ABSTRACT


Airway remodeling observed in chronic inflammatory diseases of the respiratory system is characterized by mucous cell hyperplasia, smooth muscle cell proliferation and subepithelial fibrosis. Interleukin (IL)-13 is a central mediator in the pathogenesis of inflammatory airway diseases playing a significant role in epithelial remodeling. In this study, we examined mechanisms through which IL-13 induces proliferation of normal human bronchial epithelial (NHBE) cells maintained in air/liquid interface culture in vitro, a model that allows for the maintenance of differentiated structure and function. It was found that IL-13-induced proliferation of NHBE cells is mediated by the autocrine/paracrine action of transforming growth factor-α (TGFα) produced by the epithelial cells which subsequently binds to the epidermal growth factor receptor (EGFR) on these cells. IL-13-induced release of TGFα involves the rapid mobilization of intracellular stores of TGFα to the apical cell surface, where membrane-bound TGFα is cleaved by tumor necrosis factor-α converting enzyme (TACE), resulting in released growth factor. Both neutralizing anti-TGFα antibodies and the EGFR tyrosine kinase inhibitor AG1478 block IL-13-induced proliferation in concentration-dependent manners. Additionally, IL-13 induces activation of the mitogen activated (MAP) kinase pathway and the phosphatidylinositol 3-kinase (PI3K) pathway, both of which are required for IL-13-induced proliferation. However, activation of the MAP kinase pathway by IL-13 does not appear to be TGFα mediated, as neutralizing anti-TGFα antibodies and EGFR tyrosine kinase inhibitors have little effect on IL-13-induced MAP kinase. PI3K was
found to be involved in IL-13-induced release of TGFα as the specific inhibitor of PI3K, LY294002, blocked release of the growth factor. In summary, IL-13-induced proliferation of NHBE cells involves the intracellular redistribution and subsequent TACE-mediated release of TGFα, an event mediated by PI3K. This proliferation then involves the interaction of TGFα with the EGFR and requires activation of both MAP kinase and PI3K signaling cascades. These data establish a mechanistic framework for further study into the effects of IL-13 on bronchial epithelial cells as well as additional cell types that may interact with IL-13, or growth factors induced by this cytokine, in an in vivo setting.
MECHANISMS OF INTERLEUKIN-13-INDUCED
PROLIFERATION OF NORMAL HUMAN BRONCHIAL
EPITHELIAL CELLS IN VITRO

by

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List of Abbreviations

ADAM-a disintegrin and metalloproteinase
BEGM-bronchial epithelial growth media
BSA-bovine serum albumin
DMEM-Dulbecco’s modified Eagle’s medium
ECM-extracellular matrix
EGF-epidermal growth factor
EGFR-epidermal growth factor receptor
ELISA-enzyme linked immunosorbent assay
ERK-extracellular regulated kinase
GM-CSF-granulocyte macrophage colony stimulating factor
HB-EGF-heparin binding EGF-like growth factor
IL-4-interleukin-4
IL-13-interleukin-13
LDH-lactate dehydrogenase
MAP kinase-mitogen activated protein kinase
M-CSF-macrophage colony stimulating factor
MEK-MAP kinase kinase
MMP-matrix metalloproteinase
NHBE-normal human bronchial epithelial
PAGE-polyacrylamide gel electrophoresis
PBS-phosphate buffered saline
PBST- phosphate buffered saline plus 0.05% Tween-20
PDGF-platelet derived growth factor
PI3K-phosphatidylinositol 3-kinase
PKB-protein kinase B
PKC-protein kinase C
PMA-phorbol 12-myristate 13-acetate
SCF-stem cell factor
STAT6-signal transducer and activator of transcription 6
TACE-tumor necrosis factor-α converting enzyme
TCA-trichloroacetic acid
TF-transcription factor
TGFα-transforming growth factor-α
TNF-α-tumor necrosis factor-α
INTRODUCTION

The research findings discussed in this dissertation enhance the knowledge base regarding mechanisms responsible for airway epithelial remodeling observed in chronic inflammatory diseases of the respiratory system. Specifically, the intention of the study was delineation of molecular mechanisms regulating development of airway epithelial cell hyperplasia in response to the inflammatory mediator interleukin-13 (IL-13). Focus on this cytokine as the inducer of epithelial cell responses is due to its implication as a key molecule in the pathogenesis of asthma (Grunig, 1998; Wills-Karp, 1998; Zhu, 1999). As such, IL-13 is involved with induction of the associated phenomenon of airway remodeling, complete with mucous cell hyperplasia, smooth muscle cell proliferation and subepithelial fibrosis (Black, 2001). Previous experimentation suggesting this link between IL-13 and airway remodeling was carried out in a murine model of allergic asthma (Grunig, 1998; Wills-Karp, 1998), as well as in signal transducer and activator of transcription (STAT) 6 knockout mice that have the capacity for reconstitution of IL-13/STAT6 responses only in the airway epithelium (Kuperman, 2002). The study presented in this document augments the base of knowledge regarding IL-13 and its role in asthma via mechanistic studies in human airway epithelial cells wherein the effects of IL-13 on epithelial cell proliferation are explored.

In these studies, normal human bronchial epithelial (NHBE) cells were used as an in vitro model. When grown in a biphasic air/liquid interface (ALI) culture, NHBE cells develop morphological and functional characteristics identical to those observed in airway epithelial cells in vivo (Krunkosky, 2000). This culture system provides an excellent model for recreating in vivo conditions such as inducible mucus secretion (Li,
2001) and ciliary beat (Laoukili, 2001). Thus, it allows one to avoid the use of immortalized cell lines and their associated pitfalls. While such cell lines may provide some insight into the workings of the airway epithelium, they are in fact immortalized, either malignant or virally transformed cells. Such genetic alteration, while providing an immortal cell system, inherently changes the phenotype of any given cell as well as the relationship of signaling pathways within that cell. These alterations may result in gene expression or repression that differs from that regularly observed in normal cells. Outside of an actual *in vivo* human model, the ALI culture system provides the best model for investigating the mechanisms of airway remodeling in human airway epithelial tissue.

The general hypothesis addressed herein is that IL-13 is a pivotal molecule for initiation of proliferation of airway epithelial cells. It is proposed, and supported with experimental evidence, that IL-13 induces proliferation of airway epithelial cells, an effect mediated by transforming growth factor-α (TGFα) produced and released by these epithelial cells. TGFα subsequently interacts in an autocrine manner with its receptor, the epidermal growth factor receptor (EGFR). Furthermore, the induced release of TGFα involves rapid mobilization of intracellular stores of TGFα to the cell surface, where the growth factor interacts with a protease, TNFα converting enzyme (TACE), which cleaves TGFα, allowing its migration to nearby epithelial receptors and to other airway cells where it promotes proliferation. Mobilization of cytoplasmic growth factor stores in response to cytokine stimulation of epithelial cells has not been demonstrated previously. This interaction may provide a target for therapeutics aimed at treatment of airway diseases, other inflammatory diseases, and potentially, autoimmune diseases or cancer.
The proliferation induced by released TGFα occurs via the mitogen activated protein (MAP) kinase signal transduction pathway, a pathway which can be activated when exogenous TGFα binds its receptor, the EGFR, on epithelial cells. Activation of the phosphatidylinositol 3-kinase (PI3K) pathway also appears to be required for IL-13 or TGFα-induced proliferation, although this effect may not occur solely downstream of the EGFR in IL-13-exposed cells, as PI3K has also been found to play a role in the release of TGFα from these cells. Such initiation of multiple intracellular signal transduction pathways by IL-13 may have implications beyond regulation of cellular proliferation. For example, in these epithelial cells, IL-13 and TGFα were also found to activate overlapping but distinct groups of transcription factors allowing for regulation of a large variety of genes. Modulation of such gene expression might be further regulated by crosstalk between IL-13-induced and autocrine-activated signaling pathways in these cells.

Organization of this dissertation leads the reader first through introductory material (chapter 1) focused on the general phenomenon of airway remodeling and the role of IL-13 in allergic asthma wherein this cytokine exerts specific effects on airway tissues during chronic inflammation. Three data chapters follow; the first presents a previously published study examining the autocrine mechanism whereby IL-13 induces proliferation of differentiating NHBE cells in vitro. The next chapter presents data that reveals activation of intracellular proliferative signal transduction pathways by IL-13. The final data chapter examines the role of TGFα and TACE in the proliferation of NHBE cells, with specific attention paid to intracellular movement of the growth factor (TGFα) and the mechanism of its cleavage. A concluding chapter places this original
research in the context of the broader field of airway epithelial remodeling, setting the
stage for future studies that may emerge from this work. Appendices contain additional
data regarding activation of transcription factors induced by IL-13 and TGFα in NHBE
cells and an extensive review of the varied roles of TGFα throughout the mammalian life
span.

AIRWAY REMODELING

The normal mammalian airway undergoes continuous cycles of injury followed
by repair (Warburton, 2001). Injuries to the airway epithelium are caused by a number of
different agents including, but not limited to, components of air pollution, such as ozone,
asbestos and cigarette smoke; bacteria and other virulent organisms; oxidants; mechanical
injuries such as those induced by a ventilator or a crushing force; and the inflammatory
mediators that arise in response to these injuries and during allergic responses (Fahy,
2001; Jeffery, 2001; Caughey, 2001; Tschumperlin, 2001). When these injury-repair
cycles continue in a chronic fashion they result in tissue changes collectively known as
airway remodeling. This pathological alteration includes changes throughout the airway
wall, including those affecting the epithelium (Fahy, 2001), basement membrane (Jeffery,
2001), airway vasculature (McDonald, 2001), and airway smooth muscle (Black, 2001).
Specifically, these changes include desquamation of the epithelium, increases in
basement membrane and smooth muscle thickness, and proliferation of endothelial cells
(McDonald, 2001). Epithelial mucous cell hyperplasia can also result from chronic
airway inflammation (Harkema, 2002).
A variety of studies have examined airway remodeling following exposure to air pollution. Chronic exposure to high levels of particulate matter results in remodeled airways consisting of abnormally small lumens, fibrotic walls and excess smooth muscle (Churg, 2003). The transitional metal vanadium, found in particulates common to the petrochemical industry and in lower concentrations in air pollution, induces airway fibrosis in rats (Bonner, 2000) and affects bronchial epithelial cells (Zhang, 2001). Specifically, vanadium stimulation of epithelial cells results in an increase in HB-EGF gene and protein expression. Once released from the epithelial cells, HB-EGF is able to initiate a FGF-2 autocrine loop in fibroblasts resulting in their proliferation (Zhang, 2001).

