune system and the neurotrophins could be the possible mediator between those two systems. There is growing evidence that neurotrophins involved in the pathogenesis of allergic disease, and also neurotrophic activities, are strongly up-regulated in allergic conditions. But the sources of the neurotrophins in allergic diseases and in particular the airway allergic diseases still under intensive investigation, and from our side we were investigating the actual role of airway epithelial cells in regulation the neurotrophins production and in turn investigating the possible role of this neuroimmune interaction in the pathogenesis of the allergic airway diseases like asthma . from this point arise the need to use a culturing model which could offer in vitro a suitable atmosphere resemble for what we could see in the lung itself, while the epithelial cells will be in touch with the air from the top and with the fluid (with all the nutrients and other chemical products) from the bottom, and that is almost exactly what normally see in the pulmonary vesicles.

The job for investigating the role of the respiratory epithelial cells in regulation of neurotrophins expression in allergic airway diseases was started (after establishing the protocol for isolating and culturing mouse tracheal epithelial cells) by investigating the normal and basal production of NGF, BDNF, NT-3, and NT-4 from the primary cultured tracheal epithelial cells. The ELISA measurements of the neurotrophin`s content in the collected culture mediums after 6, 12, 18, 24, 48, 72 hours incubation times was shown the presence of NGF in detectable amount starting by in average 128, 64 ng/ml after 6 hours incubation time , and that was a very good starting points for the work to continue . And it was also a very good mark that those cells could produce NGF like other structural cells of the lung like fibroblasts (73) or smooth muscle cells (37). And, as was mentioned before (in the result section), there were up regulation in the production of the NGF in time dependent manner. That result was very consistent with the end result of scientific work of Fox and his colleagues who has investigated the production of the NGF by the human pulmonary epithelial cancer cell line (A 549) and he found that under basal conditions those cells generate NGF in a time dependent fashion. Also, the result was in agreement with the final result for the work of Pons and his colleagues who has worked also with (A549) cancer cell

line and he has reached to the same result. The main advantage from the result was that the primary cultured cells were used, not cancer cells like others. And by comparing that result by other results, it was concluded that the behaviour of the primary cultured tracheal epithelial cells under normal and basal condition was exactly similar to the behaviour of the cultured human pulmonary epithelial cancer cell line. Not only, there were a production of NGF by the primary cultured tracheal epithelial cells but also there were a production of BDNF. The average production of BDNF after 6 hours was in average 59, 52 ng / ml. The total production of the BDNF was up regulated in time dependent fashion exactly like the NGF production, and that was a new result in the field of the neurotrophins production investigations. At the same time and after doing the same procedures for measuring the total production of NT-3 and NT-4 by the primary culture tracheal epithelial cells, the result was negative and there were no production under normal conditions. It was concluded that the tracheal epithelial cells were not the sources of NT-3 and NT-4 under normal situation.

In order to prove the results, measurements were done by using the RT-PCR for detection of the neurotrophins mRNA and were able to detect specific bands encoding for NGF, BDNF, but at the other hand there were a negative result concerning the NT-3 and NT-4. And actually this result was consistent with the result which was obtained by the ELISA measurements. At the same time there were support for this result from the work of Braun and his colleagues (11) who has investigated production and cellular sources of BDNF and he found that there a constitutive expression of BDNF messenger RNA (mRNA) was observed in the respiratory epithelium of nonsensitized mouse lungs.

It is clear now that primary cultured mouse tracheal epithelial cells can produce NGF and BDNF under basal conditions, but the big question was about the behaviour of the primary cultured cells in different situation and under influences of a well known activator factors which also have a critical role in the pathogenesis of allergic asthma. The characteristic features of allergic bronchial asthma are chronic airway inflammation, development of airway hyperactivity and recurrent reversible airway obstruction. There is considerable evidence to support a role for T cells in asthma; particularly the involvement of Th2 cells ($CD4^+$ T lymphocytes can differentiate toward a T helper type 1(Th1) or Th2 phenotype (1, 71) both in atopic allergic asthma and in non atopic and occupational asthma. It is well known that allergen specific IgE