Chronic airway remodeling has been examined for association with airway diseases involving chronic inflammation, such as asthma, both allergic and non-allergic (Fahy, 2001; Zhu, 2001); chronic obstructive lung disease (COPD) (Jeffery, 2001), and cystic fibrosis (CF) (Hardie, 1997). During the remodeling process associated with these diseases, inflammatory cells infiltrate the airway and release various cytokines, chemokines, and cytotoxic agents to “defend the host” and “destroy the destructive agent” whether it be a particulate or an invading organism. Unfortunately, in these diseases the presence of inflammatory cell infiltrates continues even when the offending agent is no longer present. These infiltrating inflammatory cells including neutrophils, mast cells, macrophages, eosinophils, basophils, monocytes, T and B cells, and other components of the immune system, provide the molecular triggers that induce the mechanistic cellular cascades that lead to the phenotype changes characteristic of the remodeled airway (Sastre, 2003).
REMODELING IN ASTHMA

In asthmatics, damage and shedding of the airway epithelium has been reported. Clusters of sloughed epithelial cells, Creola bodies, are observed in asthmatic sputa along with an increase in epithelial cells in bronchoalveolar lavage (BAL) fluid from affected patients (Fahy, 2001). Such epithelial damage is highly variable: some airways have intact surface epithelium even in the presence of marked inflammation and other structural changes while others are heavily denuded under similar circumstances (Jeffery, 2001). Generally, loss of epithelium induces a healing or repair process resulting in the restoration of the columnar/cuboidal epithelium that ultimately gives rise to a restored pseudostratified epithelium with a normal ratio of goblet and ciliated cells. However, if the injury is repeated chronically, squamous cell metaplasia and/or goblet cell hyperplasia result. This hyperplasia consists of a change in the mucous cell/ciliated cell ratio in the airway leading to an overall increase in mucus while simultaneously decreasing the ability for an individual to clear mucus. This decreased clearance can lead to mucus plugging of the airways, a condition that can be fatal (Shapiro, 2002).

Airway inflammation in asthma appears to be necessary for development of epithelial airway remodeling. In allergic asthma, the initial response is triggered by antigen-specific activation of mast cells via the Fcɛ receptors (FcɛRI) (Koybayashi, 1998). The subsequent degranulation of mast cells initiates early phase airway inflammation through the release of various chemical mediators, such as histamine, PGD₂, LTC₄ and proteases (Koybayashi, 1998). The late-phase response of airway inflammation is characterized by the infiltration of activated T lymphocytes (T cells) and
eosinophils. These T cells include the CD4+ subset, which regulate the inflammatory process through their release of cytokines (Janeway, 1997). These CD4+ T cells can be divided into 2 groups, TH1 and TH2, depending on the cytokines they produce. TH1 cells secrete interleukin-2 (IL-2), tumor necrosis factor-α (TNFα) and interferon-γ (IFN-γ); while TH2 cells release IL-3 (Chomarat, 1998), IL-4, IL-5, IL-6, IL-10, IL-13 (Opal, 2000) and granulocyte colony stimulating factor (GM-CSF) (Doucet, 1998). Some immunologists suggest the balance between TH2 and TH1 cytokines dictates the direction of the immune response with TH1 cytokines steering toward acute inflammation while the TH2 mediators generally aid in the resolution of inflammation. Interestingly, these TH2 cytokines, in particular IL-13, appear to be the major inducers of airway remodeling (Larche, 2003).

INTERLEUKIN-13

The cytokine IL-13 is produced by activated T cells, mast cells (Kobayashi, 1998) and basophils (Kelly-Welch, 2003). It is translated as a 132 amino acid precursor protein with a final secreted product of 112 amino acids in length and a molecular weight of 12 kDa. IL-13 and IL-4 are closely related cytokines sharing a 20-25% homology at the amino acid level and belonging to the same α-helix superfamily. Both are classified as TH2 cytokines and are transcribed from the same chromosome (5q) (Chomarat, 1998). IL-4 and IL-13 both bind to, and initiate signal transduction pathways through the common IL-4Rα receptor chain. They share many functional properties including their ability to up-regulate expression of major histocompatibility complex class II molecules and CD23 antigen expression on monocytes (Minty, 1993; Zurawaski, 1994), the
induction of IgE synthesis, CD23 expression by B cells (Punnonen, 1993), and the induction of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Bochner, 1995). IL-4 has been shown to contribute to the pathogenesis of asthma (Corry, 1998), but the IL-4Rα has been shown to be a greater contributor (Grunig, 1998), suggesting a more potent effect of IL-13 compared to IL-4 in the development of asthma.

IL-13 binds to the IL-4Rα/IL-13Rα1 heterodimer receptor complex, or to monomers of the IL-13Rα2 transmembrane receptor proteins alone, on the surface of cells (Jiang, 2000). The IL-4Rα is a 140-kd transmembrane protein that is expressed ubiquitously while the 75-80 kDa IL-13Rα1 is present on a limited number of cell types including B cells, monocytes, macrophages, basophils, mast cells, endothelial cells and epithelial cells. IL-13Rα1 is not expressed on human T cells (Chomart, 1998; Jiang, 2000).

When IL-13 binds the IL-4Rα/IL-13Rα1 complex, intracellular signal transduction pathways are initiated. These signal transduction pathways include the Janus kinase (JAK) family members JAK1 and TYK2 as well as signal transducer and activator of transcription-6 (STAT6). JAK1 associates with the IL-4Rα chain while TYK2 associates with the IL-13Rα1 chain with both kinases capable of phosphorylating STAT6 (Chomart, 1998), which then translocates to the nucleus of the cell where it acts as a transcription factor. IL-13 also has the ability to induce phosphorylation of IRS-2 in colonic epithelial cells (Wright, 1997), which, in turn, binds the p85 regulatory subunit of PI3K or the adapter protein Grb2, that is constitutively associated with Sos. Grb2 then activates Ras leading to Raf activation and subsequent activation of the MAP kinase family members MEK and Erk (L’Allemain, 1994; Cobb, 1999).
Since the heterodimer of IL-4Rα/IL-13Rα1 is sufficient for IL-13 induced signaling, the IL-13Rα2 has been hypothesized to act as a decoy receptor. The IL-13Rα2 has an extremely short cytoplasmic domain consisting of 17 amino acids in humans. Such a short cytoplasmic tail allows the receptor to be classified as a non-signaling peptide since it contains no box 1 or box 2 signaling motifs. Expression of IL-13Rα2 in vitro is not sufficient to render cells responsive to IL-13, signal transduction activation was not observed, even with the additional presence of IL-4Rα. Additionally, intracellular stores of the IL-13Rα2 have been observed in monocytes and nasal epithelial cells with these stores mobilized to the cell surface in response to the TH1 cytokine IFN-γ (Daines, 2002). Such upregulation of IL-13Rα2 surface expression would enhance binding of any free IL-13, neutralizing it and allowing the TH1 response to dominate. Guinea pigs pretreated with soluble IL-13Rα2 were protected from developing antigen-induced airway hyperresponsiveness (Morse, 2002) again demonstrating the functional role of IL-13Rα2 as a decoy receptor.

With regard to activation of signal transduction pathways by IL-13 in epithelia, IL-13 stimulation of human bronchial epithelial cell cultures resulted in apparent activation of MAP kinase and PI3K pathways that result in a change in goblet cell density (Atherton, 2003). In intestinal epithelial cells, IL-13 induced PI3K activation, but not STAT6 activation, has been implicated in increased epithelial permeability as detected by transepithelial resistance (Ceponis, 2000). Additionally, activation of PI3K by IL-13 inhibits inducible nitric oxide synthase (iNOS) and inhibits apoptosis in colon carcinoma cells (Wright, 1997, 1999).
ROLE OF INTERLEUKIN-13 IN AIRWAY REMODELING

IL-13 has been shown to be a central mediator of allergic asthma (Wills-Karp, 1998; Grunig, 1998) with targeted transgenic expression of IL-13 in the lungs of mice causing inflammation, mucus hypersecretion, subepithelial fibrosis, and eotaxin production (Zhu, 1999) similar to those observed in human asthmatics. Inhibition of IL-13 results in a complete reversal of antigen-induced airway hyperresponsiveness. Transgenic mice expressing STAT6 only in lung epithelial cells have recently provided insightful information on the functions of IL-13. In mice that do not express STAT6, IL-13 does not induce any pulmonary effects associated with asthma. The reconstitution of STAT6 only in airway epithelial cells restored the increase in mucus production and airway hyperresponsiveness but not the other hallmarks of asthma including underlying pulmonary fibrosis, increase in inflammation or emphysema (Kuperman, 2002). These findings suggest that at least two asthma-related functions are produced through direct interaction of IL-13 with the airway epithelium.

In mouse models of asthma, IL-13 regulates epithelial mucus production induced by CD4+ T cells, with mucus production in the epithelium observed following activation of T cells in response to inhaled antigen. A variety of experimental approaches have solidified the central role of IL-13 in this process. IL-13 and IL-9 both stimulate mucus production via an IL-13/IL-4Rα pathway, where mucus cannot be produced without IL-4Rα activation (Cohn, 2002). Unlike mucin induction by gram-negative bacteria, NFκB induction is not required for IL-13-induced mucus production (Whittaker, 2002). Additionally, the lungs of transgenic mice overexpressing IL-10 exhibit mucus cell metaplasia, inflammation and airway remodeling, and IL-10 induces these outcomes
through an IL-13-dependent pathway (Lee, 2002). In addition, the very nature of the
allergic inflammatory response appears to require IL-13, as the cytokine has been shown
to be required for accumulation of intraepithelial eosinophils (Kumar, 2002), presumably
through the stimulation of airway epithelial cells to produce eotaxin, a chemoattractant
for eosinophils (Li, 1999).

During airway remodeling, IL-13 has also been shown to act directly on lung
fibroblasts (Doucet, 1998), with IL-13 exposed mouse models having subepithelial
fibrosis as well as epithelial thickening. Stimulation of lung fibroblasts to IL-13 results
in expression of VCAM-1, up-regulation of $\beta_1$ integrin, and production of macrophage
colony stimulating factor (M-CSF), GM-CSF, stem cell factor (SCF), IL-6, and IL-11.
The IL-13-induced increase in VCAM-1 on these fibroblasts also encourages the
infiltration of eosinophils into tissues surrounding the airways, while increased
expression of $\beta_1$ integrin on fibroblasts modifies matrix-fibroblast interactions. The
added influx of eosinophils results in an increase in cytokines and growth factors within
the airways, while up-regulation of $\beta_1$ integrin by IL-13 leads to modifications that affect
cell-cell interactions, allowing for proliferation and migration of cells during the healing
process.

**MECHANISMS OF IL-13-INDUCED PROLIFERATION**

Prior to the study set forth in this dissertation, the specific ability of IL-13 to
induce proliferation of NHBE cells or mechanisms by which this occurs had not been
elucidated. However, as mentioned previously, IL-13 has been found to activate the
PI3K signaling pathway leading to a resultant increase in epithelial permeability
Increased epithelial permeability allows infiltration of large numbers of immune cells, such as neutrophils, into the airway, but increased permeability can also be associated with cell growth and proliferation. Altering gap junctions between cells allows cells to freely migrate into wounded areas initiating the healing process, while at the same time apically expressed growth factors can move through the loosened junctions to interact with basally expressed receptors. By easing tension on the junctions, cells are capable of “rolling” or shifting surface expression of various proteins, such as growth factors, as the cells shape change. This rolling allows surface expressed growth factor to migrate within the extracellular membrane until the ligand encounters its receptor whether in an autocrine/juxtacrine or paracrine manner (Vermeer, 2003).

**EGFR IN MUCIN PRODUCTION**

The epidermal growth factor receptor (EGFR), of importance in a variety of proliferation events, has also been linked to regulation of mucin expression in airway epithelium. Specifically, stimulation of EGFR induces goblet cells to secrete mucus (Kanno, 2003), and upregulates mucin gene expression and mucin production. EGF family ligands (including TGFα) (Perrais, 2002), neutrophil elastase (Kohri, 2002), N-formyl-methionyl-leucyl-phenylalanin (fMLP) (Lee, 2001), oxidative stress (Takeyama, 2000), bacterial infection (Kohri, 2002) and cigarette smoke (Takeyama, 2001; Basbaum, 2002) all can induce changes in mucin expression via EGFR in airway epithelial cell lines. In most of these cases, EGFR activation occurs following binding of TGFα, resulting in MAP kinase pathway activation, demonstrating a potential, non-proliferative role for this pathway.
TRANSFORMING GROWTH FACTOR-α IN AIRWAYS

TGFα, a member of the epidermal growth factor (EGF) superfamily of growth factors including the aforementioned EGF, heparin binding EGF-like growth factor (HB-EGF), betacullin (BTC), epiregulin (EPR), amphiregulin (AR), heregulin, the neuregulin subfamily and epigen (Harris, 2003). TGFα acts as a ligand for the EGF receptor (EGFR). The EGFR is a member of a family of four transmembrane receptor chains designated erbB1-4 or in humans HER1-4. TGFα normally binds to HER1 (EGFR). Upon binding of TGFα to HER1, the receptor homodimerizes with HER1 or forms heterodimers with an additional family member. Once the dimer is formed, the receptor chains become phosphorylated initiating intracellular signal transduction pathways. In crystal structures of the EGFR complex bound with TGFα, the ligand is bound exclusively to a single receptor molecule suggesting that upon ligand-receptor interaction a conformational change ensues in the receptor allowing dimerization of two HER monomers instead of preexisting dimers binding the available ligand (Schlessinger, 2002).

Cellular proliferation is an important aspect of airway remodeling and repair, as it is in other instances of cellular injury and healing. In airway remodeling, such proliferation is thought to involve complex autocrine/paracrine interactions between different cell types in the lung (Korfagen, 1994). TGFα and EGFR have been localized in human airway epithelium via immunohistochemistry from the trachea to distal airspaces throughout the entire period of fetal development, a time when cellular proliferation is abundant (Ruocco, 1996). Transgenic mice overexpressing TGFα display
morphologic alterations of several organs including the lungs where abnormal development results in large alveolar spaces, pulmonary fibrosis (Hardie, 1997) and hysteresivity (Pillow, 2001), suggesting that an overabundance of growth factor can lead to marked irregularities. Chronic lung injury that develops in some neonates is characterized by simple, evenly distributed terminal air spaces. Since the lungs are one of the last organs to fully develop and TGF\(\alpha\) is involved in the injury/repair process (see below), the injuries developed by some neonates may in part be due to an overabundance of TGF\(\alpha\) (Strandjord, 1995).

Increased levels of TGF\(\alpha\) have also been shown to correlate with a variety of epithelial injuries including wounded colonic epithelium (Wilson, 1999), asbestos-induced and oxidant injury of the airway epithelium (Vivekananda, 1994; Liu, 1996), and injury of corneal epithelial cells (Goke, 2001). In such cases, this increased expression appears to be a component of the healing process, a process that requires cellular proliferation.

In general, the epithelial repair process can be divided into two phases, migration and proliferation. First, epithelial cells adjacent or just beneath the injured surface migrate into the wound to cover the denuded area. This process does not require proliferation, only migration and the deposition of extracellular matrix (ECM). Epithelial proliferation and differentiation then occur to replenish the decreased cell pool (Dignass, 1996). Some of the increases in growth factor levels observed in response to an injury are attributable to the influx of inflammatory cells, such as neutrophils and macrophages, which produce and secrete these factors, while additional growth factors are generated by the affected tissues. For example, fibroblasts do not normally generate TGF\(\alpha\), but in
response to oxidative stress fibroblasts from normal lungs synthesize and release TGFα (Vivekananda, 1994) that can, in turn affect the surrounding epithelium and other fibroblasts.

The controlled release of TGFα, or any growth factor, is essential for numerous biological functions such as development, injury repair and immunological responses. By initiating release of TGFα from within NHBE cells, IL-13 can influence these biological functions by controlling TGFα and its interactions with EGFR and the subsequent events following ligand receptor interactions (Booth, 2001). Following this reasoning, if during development a TH2 immune response is mounted, this IL-13/TGFα interaction could impact the normal development of the lungs or perhaps other organs and tissues. Furthermore, during prolonged or chronic IL-13 exposure, elevated TGFα levels and excessive proliferation would be expected. Such is the case in asthma and other inflammatory diseases of the airway. Increased epithelial thickness and pulmonary fibrosis are the visible results of this excessive proliferation due to chronic inflammation (Harkema, 2002; Hardie, 2003).

Knowing that both TGFα and EGFR are involved during airway remodeling, it is likely that an autocrine/paracrine mechanism is involved in proliferative events in vivo. These mechanisms exist in numerous other biological processes including various forms of cancer. Specifically, autocrine activity of TGFα is associated with the progression of transformed properties in human colon carcinoma cells (Ziobert, 1993; Shvartsman, 2002) as well as migration of normal colonic epithelial cells (Wilson, 1999). Autocrine TGFα actions also affect fetal lung development (Ruocco, 1996), lung fibrosis (Korfhagen, 1994) and responses to IL-13 in vitro (Booth, 2001).
TGFα and its many biological roles are reviewed in detail in appendix A of this dissertation.

**TUMOR NECROSIS FACTOR-α CONVERTING ENZYME**

For TGFα to play a role in airway remodeling or injury-repair, it must be shed by an appropriate protease. One such protease is tumor necrosis factor-α converting enzyme (TACE), an α-secretase and member of the ADAM (a disintegrin and metalloproteinase) family of proteases, classified as ADAM17. The ADAMs are a group of unique zinc-binding transmembrane metalloproteinases, with TACE responsible for the cleavage of a number of membrane bound pro-proteins, including TGFα, via a process known as shedding (Peschon, 1998). Cleaved proteins include TGFα, TNF-α, TNF-α p75 receptor, l-selectin (Peschon, 1998; Black, 1997), erbB4/HER4 (Rio, 2000), β-amyloid precursor protein, IL-6 receptor, and FAS ligand (Arribas, 1997). As is the case with TGFα, TACE converts membrane-anchored growth factors into diffusible proteins, membrane receptors into soluble competitors for their own ligand or accessories to ligand binding, and cell adhesion receptors into products no longer capable of mediating physical interactions with other cells or the extracellular matrix. TACE has been shown to be present on the surface of cells, but the majority of TACE is localized to a perinuclear compartment similar to TNF-α in human monocytes and primate kidney cells (Schlondorff, 2000). This finding raises the question of whether or not TACE-mediated ectoderm shedding occurs on the cell surface as well as within the cell.

Activation of TACE requires proteolysis. The protease or proteases involved in the activation process of TACE are unknown at this time, but TACE activation is
mediated by protein kinase C (PKC) (Black, 1997), nitric oxide (NO) (Zhang, 2000) and
the MAP kinase pathway family member Erk (Diaz-Rodriguez, 2002; Fan, 2003).
Activation requires oxidation and dissociation of the cysteine thiol-linkage from the
latent enzymatic site; this complex is known as the cysteine zinc switch (Zhang, 2000).

Interestingly, the inflammatory mediator IL-13 has been shown to affect various
reactive oxygen species, including NO. IL-13 has also been shown to activate signal
transduction pathways that include insulin receptor substrate and phosphatidylinositol 3-
kinase (Wright, 1997), both of which are known to be upstream of PKC and/or Erk in
various signaling pathways. However, in NHBE cells, IL-13 does not appear to
dramatically affect TACE protein levels or gene expression (Chap 4). Rather, IL-13
induces TACE to coalesce into dense, presumably enzymatic areas near the cell surface.
These areas of concentrated TACE are the endpoint for intracellular TGFα movement.

TACE−/− mice develop very similar phenotypes to TGFα−/− mice. In both cases the
lungs are grossly underdeveloped with large interstitial spaces and impaired branching,
the eyelids do not fuse and the animal’s fur is wavy and coarse (Peschon, 1998). In vitro
lung explant experiments utilizing TACE antisense oligonucleotide-treated lungs have
demonstrated TACE’s necessity for normal lung development. The addition of TGFα to
the antisense treated cultures rescued the cultures from both inhibition of lung branching
morphogenesis and lung epithelial cell differentiation (Zhao, 2001). Recently, TACE has
been implicated in MUC5AC mucin expression in airway epithelial cells via TGFα
shedding (Shao, 2003).

Additional members of the ADAM family and other proteases, including some
MMPs (matrix metalloproteinases), have been implicated in airway diseases and
remodeling. Recently the newly described ADAM33 has been linked to asthma through genetic screening (Van Eerewegh, 2002; Shapiro, 2002). In addition, other MMPs such as matrilysin (MMP7) (Zuo, 2002) and MMP9 (Lee, 2001) are overexpressed or upregulated during airway diseases.

**SUMMARY**

The phenomenon of airway remodeling is a consequence of repeated cycles of injury and repair occurring during a chronic state of inflammation in the airways of asthmatic patients. The inflammatory mediator IL-13, being a central molecule in the pathogenesis of asthma, contributes to the induction of remodeling. This remodeling is characterized by an increase in mucous producing cells, changes in the basement membrane and extracellular matrix, and changes in smooth muscle and airway vasculature. The epithelium is capable of producing many growth factors, including TGFα, that could contribute to the observed remodeling.

IL-13 contributes to the process of airway remodeling in many ways. The novel function of IL-13 discussed herein of initiating the intracellular redistribution of the growth factor TGFα, which ultimately results in shedding of the growth factor, may be a major mechanistic factor in airway remodeling. Proliferation and mucus production are both processes associated with IL-13 and airway remodeling, and these functions are both stimulated by TGFα. The fact that IL-13 induces this proliferation via TGFα and EGFR is key to understanding the underlying immune response involved in the onset and resolution of airway diseases such as asthma, at least for the components of the pathology that involve dynamic epithelial tissue changes. Control of excessive epithelial
proliferation could prevent deleterious effects of airway remodeling, specifically the increase in the epithelial layer that can result in a smaller airway diameter. This decreased airway diameter coupled with the increase in goblet cells contributes significantly to mucus plugging of the airways, a leading cause of mortality among asthmatics.