synthesis is T cell dependent through cognate activation of B lymphocytes and T cell-derived cytokines, such as IL-4 and IL-13 (101). Thus in atopic asthma and allergic rhinitis allergen processing and presentation to allergen-specific T cells through antigen-presenting cells are a key initiation step. Growing interest in the role of the T cell in asthma arose from the concept that, in addition to participating in IgE synthesis, T-cell products might also have direct effects on the airways through the recruitment of inflammatory cells, particularly eosinophils. A number of studies showed evidence for CD4+ T-cell activation in the peripheral blood of asthmatic patients during exacerbations (40). Sampling of the airways either with bronchial biopsy or bronchoalveolar lavage (BAL) revealed T cells with features of activation (3, 49, and 81). In some studies T-cell activation could be related both to measures of asthma severity, such as the degree of airway narrowing or AHR, and to the bronchial eosinophil response (81, 82, 105). Similarly, after the description of the TH2/TH1 dichotomy, mRNA cells for the signature TH2 cytokines IL-4 and IL-5 were detected in airway samples from atopic asthmatic patients (64). This linked IgE synthesis through IL-4 and eosinophilic airway inflammation through IL-5, together with IL-13 and GM-CSF (54,104,107). In addition, a number of investigators have isolated allergen-specific T cell lines and clones from the BAL fluid of asthmatic patients (30, 93). Recent studies have highlighted the importance of two structurally related Th2 cytokines, interleukin IL-4 and IL-13, in the events leading to allergen-induced AHR. IL-4 is required for the differentiation of T lymphocytes to a Th2 phenotype (1). Once a Th2 response has been established in the lung, IL-13 appears to play a role in the downstream events leading to AHR. Sustained inhibition of IL-13 throughout the period of allergen challenge reduces airway inflammation and mucus overproduction and abrogates AHR (42, 108). Repeated administration of IL-13 to the airways of naive mice induces airway inflammation, mucus production, and AHR (42, 108). Overexpression of IL-13 in the airways of transgenic mice also results in inflammation and mucus overproduction and leads to marked fibrosis, airway remodelling, and increased airways resistance (113). Taken together, these studies suggest that IL-13 produced by Th2 cells may help to account for many of the airway abnormalities seen in asthma. Venkayya and his colleagues have studied the possibility that Th2 cytokines might induce airway hyperresponsiveness (AHR) by acting directly on resident airway cells (100). They found that recombinant IL-4 and IL-13 both induced airway hyperresponsiveness (AHR) within 6 h. And this

induction of airway hyperresponsiveness (AHR) occurred in the absence of inflammatory cell recruitment or mucus production. And they have concluded that these results strongly suggest that products of activated Th2 lymphocytes can rapidly perturb airway function through direct effects on resident airway cells. At the same time Kuperman and his colleagues have demonstrated the importance of direct effects of IL-13 on epithelial cells in causing two central features of asthma (60). Taken together it was presumed that IL 13 and IL 4 was the target and was used as a direct stimulator for the primary cultured cells. And in order to investigate the role of other cytokines (which is not related to Th 2 cells) and have direct effects over the airway epithelial cells and link to the pathophysiology of asthma , IL-1 β was chosen as a stimulator for the cells, and actually that came from having the results of many works which have been done by different investigators ,and based on the available data shown that interleukin-1 β plays some important inflammatory role in the lung and airways during various pathological conditions, including asthma (48, 76). The effect of interleukin-1 β on NGF secretion was reported in some lung structural cells, like in primary culture including pulmonary fibroblasts (73) and airway smooth muscle cells (37). These effects have been shown also in other cell lines such as human astrocytoma and glioblastoma (34). Some other works in this field was the work of Frossard and his colleagues, they found that Nerve growth factor is released by IL-1beta and induces hyperresponsiveness of the human isolated bronchus (38). Also Fox and Pons have used IL-1 β as a stimulator for the human lung epithelial A549 cells in order to investigate them role in the production of NGF (36, 77). It was clear (based on those available data) that IL-1 β and IL-4, IL-13 might have a direct effects over the cultured tracheal epithelial cells, but was also important to know the doses which was used in order to initiate an expected influences. Three different concentrations for every type of cytokines (0, 1, 1 and 10 ng/ml) were used, and the selection was relayed over the clear function of the IL-1 β (in a similar concentration) on the cultured lung epithelial A549 cells by the work of Fox and his colleagues (36), and also experimentally in the way that those cytokines at given concentrations have a positive effects over the LA 4 cell line cultured epithelial cells. Those cytokines in this study were used in doses within the known biological range of action (10). The direct effects on neurotrophin`s production by IL-1 β , IL-4 and IL-13 at that given concentrations over the primary cultured murine tracheal epithelial cells was negative, and actually was unexpected result ,because of the well know effects of the