In the studies presented herein, the hypothesis that human bronchial epithelial cells release TGF\(\alpha\) in response to IL-13 and that TGF\(\alpha\) then binds the EGFR, initiating epithelial proliferation, is well supported. NHBE cells move intracellular reserves of TGF\(\alpha\) to the cell surface following exposure to IL-13. It is on the cell surface where the growth factor interacts with TACE resulting in growth factor shedding allowing for its migration and binding to EGFR, initiating cellular proliferation. NHBE cell proliferation following IL-13 stimulation requires both the MAP kinase and PI3K pathways. Since this is not the only known cytokine-growth factor-protease cycle that results in cellular proliferation, this TGF\(\alpha\) movement model could provide mechanistic insight upon which to base additional study regarding other inflammatory disease phenomena.
References cited


Churg, A., Brauer, M., Avila-Casado, M., Fortoul, T. & Wright, J. Chronic exposure to high levels of particulate air pollution and small airway remodeling. *Environmental Health Perspectives* 111, 714-718 (2003).


CHAPTER II
Title:

IL-13 Induces Proliferation of Human Airway Epithelial Cells In Vitro Via a Mechanism Mediated by Transforming Growth Factor-α

Authors:

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Citation:

ABSTRACT

Remodeling of the airways, as occurs in asthmatic patients, is associated with the continual presence of inflammatory mediators and Th2 cytokines, especially interleukin-13 (IL-13), during cycles of epithelial injury and repair. In this study, we examined the effect of IL-13 on well-differentiated normal human bronchial epithelial (NHBE) cells maintained in air/liquid interface culture. IL-13 induced proliferation of NHBE cells after 24 hours exposure, as reflected by $^3$H-thymidine uptake and cell counts. The effects of IL-13 were mediated through the epidermal growth factor receptor (EGF-R), as proliferation was attenuated by AG1478, an EGF-R tyrosine kinase inhibitor. Proliferation appeared to be mediated by transforming growth factor alpha (TGFα), a potent ligand for EGF-R, which was released rapidly from NHBE cells in response to IL-13. Neutralizing antibody to TGFα, but not antibodies against other potentially important growth factors (EGF, HB-EGF, PDGF), inhibited the mitogenic response to IL-13. This study provides the first experimental evidence that IL-13 can initiate a proliferative response of human airway epithelium in the absence of inflammatory cells or other cell types. The results are consistent with a mechanism whereby IL-13 induces release of TGFα from the epithelial cells, which in turn binds via an autocrine/paracrine-type action to the EGF-R, initiating proliferation. IL-13-induced airway remodeling in vivo may involve this epithelium-driven response.
INTRODUCTION

Airway remodeling, a major lesion in patients with asthma and other chronic inflammatory airway diseases, may involve perpetuation of the epithelial injury/repair cycle by the chronic presence of inflammatory mediators. Th2 cytokines, especially interleukin-13 (IL-13), have been implicated in the generation of a number of responses related to asthma and airway remodeling (1-5). IL-13 levels are greatly increased in lavage fluid from allergen-challenged asthmatic patients (6). In addition, IL-13 is associated with airway hyperresponsiveness (1), inflammation, eosinophilia, goblet cell hyperplasia, mucus hyperproduction (1,4,5) and subepithelial fibrosis (2) in murine models of asthma.

Although the precise pathogenesis remains unknown, epithelial cells have been found to undergo proliferation in remodeling airways (7-10). The epidermal growth factor receptor (EGF-R) has been shown to be important in remodeling that occurs in response to toxin-induced airway injury (11). The EGF-R also has been implicated in development of mucous cell hyperplasia in animal models (3,12).

As a potent ligand for the EGF-R (13,14), transforming growth factor alpha (TGFα) may be of major importance in the remodeling process. TGFα is produced by numerous types of epithelia in response to different insults (15,16), and the autocrine/paracrine function of TGFα and the EGF-R is a common theme in regulation of proliferating tissues, including intestinal cancers, papillary thyroid carcinomas, olfactory and mammary epithelium (17-20), and developing fetal tissue (21,22).

In this study, we hypothesized that proliferation of airway epithelium could be initiated by direct interaction between IL-13 and epithelial cells, and that this response
could be mediated by TGFα produced by the epithelial cells themselves. TGFα produced in response to IL-13 stimulation could then act, via an autocrine/paracrine-type mechanism, to bind to the EGF-R and induce proliferation. The results show that IL-13 can induce proliferation of differentiated normal human bronchial epithelial (NHBE) cells in vitro via a mechanism involving the EGF-R. This IL-13-induced proliferative response was inhibited by a neutralizing anti-TGFα antibody, but not by antibodies to other growth factors. Additionally, IL-13 provoked release of soluble TGFα from these cells. Since the epithelial cell cultures are devoid of other cell types (e.g. leukocytes), the results suggest the presence of a TGFα/EGF-R autocrine/paracrine loop in NHBE cells that can be activated by IL-13. This mechanism may relate to proliferation and remodeling in the airways of asthmatic patients.
Materials and Methods

Reagents

NHBE cells and bronchial epithelial growth media (BEGM) and supplements were purchased from Clonetics Corporation (Walkersville, MD). Transwell membranes were from Costar Corporation (Cambridge, MA). TGF\(\alpha\) ELISA kits and AG1478 were purchased from Calbiochem (La Jolla, CA). Anti-EGF and anti-HB-EGF antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while TGF\(\alpha\), anti-TGF\(\alpha\) and anti-PDGF antibodies, and goat total IgG were from R&D Diagnostics (Minneapolis, MN). All other reagents, except those used for cell culture listed below, were from Sigma (St. Louis, MO).

NHBE Cell Culture

Normal human bronchial epithelial cells from three different donors were expanded and grown as described previously (23). Briefly, following expansion, NHBE cells (passage 2) were plated at a density of approximately 35,000 cells per cm\(^2\) on 12-well transwell membranes coated for 1 hour with 50\(\mu\)g/ml rat-tail collagen, type I (Collaborative Research, Bedford, MA). Initially, the cells were kept submerged in a 50:50 mix (volume: volume) of BEGM: Dulbecco’s modified Eagle medium with high glucose (DMEM-H) and supplements including 0.13mg/ml bovine pituitary extract, 5 x 10\(^{-8}\) M all-\(\text{trans}\) retinoic acid, 1\(\mu\)g/ml bovine serum albumin (Intergen, Purchase, NY), 0.5ng/ml EGF, 0.5\(\mu\)g/ml hydrocortisone, 5\(\mu\)g/ml insulin, 10\(\mu\)g/ml transferrin, 0.5\(\mu\)g/ml epinephrine and 6.5ng/ml triiodothyronine (Clonetics). Medium was changed every other day until the cells reached confluence between days 6 to 8 in culture, at which time the
apical medium was removed while the basolateral medium was changed daily. For the purposes of this study, day 0 was defined as the day the cells reached confluence in culture. In addition, the air/liquid interface was established on this day (day 0). Experimentation was carried out on day 9 post-confluence when mature secretory cells are prominent.

[\(^{3}\text{H}\)]-Thymidine Incorporation Assay

NHBE cells were grown to confluence in air/liquid interface. Cells were grown without EGF for 24 hours prior to the \(^{3}\text{H}\)-thymidine incorporation assay. Cultures then were incubated with 1\(\mu\text{Ci/ml}\) \(^{3}\text{H}\)-thymidine (specific activity = 87.1 Ci/mmol) together with specific reagents described below. Following incubation for the indicated time period, the medium was removed and the cultures washed 3 times with ice-cold phosphate buffered saline. The entire Transwell inserts were then removed and placed in 1 ml of scintillation fluid, and incorporated radioactivity analyzed in a LKB 1209 RACKBETA liquid scintillation counter. In addition, cells were counted using a hemocytometer.

Exposures of NHBE Cells

NHBE cells were exposed on day 9. All experiments were done using cells from at least two different donors, and utilized concentrations of growth factors and inhibitors that exhibited no cytotoxicity to NHBE cells as determined by lactate dehydrogenase (LDH) assay. Determination of cytotoxicity by LDH assay was carried out as previously described for air/liquid interface cultures (24).
Proliferation in response to IL-13: IL-13 (10ng/ml) was added both basolaterally and apically to day 9 cells for 24 hours, after which time $^3$H-thymidine incorporation and cell counts were assessed.

Role of EGF-R in IL-13- and TGFα-induced proliferation: Cells were exposed for 24 hours to IL-13 (10ng/ml) in the presence or absence of the specific EGF-R tyrosine kinase inhibitor AG1478 (0.1, 1, 5µg/ml) (25,26), after which thymidine incorporation was measured as described above.

Role of TGFα in IL-13-induced proliferation: To determine whether or not TGFα was involved in the proliferative response to IL-13, effects of neutralizing antibodies to TGFα and a number of other potentially important growth factors (e.g. epidermal growth factor [EGF]; platelet-derived growth factor [PDGF]; and heparin-binding epidermal growth factor-like growth factor [HB-EGF]) on IL-13-induced proliferation were assessed. Initial studies utilized a range of concentrations (50, 5, 0.5µg/ml; data not shown) to determine an optimal concentration of anti-TGFα antibody that affected proliferation in response to IL-13. Thus, this optimal concentration (0.5µg/ml) was used for all subsequent neutralizing antibody studies. Day 9 (well-differentiated) cells were exposed to 0.5µg/ml of the above neutralizing antibodies together with IL-13 (10ng/ml) and $^3$H-thymidine, as described above for 24 hours, at which time proliferation was measured as described above.
Release of TGFα by NHBE Cells in Response to IL-13

Day 9 cells were exposed to IL-13 (10ng/ml) in the presence of complete culture medium apically and basolaterally for either one or three hours, at which time apical media were analyzed for the presence of soluble TGFα using a commercially available ELISA (Calbiochem, LaJolla, CA) according to the manufacturer’s guidelines.

Data Analysis

The SigmaStat 2.03 software package from SPSS, Inc. (Chicago, IL) was used for all analyses. For analysis of 3H-thymidine uptake data, one-way analysis of variance (ANOVA) with appropriate post-test correction for multiple comparisons was performed. Soluble TGFα ELISA data were analyzed using a Student’s t-test. Data were considered significant at p<0.05. Replicates (2 to 8) of entire experiments were performed, and representative data are presented as means ± standard error of the mean (SEM).
Results

Proliferative response to IL-13

To determine whether IL-13 can induce proliferation of NHBE cells, $\text{^3H}$-thymidine incorporation in primary cultures exposed to IL-13 was examined. $\text{^3H}$-thymidine incorporation was increased significantly following a 24-hour exposure to IL-13 in day 9 cells ($p<0.001$, $n=12$) (Figure 1). This finding correlated with a significant increase in total cell number in day 9 cells exposed to IL-13 (10ng/ml) for 24 hours as assessed by counting. These data demonstrate that NHBE cells proliferate in response to IL-13.