IL-1 β over the cultured lung epithelial A549 cell line cells. (36,77) At the same time The direct effects on neurotrophin`s production by IL-1 β , IL-4 and IL13 at that given concentrations over the LA 4 cell line cultured epithelial cells was positive and that was totally in agreement with findings of Fox and Pons, and if we try to figure out the possible reason that make this unagreement between the two findings we could find only that the cultured cells in first occasion was a primary and normal mouse tracheal epithelial cells, but by the other experiments was only cancer epithelial cell line, not normal cells and that could be the possible cause of the differences in the reacting of those cells towards the stimulation by IL-1 β , IL-4 and IL-13. On the other hand and up till now there is no single data investigating the direct effects of those cytokines over primary and normal cultured airway epithelial cells. Other possible reasons for this negative result could be the technique itself in four ways: the culturing model, the concentrations, and the incubation time or finally the type of stimulator.

Concerning the first possible reason (culturing model) for this negative result, the same experiment were done but by using another culturing method (just normal and simple culturing protocol in the 24 well culture dish preplated with type I collagen without using inserts) and also the basal production was measured, the result was almost identical for that result which was obtained before. After stimulation those cultured cells with IL-1 β , IL-4 and IL-13 at different concentrations, a negative result was also obtained, there were no upregulation in the production of NGF and BDNF. From the work of Fox and Pons, it was noticed that they have used both culturing methods and they have got a positive result. From all of that it was concluded that culturing model is not the reason for having that unexpected negative result.

Concerning the two other possible reasons for that negative result (concentrations ,incubation time), the positive result by using the LA 4 cell line cultured epithelial cells which was activated with the same type of cytokines and at the same concentrations and which was also in agreement with findings of Fox and Pons , could make every body believe that those cytokines concentrations was within its effective range and also the incubation time was long enough to show up them effect over cultured cells. From all of that it was excluded those two reasons as possible reasons for that negative result.

The final possible reason for that negative result may be could the answer for that question. As was illustrated before that the whole work was done by using normal primary cells not cancer cells in contrast to the other works which have used cancer

cell line in them experiments and even in the second part of the present work (LA 4 cell line) and both works have got a positive result against those cytokines at that concentrations. May be there is different in the behaviour of the primary normal cells and even in the type of receptors or the activation mechanisms. Starting from this point and in order to verify the work, the look for a new stimulator which could have a direct effect over the primary cultured cells and at the same time has an important role in the pathophysiology of the allergic asthma was started directly after the end of the present work. IL -5 could be the next target and other similar cytokines, depending on the results which was got by using the primary cultured murine tracheal epithelial cells.

It was hard to say whether the airway epithelial cells are one of the major sources for NGF and BDNF or other neurotrophins and also it was hard to say what type of role those cells could have in the pathophysiology of the allergic asthma from the side of the possible role of neurotrophins in the pathology of allergic asthma. But from the other hand it could not exclude 100 % the possible role for the respiratory epithelial cells

6. Conclusion

It was reported that tracheal epithelial cells and under basal conditions were capable of producing NGF and BDNF, this production was upregulated in time dependent manner, and at the same time those cells were not able to produce the other neurotrophins like NT-3 and NT-4.

The pro-inflammatory and asthma-associated cytokines like IL-1 β , IL-13, IL-4, have no effects over neurotrophin`s production by the primary cultured tracheal epithelial cells. On the other hand the overall production of NGF and BDNF by the cultured airway epithelial LA 4 cancer cell line, under the influence of IL-1 β , IL-13, IL-4, upregulated in concentration dependent manner. From all of that, it was hard to figure out the actual role of the airway epithelial cells in the regulation of neurotrophins expression in allergic airway diseases.

7. Zusammenfassung

Das Allergisches Asthma bronchiale (BA) ist eine Modellkrankheit für neuro-immunologische Interaktionen, und ist charakterisiert durch eine chronische Entzündung der Atemwege, Entwicklung von Atemwegshyperaktivität und reversibler Broncho-Obstruktion. Neurotrophine (NGF, BDNF, NT-3 und NT-4) sind wichtige Mediatoren auch im Immunsystem, und es wird vermutet, dass sie Neuro-Immunitätsinteraktionen in der chronischen Entzündung der Atemwege steuern. Untersuchungen an Tieren haben gezeigt, dass bei allergischer Entzündung der Atemwege Neurotrophine hochreguliert werden. Es wurde auch gezeigt, dass menschliche Lungen-Epithelzellen (A549) NGF produzieren können.

Ziel dieser Studie war, die Rolle von Atemwegs-Epithelzellen in der Regulation der Neurotrophinproduktion bei allergischen Atemwegserkrankungen zu untersuchen.

Um die Produktion von Neurotrophinen von Maus-Trachealepithelzellen zu untersuchen, wurde ein Protokoll für das Züchten der Mäuse-Trachealepithelzellen unter Air-liquid Interface Bedingungen entwickelt. Hierfür wurde ein früher publiziertes Protokoll modifiziert. Epithelzellen der Trachea von Wildtypmäusen wurden mit einem spezifischen Medium dissoziiert, das eine DNase und Pronase enthält. Diese Zellen wurden über der semipermeablen Membran des Zellkultureinsatzes in Anwesenheit von Wachstumsfaktoren gezüchtet. Um die Reinheit jener Zellen zu untersuchen, wurden kultivierte Zellen mit Pancytokeratin (Epithelzellen Marker) Einzelzellen zu unterschiedlichen Zeitpunkten gefärbt. Die basale Produktion von Neurotrophinen wurde zu unterschiedlichen Zeitpunkten gemessen (6, 12, 24, 48, und 72 Stunden). Der Effekt der Zytokine IL-1 β , IL-13, und IL-4 auf die Neurotrophinproduktion wurde dosisabhängig untersucht und quantitativ durch Enzyme-linked Immunosorbent Assay (ELISA) gemessen. Das gleiche Protokoll wurde auf murine LA 4 Epithel-Krebszellen angewendet.

Es wurde festgestellt, dass >95% der kultivierten Zellen Pancytokeratin positiv waren, und für mehr als vierzig Tage in der Kultur überlebten. Indem wir die basale Produktion von Neurotrophinen durch diese Zellen untersuchten, fanden wir heraus, dass NGF und BDNF Produktion zeitabhängig hochreguliert wurden, während NT-3 und NT-4 nicht produziert wurden. Spezifische mRNA für NGF, BDNF waren in allen Proben durch RT-PCR nachweisbar. In kultivierten Primärzellen konnte keine Effekte von IL-1 β , IL-13, und IL-4, auch bei ansteigenden Konzentrationen, auf die

Neurotrophinproduktion nachgewiesen werden. Allerdings hatten diese Zytokine Effekte auf die Neurotrophin-Produktion von LA-4 Epithelzellen. Es gab eine konzentrationsabhängige Hochregulation von NGF und BDNF nach Inkubation mit IL-1 β , IL-13, und IL-4, in LA 4 Zellen.