IL-13 induces proliferation via EGF-R

To determine whether or not the epidermal growth factor receptor is involved in IL-13-induced proliferation, NHBE cells were incubated for 24 hours with IL-13 (10ng/ml) together with the compound AG1478, a specific inhibitor of EGF-R tyrosine kinase (25). AG1478 blocked $\text{^3H}$-thymidine incorporation in these cultures in a concentration-dependent manner (Figure 2A). When AG1478 was added together with TGF$\alpha$ (5ng/ml), a concentration-dependent inhibition of $\text{^3H}$-thymidine incorporation was observed similar to the effects of AG1478 on IL-13-induced proliferation (Figure 2B). These data indicate that both IL-13- and TGF$\alpha$-induced proliferation of NHBE cells is mediated by the EGF-R.
TGFα mediates IL-13-induced proliferation

Since IL-13 is not known to serve directly as a ligand for the EGF-R, we examined the ability of a variety of neutralizing antibodies against known growth factors to block IL-13-induced proliferation of NHBE cells. Anti-TGFα antibody at a concentration of 0.5µg/ml completely blocked IL-13 induced proliferation (p<0.005, n=6); other neutralizing antibodies (anti-EGF, anti-HB-EGF, anti-PDGF and total IgG) at the same concentration had no significant effect (Figure 3). As an additional control, anti-TGFα antibody at 5µg/ml also blocked ³H-thymidine incorporation in these cells in response to exogenously applied TGFα (5ng/ml), indicating specificity of the neutralizing antibody for TGFα (Figure 3). Thus, IL-13-induced proliferation of NHBE cells appears to be mediated by TGFα.

TGFα is a member of the EGF family of ligands, and increased levels of TGFα have been documented in several hyperproliferative epithelial disorders (27-29). Following short incubations of NHBE cells with IL-13, ELISA was used to examine media for soluble TGFα released by the cells. Within one hour, and maintained through 3 hours of exposure, a significant (p<0.05, n = 3) increase in soluble TGFα compared to control cultures was observed (Figure 4). Thus, IL-13 induces a rapid increase in release of soluble TGFα from NHBE cells, and this growth factor appears to mediate subsequent proliferation of these cells.
Discussion

IL-13 has been shown to play a key role in the pathogenesis of asthma and remodeling of inflamed airways (1-5). This report provides the first evidence that IL-13 can directly initiate a proliferative response in human airway epithelium. The mechanism responsible for this proliferation appears dependent on IL-13-induced release of epithelial-derived TGFα, a growth factor that can bind to the EGF-R and initiate proliferation. Importantly, the results indicate that proliferation of epithelial cells in response to IL-13 can occur in the absence of other cell types, such as neutrophils or eosinophils, and that the epithelium itself is a source of TGFα.

While this response occurred in vitro, one would anticipate similar effects of IL-13 in vivo, where its increased levels in airways of allergen-challenged asthmatics have been documented (6). Upon allergen-challenge, the level of IL-13 in lavage fluid from asthmatics has been shown to be in the range of 0.4 - 3ng/ml (6). Hence, the concentration of IL-13 (10ng/ml) used in these studies is comparable to the concentration of IL-13 available to interact with airway epithelium in vivo in diseased airways. As illustrated in Figure 4, IL-13 induces release of 100 - 300pg/ml of TGFα from NHBE cells in vitro after one-hour exposure. We have observed that 500pg/ml TGFα can cause direct proliferation of these cells, further confirming the ability of TGFα to act as the sole ligand mediating IL-13-induced NHBE cell proliferation. This potential mechanism is further confirmed, as the mitogenic effects of even a 10-fold higher concentration of TGFα (5ng/ml) were attenuated by neutralizing antibodies against this growth factor. IL-13-induced proliferation also was inhibited by concentrations of the EGF-R tyrosine kinase inhibitor AG1478 (1, 5µg/ml) similar to those found to be effective in attenuating
activity of this kinase in primary pulmonary myofibroblasts exposed to TGFα (26). Taken together, these observations suggest that the cytokines, growth factors and other reagents involved in these studies of IL-13-induced proliferation of NHBE cells in vitro appear to do so at (patho)physiological concentrations relevant to the in vivo situation.

Based on the results of these studies, the most likely scenario for IL-13-induced proliferation of NHBE cells is that IL-13 induces release of soluble TGFα, that in turn binds to the EGF-R on these cells and initiates proliferation. The EGF-R exists as a heterodimer, and the various heterodimeric receptor chains have different affinities for ligands composing the EGF growth factor family. The EGF-R chain with the highest affinity for TGFα is the EGF-R (or HER1) polypeptide chain (13,14). The EGF-R is capable of forming homodimers or heterodimers with HER3 or HER4 chains. Upon binding TGFα, initiation of tyrosine phosphorylation of these chains is dependent on cell type (14). The inhibitory effects of AG1478 and neutralizing TGFα antibodies on IL-13-induced proliferation observed in this study suggest TGFα is involved integrally in this proliferative response.

Inflammatory mediators may be present for prolonged periods of time in diseased airways, and it is believed that this chronic inflammation contributes to the profound airway remodeling observed in patients with long-term respiratory illnesses. In preliminary studies from our laboratory, we have reported that long-term exposure of NHBE cells to IL-13 also provokes release of soluble TGFα (30). In addition, long-term IL-13 exposure increases the percentage of Alcian blue/PAS positive mucus-producing cells in these cultures (31), suggesting a possible link between the long-term presence of IL-13 and development of a mucous phenotype in airway epithelium. Although both IL-
13 and the EGF-R have been implicated in development of mucous cell hyperplasia during airway remodeling (3), it remains to be seen whether the TGFβ/EGF-R proliferative response described in this study plays a direct role in development of this lesion.

While the experiments reported here were carried out in vitro, the model epithelial system used develops well-differentiated epithelial cells, similar in structure and function to human airway epithelium in vivo. Recognizing the limitations of this in vitro system, the results of this study highlight the potential contribution of the airway epithelium itself to possible mechanisms of airway remodeling. While precise mechanisms regulating all aspects of airway remodeling remain to be elucidated, it appears that interactions between IL-13 and the human airway epithelium have the potential to provoke a number of responses related to remodeling, and to do so in the absence of additional inflammatory cells.
REFERENCES


Figure 1. IL-13 induces proliferation of NHBE cells in vitro. $^3$H-thymidine incorporation in NHBE cells following exposure to IL-13. Cultures were maintained for 24 hrs in EGF-free media and then exposed to IL-13 (10ng/ml, 24 hrs) in the presence of the radioactive label. Error bars indicate SEM. $n = 12$, * = significantly different from control, p<0.001.
A) 70000

Cpm/culture

0 0 0.1 1 5 AG1478 µg/ml

Con IL-13 10 ng/ml

B) 70000

Cpm/culture

0 0 0.1 1 5 AG1478 µg/ml

Con TGF-α 5 ng/ml
**Figure 2.** Inhibition of EGF-R tyrosine kinase activity attenuates IL-13-induced $^3$H-thymidine incorporation in NHBE cells. AG1478, a specific EGF-R tyrosine kinase inhibitor, was added to NHBE cells for 30 minutes prior to addition of IL-13 (A) or TGFα (B) plus $^3$H-thymidine. The cultures were then incubated for 24 hours with all reagents present. Error bars indicate SEM. $n = 4$ to 10, * = significantly different from control, $p<0.05$. † = Significantly different from primary treatment, $p<0.01$.

**Figure 3.** Effect of neutralizing antibodies on IL-13-induced proliferation. NHBE cell cultures were exposed to IL-13 (10ng/ml, 24 hrs) in combination with $^3$H-thymidine and the appropriate antibody (0.5µg/ml; T= anti-TGFα, I= IgG, P= anti-PDGF, E= anti-EGF, H= anti-HB-EGF). As a further control, TGFα (5ng/ml, 24 hrs) alone induced a proliferative effect that was attenuated by addition of anti-TGFα antibody (0.5µg/ml; T). Error bars indicate SEM. $n=6$; * = significantly different from control, $p<0.05$; † = significantly different from IL-13 treatment alone, $p<0.05$; ‡ = significantly different from TGFα treatment alone, $p<0.05$. 

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Figure 4. Release of soluble TGFα from NHBE cells is increased by exposure to IL-13. Differentiating NHBE cells (day 9) were exposed to IL-13 (10ng/ml) or control media for 1 or 3 hrs. Release of soluble TGFα during this incubation time was measured by ELISA. Error bars indicate SEM. n = 3 for each group, * = significantly different from control; p<0.05.
CHAPTER III
IL-13-Induced Proliferation of Human Airway Epithelial Cells Requires Activation of MAP Kinase and PI3Kinase/Akt Pathways

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Abstract

Involvement of interleukin (IL)-13 in allergic asthma and resulting airway remodeling is well documented. Previous work from our laboratory provided mechanistic evidence implicating airway epithelium as an active participant in IL-13-induced epithelial cell proliferation during remodeling. Specifically, we demonstrated that IL-13 induces proliferation of normal human bronchial epithelial (NHBE) cells through a transforming growth factor-α (TGFα)/epidermal growth factor receptor (EGFR) autocrine/paracrine loop. Here, we expand the mechanistic understanding of IL-13-induced NHBE cell proliferation by demonstrating that IL-13 activates the mitogen activated protein kinase kinase/extracellular-signal regulated kinase (MEK/Erk) signaling cascade as well as the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway. We also demonstrate that both pathways are required for IL-13-induced NHBE cell proliferation, as inhibition of either pathway blocks proliferation. IL-13-induced activation of the MEK/Erk pathway, however, does not appear to be inhibited by neutralizing anti-TGFα antibodies nor by an inhibitor of EGFR tyrosine kinase, suggesting MEK/Erk activation is not mediated solely by TGFα or EGFR. Additionally, PI3K is involved in IL-13-induced release of TGFα from the epithelial cells. These results demonstrate that IL-13 activates MEK/Erk and PI3K/Akt pathways, both of which are important for NHBE cell proliferation. The IL-13-induced TGFα/EGFR interaction, however, is unlikely the sole mediator for coordinating activation of these pathways.
INTRODUCTION

Epithelial cell injury and repair occurs frequently in airway diseases, such as asthma, that are characterized by recurrent periods of airway inflammation. These continuing cycles of injury and repair are thought to induce structural changes in the airway walls referred to collectively as airway remodeling (1-4). These changes appear similar regardless of the injury source, be it pollutants, bacterial products, ventilator-induced mechanical force, crushing force, or inflammatory mediators that arise in response to pathogens (2,3,5). While airway structural changes include alterations of multiple cell types that comprise the airway wall, the epithelium in particular develops an overall thickening (2) likely, in part, as a result of cellular proliferation. In addition, an increase in goblet cell number occurs, changing the ratio of mucus-producing to ciliated cells (6,7).

Using murine models of allergic asthma, interleukin (IL)-13 has been identified as a central mediator of numerous characteristics of the asthmatic phenotype including airway hyperreactivity, goblet cell metaplasia and mucus overproduction (8-10). More recently, IL-13 has also been linked to the development of asthma in humans with genetic polymorphisms discovered within the IL-13 gene showing a significant association with atopic and non-atopic asthma (11-14).

We have demonstrated previously that IL-13 induces proliferation in normal human bronchial epithelial (NHBE) cells in vitro, a finding that may help explain in vivo observations of epithelial hypertrophy in IL-13 transgenic mice (15). In our NHBE cell model, IL-13 induces proliferation via an autocrine growth loop involving transforming growth factor-α (TGFα) and its receptor, the epidermal growth factor receptor (EGFR)
While the effects on pathways downstream of EGFR in NHBE cells exposed to IL-13 are currently unknown, ligand-mediated stimulation of EGFR is known to initiate mitogen activated protein (MAP) kinase signaling cascades in numerous cell types (17), with such cascades shown to be activated by IL-13 in human keratinocytes (18) and airway smooth muscle cells (19,20).

Like MAP kinase signaling cascades, the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is also recognized as a potent regulator of cell proliferation (21). In intestinal epithelial cells, this PI3K/Akt signaling pathway has been found to induce survival mechanisms following activation by IL-13 (22,23).

In this study, we have focused on characterization of the intracellular signaling pathways that control IL-13 induced proliferation of NHBE cells. Specifically, the involvement of well-recognized proliferative cascades that employ MAP kinases and PI3K was examined. Our results demonstrate that both pathways, MAP kinase and PI3K/Akt, are required for IL-13 induced proliferation of NHBE cells \textit{in vitro}, with exposure to the cytokine resulting in increased phosphorylation of extracellular-signal regulated kinase (Erk) and Akt, respectively. Activation of the MAP kinase pathway, however, does not appear exclusively dependent on the autocrine action of TGF\(\alpha\), as neutralizing anti-TGF\(\alpha\) antibodies and an EGFR tyrosine kinase inhibitor that effectively block IL-13-induced proliferation, do not similarly affect phosphorylation of Erk.

Involvement of the PI3K/Akt cascade in IL-13-induced proliferation of NHBE cells also appears complex; while the PI3K/Akt pathway is activated when exogenously added TGF\(\alpha\) binds to EGFR, PI3K also mediates IL-13 induced release of TGF\(\alpha\), an event upstream of the EGFR in NHBE cells following IL-13 exposure. These data point to a
complex system of interacting signal transduction pathways that are required for IL-13-induced proliferation of NHBE cells.
Materials and Methods

Reagents

NHBE cells, bronchial epithelial growth media (BEGM), and supplements were purchased from Cambrex (Walkersville, MD). Transwell membranes were purchased from Costar Corporation (Cambridge, MA). AG1478, LY294002, PD98059 and U0126 were purchased from Calbiochem (La Jolla, CA). IL-13, TGFα, and neutralizing anti-TGFα antibodies were purchased from R&D Diagnostics (Minneapolis, MN). Anti-Erk, anti-phospho-EGFR, and anti-EGFR were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Erk, anti-Akt, and anti-phospho-Akt were from Cell Signaling Technology (Beverly, MA). Secondary antibodies were from Santa Cruz Biotech (Santa Cruz, CA). Protein A-Sepharose beads were purchased from Roche Diagnostics (Indianapolis, IN). All other reagents, except those needed for cell culture as referenced below, were purchased from Sigma (St. Louis, MO).

Methods

NHBE Cell Culture

NHBE cells (Cambrex, Walkersville, MD) were grown on transwell membranes as described previously (24). Briefly, following expansion, NHBE cells (passage 2) were plated at a density of approximately 35,000 cells per cm² on transwell membranes coated for 1 hour with 50 µg/ml rat-tail collagen, type I (Collaborative Research, Bedford, MA). Initially, the cells were kept submerged in a 50:50 mix (volume: volume) of BEGM: Dulbecco’s modified Eagle medium with high glucose (DMEM-H) supplemented with 0.13mg/ml bovine pituitary extract, 5 x 10⁻⁸ M all-trans retinoic acid (Sigma), 1 µg/ml
bovine serum albumin (Intergen, Purchase, NY), 0.5 ng/ml EGF, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin, 0.5 µg/ml epinephrine, 6.5 ng/ml triiodothyronine (Cambrex), and 44.0 IU/ml nystatin (Amresco, Solon, OH). Medium was changed every other day until the cells reached confluence between days 6 to 8 in culture, at which time the apical medium was removed to establish an air/liquid interface (ALI) after which time the basolateral medium was changed daily.

**Western blot**

Total cellular protein was extracted from NHBE cells using RIPA extraction buffer (1x phosphate buffered saline, % NP-40; 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Protein concentration was determined using the Bradford dye-binding procedure (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA). Equal amounts of protein per sample were prepared for electrophoretic separation by mixing 1:1 with 2X SDS gel loading buffer (100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM β-mercaptoethanol), and then boiling for 5 minutes. Samples were then fractionated on 10-20% gradient precast SDS-PAGE gels (Bio-Rad) followed by transfer to nitrocellulose membranes. Membranes were blocked for 1h in PBST (phosphate buffered saline with 0.1% Tween-20) containing 5% nonfat milk and then incubated overnight at 4°C with primary antibodies diluted 1:1000. The membranes were then washed twice with PBST (30 min each wash) followed by incubation for 1h at room temperature with HRP-conjugated secondary antibody diluted 1:5000 in 5% nonfat milk/PBST. After two, 30-min washes with PBST, the immunoreactive protein
complexes were visualized using enhanced chemiluminescence (ECL) (Amersham Biologicals, Buckinghamshire, England) as the detection method. To visualize multiple proteins using the same blot, membranes were stripped using a Re-Blot Western Blot Recycling Kit (Chemicon Int., Temecula, CA), and then reprobed with appropriate antibodies.

*Immunoprecipitation and EGFR analysis*

Total cellular protein was extracted from NHBE cells using RIPA extraction buffer as described above. Lysates were immunoprecipitated overnight at 4°C in the presence of anti-EGFR antibody and Protein A-Sepharose beads on a rocking platform. The antigen-antibody-Protein A-Sepharose bead complexes were pelleted by centrifugation and washed three times with ice-cold extraction buffer. Following the final wash, the complexes were resuspended in gel loading buffer, boiled for three minutes, and then resolved by SDS-PAGE. Immunoblotting with specific antibody was carried out as described above.

*Measurement of TGFα by ELISA*

Media collected from the basolateral surface of NHBE cell cultures was analyzed for the presence of TGFα using a commercially available ELISA (Calbiochem, La Jolla, CA) according to the manufacturer’s guidelines.
Experimental protocols

For the purpose of this study, day 0 was defined as the day the cells reached confluence in culture. In addition, the ALI was established on this day (day 0). Experimentation was carried out on days 7-9 post-confluence when mature secretory cells are prominent. All experimentation was repeated using epithelial cells isolated from at least two different donors.

Concentration of IL-13

The optimal proliferation-inducing concentration of IL-13, 10 ng/ml, was determined by performing [³H]-thymidine incorporation assay using a broad range (0.1-100 ng/ml) of IL-13 concentrations. The day of optimal IL-13 induced proliferation was determined for each donor-derived set of cells using a [³H]-thymidine incorporation assay. Optimal day of proliferation was always Day 8 or 9 post-confluence. For all [³H]-thymidine incorporation studies, NHBE cells were incubated with EGF-free media for the 24 hrs prior to IL-13 exposure. Cells were treated with IL-13 from the basolateral surface only in all experiments. All inhibitors and neutralizing antibodies were added 30 min prior to IL-13 exposure. The concentration of neutralizing anti-TGFα used was determined previously (16), and all inhibitors were used in a range of concentrations to determine the optimal concentration of each inhibitor.

Proliferation Studies

NHBE cells grown in 50:50 media without EGF for 24 hrs. The NHBE cells were then incubated with 1 µCi/ml [³H]-thymidine (specific activity = 87.1 Ci/mmol) (Perkin
Elmer, Boston, MA) in the presence of specific reagents as described below. Following incubation for the indicated time period, the medium was removed and the cultures washed with ice-cold PBS and the cells treated with 10% TCA. The entire Transwell insert was then removed and placed in 4 ml of scintillation fluid, and incorporated radioactivity analyzed in a LKB 1219 RACKBETA liquid scintillation counter. Proliferation was further characterized by performing cell counts. To perform manual cell counts, NHBE cells were liberated by applying warm Versene (Invitrogen, Grand Island, NY) for 5 min and then counted using a hemacytometer.

**Exposure for Western Analyses**

Cells used in experiments to examine phospho-Erk were exposed to inhibitors or neutralizing anti-TGFα antibody for 30 min prior to addition of IL-13 or TGFα. Proteins isolated from cells used to analyze phospho-Akt were first isolated at time 0, the time point right before addition of any inhibitors or inducing agents. A time course of total cellular proteins was then isolated 5, 15, 30, and 60 min after the simultaneous addition of IL-13 or TGFα, with or without the inhibitor LY294002.

**Sample collection for ELISA analysis**

NHBE cells were exposed to IL-13 (10 ng/ml) in the presence of complete culture medium for one hour, after which time media samples were collected from the basolateral compartment and stored overnight at 4°C. The samples were then analyzed using a commercially available TGFα ELISA kit (Calbiochem).
Statistical analysis of data

The SigmaStat 2.03 software package from SPSS, Inc. (Chicago, IL) was used for all analyses. For analysis of proliferation data, one-way analysis of variance (ANOVA) with appropriate post-test correction (Tukey) for multiple comparisons was performed. Data were considered significant at $p<0.05$. Replicates (2 to 6) of entire experiments were performed, and representative data are presented as means ± standard error of the mean (SEM).
RESULTS

Requirement of MAP kinase pathway in IL-13-induced proliferation

Previous work from our laboratory demonstrated that IL-13 induces proliferation of NHBE cells in vitro (16). To further characterize this proliferative mechanism, we set out to examine intracellular signal transduction pathways known to be involved in cellular proliferation with the goal of discerning whether such pathways regulate IL-13-induced airway epithelial cell proliferation. To determine whether or not IL-13-induced proliferation of NHBE cells is mediated by the MAP kinase signal transduction pathway, the inhibitor PD98059 was co-incubated with IL-13 in a [3H]-thymidine incorporation assay. PD98059 acts by inhibiting MAP kinase kinase (MEK), thereby blocking the activation of Erk. PD98059 inhibited IL-13-induced proliferation in a concentration-dependent manner (20 and 0.2 µM; Fig. 1A). Similarly, another MAP kinase inhibitor, U0126 (100 and 25 nM), which inhibits MEK1 and MEK2 activity, also inhibited IL-13-induced proliferation as assayed using [3H]-thymidine incorporation (Fig. 1B). Cell counts taken following inhibitor treatment further confirm that IL-13 induces proliferation of NHBE cells while PD98059 and U0126 block this event (Fig. 1C). These data indicate that the MAP kinase pathway mediates IL-13-induced NHBE cell proliferation in vitro. In addition, we noted that some maintenance level of epithelial cell proliferation also appears to be mediated by the MAP kinase pathway as MEK inhibitors alone reduced constitutive levels of [3H]-thymidine incorporation or total cell numbers (Figs. 1A, B and C).
**PI3K is also necessary for IL-13-induced proliferation**

Another signaling cascade often involved in cellular proliferation is the PI3K pathway. We next investigated whether or not this pathway was also involved in IL-13-induced proliferation of NHBE cells *in vitro*. An inhibitor of PI3K, LY294002 (3, 0.3 μM), was co-incubated with IL-13 in a [³H]-thymidine incorporation assay. LY294002, while having no impact on constitutive proliferation, did inhibit IL-13-induced proliferation of NHBE cells in a concentration-dependent manner (Fig. 2). This result indicates that PI3K is also required for IL-13-induced proliferation of NHBE cells *in vitro*.