Zusammenfassend zeigen diese Resultate, dass Primärzellkulturen mit murinen Tracheal-Epithelzellen angelegt werden können. Diese Zellen sind funktionell aktiv und produzieren NGF sowie BDNF. Die Zytokine IL-1 β , IL-13 und IL-4 spielen keine Rolle in der Regulation dieser Neurotrophinproduktion.

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9. Contents

1. Introduction.....	3
1.1. Neurotrophins and their receptors.....	3
1.2. Neuroimmune interaction.....	5
1.2.1. Distribution of neurotrophins and their receptors in the immune system.....	6
1.2.2. Function of neurotrophins in the immune system.....	6
1.3. Neurotrophins in allergic Asthma.....	6
1.3.1. Allergic Asthma.....	6
1.3.1.1. Immunopathology of allergic Asthma.....	6
1.3.1.2. Neurogenic inflammation.....	7
1.3.1.3. Neuronal plasticity.....	8
1.3.2. Cellular sources of neurotrophins in allergic Asthma.....	9
1.4. Airway Epithelial cells and Asthma.....	11
1.4.1. Histological structure of the airway and the airway epithelium.....	11
1.4.2. Epithelium changes in allergic Asthma.....	13
1.4.3. Neurotrophins and their receptors in the airway epithelial cells.....	14
2. Objective.....	16
3. Material and Methods.....	17
3.1. Isolation and culturing the mouse tracheal epithelial cells.....	17
3.1.1. Medium preparation.....	17
3.1.2. Coating protocol of the insert with Rat Tail Collagen, Type I.....	18
3.1.3. Isolation and culturing protocol.....	19
3.2. Identification of cultured cells.....	21
3.2.1. Preparation of working solutions.....	21
3.2.2. Staining Procedures.....	22
3.3. Detection of tracheal epithelial cells NGF and BDNF by PCR.....	23
3.3.1. Isolation RNA from the primary cultured cells.....	23
3.3.2. DNA-Digestion.....	24
3.3.3. First strand cDNA synthesis.....	24
3.3.4. PCR.....	25
3.4. Measuring the Neurotrophin basal production of the primary cultured mouse tracheal epithelial cells.....	26
3.4.1. Collecting Protocol.....	26
3.4.2. ELISA measurement procedures.....	28
3.5. Measuring the Neurotrophin production after stimulating the primary cells by different types of cytokines.....	32
3.5.1. Collecting Protocol.....	32
3.5.2. ELISA measurement procedures.....	35
3.6. Measuring the Neurotrophin production by LA 4 cell line after stimulating by different types of cytokines.....	35
3.7. List of using material.....	35
4. Results.....	39
4.1. Isolation and culture Tracheal epithelial cells at air liquid interface.....	39
4.1.1. Isolation step.....	39
4.1.2. Culturing step.....	40
4.2. Identification of cultured cells.....	41
4.3. RT-PCR detection of tracheal epithelial cells NGF and BDNF.....	43

4.4. Neurotrophin basal production by the primary cultured mouse tracheal epithelial cells.....	44
4.4.1. NGF basal production.....	44
4.4.2. BDNF basal production.....	45
4.4.3. NT3, NT4 basal production	46
4.5. Neurotrophin production after stimulating the primary cells by different Types of cytokines	47
4.5.1. NGF production after stimulation the epithelial cells by IL1 β , IL4 and IL13 at different concentrations.....	47
4.5.2. BDNF production after stimulation the epithelial cells by IL1 β , IL4 and IL13 at different concentrations.....	47
4.6. Neurotrophin production by LA 4 cell line after stimulating by different types of cytokines.....	50
4.6.1. NGF production after stimulation the LA 4 cell line by IL1 β , IL4 and IL13 at different concentrations.....	50
4.6.2. BDNF production after stimulation the LA 4 cell line by IL1 β , IL4 and IL13 at different concentrations.....	50
5. Discussion.....	53
6. Conclusion.....	60
7. Zusammenfassung.....	61
8. References.....	63
9. Contents	74