**Involvement of MEK/Erk in IL-13-induced proliferation**

Thus, as inhibitors of either the MAP kinase or the PI3K pathway block IL-13-induced proliferation of NHBE cells in vitro, it appears that both of these pathways are required for this IL-13-induced proliferation. Next, we wanted to examine the effect of IL-13 induction on common substrates in these pathways in an effort to establish a hypothetical pathway through which this proliferation is mediated. With regard to the MAP kinase pathway, IL-13 and TGFα each induced phosphorylation of Erk within 15 min of exposure, an event that was not observed 15 min after a media change in untreated cells (Fig. 3A). Furthermore, phosphorylation of Erk remained elevated for at least 30 minutes post-treatment. Both the IL-13- and TGFα-induced phosphorylation events could also be blocked by the MEK inhibitor U0126 (25 nM; Figs. 3B and C, respectively). Since MEK is known to phosphorylate Erk, these results demonstrate that both stimuli, IL-13 and TGFα, activate the MAP kinase pathway upstream of MEK.
Role of TGFα/EGFR loop in IL-13-induced Erk activation

We have shown previously that the autocrine release of TGFα mediates IL-13-induced proliferation of NHBE cells in vitro (16). Thus, we wished to determine whether IL-13 affected the MAP kinase pathway through this autocrine/paracrine action of TGFα. As noted above, following NHBE cell exposure to IL-13 or TGFα for various lengths of time (5, 15, 30 and 60 min), the phosphorylation of Erk was investigated. IL-13 and TGFα both induced the phosphorylation of Erk above the level observed in untreated cells. The presence of neutralizing anti-TGFα antibody had little effect on IL-13-induced Erk phosphorylation, although neutralizing anti-TGFα antibody did attenuate TGFα-induced Erk activation by 30 min. post-exposure (Fig. 3A).

Another element of the autocrine mechanism governing IL-13-induced proliferation of NHBE cells is the binding of TGFα to its receptor, the EGFR, on these same cells. As shown in Fig. 4A, following a 15-minute exposure of NHBE cells to IL-13 (10 ng/ml) or TGFα (5 ng/ml), proteins immunoprecipitated from these cells using an anti-EGFR antibody, displayed a significant increase in the phosphorylation state of the EGFR when compared to phosphorylated-EGFR from untreated cells.

To further examine the role of EGFR in MAP kinase signaling during IL-13-induced proliferation, NHBE cells were exposed to the EGFR tyrosine kinase inhibitor AG1478 at 1 µM, a concentration previously shown to block IL-13-induced proliferation (16). IL-13 induced phosphorylation of Erk by 15 min post-exposure and this level of Erk phosphorylation remained constant whether or not the inhibitor was present (Fig 4B). Presence of the inhibitor alone did not induce phosphorylation of Erk (Data not shown). Since neither inhibition of the kinase activity of EGFR nor neutralization of TGFα using
anti-TGFα antibodies blocked IL-13-induced phosphorylation of Erk, it stands to reason that IL-13 activates the MAP kinase pathway in a TGFα/EGFR-independent manner. Taken together, these data suggest that Erk phosphorylation in response to IL-13 in NHBE cells is not mediated solely by the autocrine production of TGFα and its subsequent binding to the EGFR.

**IL-13 affects the PI3K/Akt pathway**

Having demonstrated that IL-13 initiates a proliferative response in NHBE cells mediated by the PI3K pathway (Fig 2), we wanted to examine the effect of IL-13 on the common PI3K substrate, Akt/protein kinase B (PKB), as part of our effort to establish a central hypothesis governing IL-13 induced NHBE cell proliferation. Following IL-13 treatment, Akt/PKB is phosphorylated in NHBE cells (Fig. 5), with the level of phosphorylation doubling that observed in untreated cells (time 0) by 60 min post-exposure to IL-13. (Densitometry data not shown). Addition of the PI3K inhibitor LY294002 blocks the constitutive and stimulated phosphorylation of Akt/PKB. Similarly, stimulation of NHBE cells with TGFα also results in phosphorylation of Akt/PKB, an event that can be blocked with LY294002 (Fig 5). These data indicate that both IL-13 and TGFα can provoke the phosphorylation of Akt/PKB via PI3K.

To further characterize the role of PI3K in IL-13-induced proliferation of NHBE cells, we wanted to determine the location of PI3K involvement in this proliferative pathway. We have demonstrated already that PI3K is affected by IL-13 (Figs 2 and 5) and it is also affected following TGFα stimulation leading us to believe that PI3K is involved in NHBE cell proliferation following TGFα binding to its receptor EGFR. As
verified in Figure 6A, LY294002 blocks TGFα-induced proliferation in a concentration-dependent manner further supporting the possibility that PI3K involvement in NHBE cell proliferation occurs downstream of EGFR. However, LY294002 also inhibits IL-13-induced release of soluble TGFα by NHBE cells (Fig 6B) suggesting that PI3K is involved downstream of the IL-13 receptor complex but prior to TGFα interacting with its receptor. Taken together, the data suggest that PI3K is involved in multiple stages during IL-13-induced proliferation of NHBE cells. First, PI3K is involved in the release of TGFα from NHBE cells following IL-13 exposure, and, second, PI3K is downstream of the EGFR, mediating the proliferative pathway initiated by the autocrine action of TGFα.
Discussion

IL-13 has been implicated in allergic asthma where it is associated, among other phenotypes, with the phenomenon of airway remodeling in response to chronic airway inflammation. Chronic inflammation perpetuates epithelial cell proliferation, possibly inducing mucous cell metaplasia, and can also induce proliferation of underlying fibroblasts leading to subepithelial fibrosis. This overall remodeling process can be thought of as a multi-step event that includes migration of epithelial cells into wounded or denuded areas, followed by proliferation and differentiation, and finally by reduction of excess epithelial cells which resulted from the proliferation. Interruption of these steps may result in permanent alterations such as those observed in airway tissue recovered from severe asthmatics and chronic bronchitis postmortem (3, 25).

During the remodeling process associated with chronic airway inflammation, the presence of TH2 cytokines, in particular IL-13, plays an integral role in inducing epithelial changes. In transgenic mouse models of asthma, IL-13 has been linked to the development of mucous cell metaplasia (15), an event that is STAT6-dependent in vivo (10). Furthermore, IL-13 appears to also direct epithelial cell differentiation toward the formation of a goblet metaplastic phenotype in human epithelial cells in vitro (6). Such an increase in goblet cells, accompanied by a corresponding decrease in ciliated cells (7), could result in an accumulation of mucin within the airways. Due to the effect of IL-13 on the reduction of ciliary beating, this mucin would not be as readily cleared as in a normal, non-remodeled airway (7).

Previous work from our laboratory provided mechanistic evidence implicating the airway epithelial cell as an active, and potentially primary, participant in IL-13-induced
epithelial cell proliferation during airway remodeling. Specifically, we showed that IL-13 induces epithelial cell proliferation through a TGFα-mediated autocrine/paracrine loop, wherein TGFα binds EGFR on epithelial cells (16). Furthermore, this proliferative mechanism was induced by direct interaction of the purified cytokine with epithelial cells, thus eliminating the requirement of additional inflammatory cells or their products in this process. In this report, we expand our mechanistic understanding of IL-13-induced proliferation of NHBE cells by demonstrating that IL-13 activates the MEK/Erk signaling cascade as well as the PI3K/Akt signal transduction pathway. We show further that both pathways are required for IL-13 induced proliferation of NHBE cells in vitro, as inhibition of either signaling pathway blocks IL-13-induced NHBE cell proliferation. Additionally, PI3K was found to play a role in IL-13-induced release of TGFα.

It has been known for some time that MAP kinase pathways can be activated by TGFα in a variety of epithelial cell types including the olfactory mucosa (26), epidermal keratinocytes (27) and intestinal epithelial cells (28), following binding of this growth factor to the EGFR. The MAP kinase signaling pathway is acknowledged generally as a proliferative pathway activated in response to various growth-related stimuli including: growth factors (EGF, PDGF, and FGF), cytokines (IL-1, -3, -5, and -12), and other compounds such as hormones (insulin and progesterone), lipids (lysophosphatidic acid) and secretagogues (nicotine and prostaglandins) (reviewed in 29). Thus, it seemed logical that since the IL-13-induced proliferative pathway in NHBE cells was initiated by TGFα acting as an EGFR ligand, this pathway would act ultimately through MAP kinases.

Our initial hypothesis for this study consisted of a linear progression in which IL-13 induced the release of TGFα from the epithelium, after which the growth factor bound
EGFR initiating the MAP kinase signal transduction pathway to control epithelial cell proliferation. Our results do demonstrate that IL-13 induces activation of EGFR via epithelium-derived TGFα, and that exposure to IL-13 or TGFα leads to phosphorylation of Erk. Neutralizing anti-TGFα and the EGF tyrosine kinase inhibitor AG1478 both block IL-13 induced proliferation; however, inconsistent with our initial hypothesis, these inhibitors did not appreciably block phosphorylation of Erk at concentrations that attenuated proliferation effectively. Thus, phosphorylation of Erk appears to be independent of TGFα/EGFR interaction, suggesting that additional signaling pathways, albeit pathways not downstream of the EGFR, may be involved in IL-13-induced phosphorylation of Erk.

While exploring this new hypothesis we discovered that MEK inhibitors, PD98059 or U0126, blocked IL-13 induced proliferation as well as the phosphorylation of Erk in NHBE cells. Taken together, these data demonstrate that the MAP kinase pathway is involved in IL-13 induced proliferation of NHBE cells even though activation of this pathway may not be dependent upon the TGFα/EGFR interaction.

Since the data we generated did not fully substantiate our initial hypothesis, we investigated the possibility that additional signaling cascades, specifically PI3K-mediated pathways, are involved during IL-13-induced proliferation of NHBE cells in vitro. Various recent findings suggest PI3K may play a prominent role in mediating epithelial cell events. For example, it was demonstrated that in addition to activation of the MAP kinase pathway, activation of the PI3K signaling pathway is required for bronchial epithelial cell proliferation following stimulation with hepatocyte growth factor (30). IL-
13 has also been shown previously to activate PI3K in colonic epithelial cells (22, 31), where the cytokine activates an Akt-dependent survival mechanism (23).

Based on results presented here, we have established a hypothesis, illustrated in Figure 7 that describes the involvement of at least two intracellular signaling pathways in the regulation of IL-13-induced proliferation of NHBE cells in vitro. Following the binding of IL-13 to its receptor complex, the MAP kinase and PI3K pathways are activated, noted as increased phosphorylation of Erk or Akt, respectively. Similarly, inhibition of either pathway blocks both IL-13 and TGF\(\alpha\) induced cell proliferation, suggesting both pathways are required during stimulated proliferation.

PI3Ks are comprised of three classes based on their subunit structure and preferred lipid substrate target. Only class Ia is phosphorylated by tyrosine kinases (32) suggesting that members of PI3K class Ia are involved in both IL-13- and TGF\(\alpha\)-induced proliferation since tyrosine kinases are associated with IL-13R complexes and the EGFR. The serine/threonine protein kinase Akt/PKB is one downstream target of PI3K Ia. Akt/PKB has proven to be a major regulator of PI3K-mediated cell proliferation in mammary epithelium following erbB receptor activation (33). Our data demonstrate that following IL-13 exposure Akt becomes phosphorylated in NHBE cells as well. The PI3K inhibitor LY294002 blocks both IL-13- and TGF\(\alpha\)-induced phosphorylation of Akt, both IL-13 and TGF\(\alpha\)-induced proliferation, and the IL-13-induced release of TGF\(\alpha\) in NHBE cells. These results suggest the likelihood that the PI3K signaling cascade serves multiple functions following IL-13 exposure. One role for this enzyme appears to be immediately downstream of the IL-13 receptor complex, where it participates in
mediating the release of TGFα. Secondly, PI3K is downstream of EGFR as
demonstrated by the ability of LY294002 to inhibit TGFα induced proliferation.

Since multiple isoforms of each PI3K class exist, it is quite feasible that PI3Ks
can function in multiple steps during IL-13-induced proliferation of NHBE cells, with
one step before and one after activation of the EGFR. Alternatively, a more complex
interplay between IL-13R- and EGFR-induced pathways might occur in the presence of
IL-13 induction. Hence, the role of PI3K downstream of EGFR might be modified in the
presence of IL-13, such that its function differs from when it is activated subsequent to
TGFα/EGFR stimulation in the absence of the cytokine.

The mechanism by which IL-13 activates PI3K may involve the insulin receptor
substrate (IRS)-1 or -2. These signaling molecules are known to interact with IL-4Rα,
one component of the IL-13 receptor complex, and to associate with the p85 subunit of
PI3K (34). Under most circumstances, once the IRS-1 or -2 bound to IL-4Rα becomes
phosphorylated, it binds Grb2, which is constitutively associated with Sos. Sos then
activates Ras, leading to Raf activation with subsequent activation of Erk. Thus,
interaction of IRS-2 with PI3K following IL-13 receptor activation could lead ultimately
to activation of Erk, providing a mechanism for the dual activation of the MAP kinase
and PI3K signaling pathways in response to this cytokine.

IL-13, EGFR and Erk, shown by our findings to be important for epithelial cell
proliferation, have also been implicated in the induction of mucin expression. In an IL-
13-overexpressing transgenic mouse model, airway epithelial cell hypertrophy, mucus
cell metaplasia, as well as the hyperproduction of neutral and acidic mucus were
observed when compared to transgene-negative littermates (35). Additionally, in studies
using the mucoepidermoid NCI-H292 lung cancer cell line, direct stimulation of EGFR by EGF or TGFα, increased MUC2 and MUC5AC mRNA in an EGFR- and Erk-dependent manner (36, 37). Direct EGFR stimulation also results in elevated mucin2 and mucin5AC protein expression in this cell line model (36). The results reported from our current study indicate that IL-13-induced MAP kinase activation is not EGFR dependent, suggesting that IL-13-induced proliferation and IL-13-induced mucin expression may be differentially regulated. Alternatively, signaling pathways activated following exposure to IL-13 may differ depending on the experimental model examined (i.e. primary NHBE cells, transgenic mice, or immortal cell lines).

Interaction of IL-13 with its receptor on NHBE cells is the initial event in what now appears to be a multi-dimensional signaling cascade that leads ultimately, among other outcomes, to proliferation of these epithelial cells. The results presented here provide the first evidence that two pathways known to govern proliferation in other cell types are both required for IL-13-induced proliferation of NHBE cells. As the manner in which these pathways interact becomes clearer, molecules beyond TGFα and the EGFR should emerge as potential therapeutic targets. Manipulation of such molecules by traditional pharmacologic or novel means may block development of the thickened epithelial layer that results from excessive epithelial cell proliferation due to the chronic presence of inflammatory mediators such as IL-13. Thus eliminating narrowing of the airway lumen should decrease the chance that airways will be obstructed by excess mucus during an acute asthmatic event, providing hope for observable decreased morbidity and mortality in chronic asthmatics.
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REFERENCES


hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. 


Figure 1. Inhibition of MAP kinase attenuates IL-13-induced proliferation of NHBE cells in vitro. (A, B) [³H]-thymidine incorporation in NHBE cells following exposure to IL-13. Cultures were maintained for 24 hrs in EGF-free media and then exposed to IL-13 (10 ng/ml, 24 hrs) and/or the (A) Mek inhibitor PD98059 (20 or 0.2 µM; 24 hrs) or (B) U0126 (100 or 25 nM; 24 hrs) in the presence of the radioactive label. Error bars indicate SEM. n = 6; * = significantly different from untreated control, p<0.05; †= significantly different from appropriate control, p<0.05. (C) Cell counts following 24 hrs exposure to IL-13 and/or PD98059 or U0126. Error bars indicate SEM. n=4; *= significantly different from appropriate control, p<0.05.

Figure 2. Inhibition of PI3K attenuates IL-13-induced proliferation of NHBE cells. [³H]-thymidine incorporation in NHBE cells following 24 hrs exposure to IL-13 (10 ng/ml) with or without the PI3K inhibitor LY294002 (3 or 0.3 µM). Error bars indicate SEM. n = 12; * = significantly different from untreated control, p<0.05, †=significantly different from IL-13 treatment alone, p<0.05.
Figure 3. IL-13 induced phosphorylation of Erk is not TGFα mediated. Representative Western blots (A; top three rows) demonstrating IL-13 induced Erk phosphorylation is not attenuated by the addition of neutralizing anti-TGFα: NHBE cells were incubated with IL-13 (10 ng/ml) with or without neutralizing anti-TGFα (0.2 μg/ml) for the times indicated after which cell lysates were collected, separated via SDS-PAGE and the transferred proteins probed with anti-phospho-Erk and anti-Erk antibodies. Representative Western blots (A; bottom three rows) demonstrating TGFα induced phosphorylation of Erk is attenuated by neutralizing anti-TGFα: NHBE cells were exposed to TGFα (5 ng/ml) with or without neutralizing anti-TGFα antibodies for the times indicated, after which cell lysates were collected and separated via SDS-PAGE. Transferred proteins were probed with anti-phospho-Erk and anti-Erk antibodies. (B) Western blots demonstrating UO126 (25 nM) blocks both IL-13 and (C) TGFα induced Erk phosphorylation. NHBE cells were exposed to (B) IL-13 (10 ng/ml) or (C) TGFα (5 ng/ml) with UO126 for times indicated, after which cell lysates were collected and separated via SDS-PAGE. Transferred proteins were probed with anti-phospho-Erk and anti-Erk antibodies.
Figure 4. While IL-13 increased phosphorylation of EGFR, inhibiting EGFR activation does not block IL-13-induced Erk phosphorylation. (A) Western blot demonstrating induction of EGFR phosphorylation by IL-13. IL-13 (10 ng/ml) or TGFα as a positive control (5 ng/ml) were added to NHBE cell cultures for 15 minutes and total protein lysates were collected and immunoprecipitated with anti-EGFR antibodies. The precipitates were analyzed via Western blot with anti-phospho-EGFR antibodies. The blots were chemically stripped and reprobed with anti-EGFR to ensure equal loading. (B) Western blot illustrating that the EGFR tyrosine kinase inhibitor AG1478 has little to no effect on IL-13-induced phosphorylation of Erk. NHBE cells were incubated with IL-13 (10 ng/ml) and/or AG1478 (1 μM) for times indicated, followed by collection of total protein lysates that were separated via SDS-PAGE. Western blot analysis was completed by hybridization of transferred proteins with anti-phospho-Erk and anti-Erk antibodies.
Figure 5. IL-13 modulates the PI3K pathway in NHBE cells. Representative Western blots demonstrating phosphorylation of the PI3K substrate Akt following IL-13 (10 ng/ml) or TGFα (5 ng/ml) exposure for the indicated times with or without the PI3K inhibitor LY294002 (LY) (3 μM).
Figure 6. Role of PI3K in TGF\(\alpha\)-induced proliferation and release of TGF\(\alpha\). (A) \([\textsuperscript{3}H]\)-thymidine incorporation in NHBE cells following 24-hour exposure to TGF\(\alpha\) (5 ng/ml) with or without the PI3K inhibitor LY294002 (3 or 0.3 \(\mu\)M). Error bars indicate SEM. \(n=12\); * = significantly different from untreated, \(p<0.05\), †††† = significantly different from TGF\(\alpha\) treated, \(p<0.05\). (B) LY294002 blocked IL-13 induced release of TGF\(\alpha\) from NHBE cells. NHBE cells were treated for 1 h with IL-13 (10 ng/ml) with and without LY294002 (3 or 0.3 \(\mu\)M) after which time media was collected and analyzed via ELISA. Error bars indicate SEM. \(n=4\); * = significantly different from untreated, †††† = significantly different from IL-13 treated, \(p<0.001\).
Figure 7. Illustration depicting signal transduction pathways involved in IL-13-induced proliferation of NHBE cells. IL-13 binds to its receptor on the cell surface initiating the PI3K-mediated release of membrane bound TGFα. IL-13 also initiates the MAP kinase pathway in a TGFα/EGFR independent manner. Following its release, TGFα is then free to bind its receptor the EGFR. Neutralization of sTGFα inhibits both NHBE cell proliferation and Erk activation. Upon the binding of TGFα to EGFR the MAP kinase and PI3K pathways are activated. The MAP kinase pathway involved includes family members MEK and Erk. Inhibitors of MEK (PD98059, U0126, in red) block phosphorylation of Erk and inhibit proliferation. The PI3K inhibitor LY294002 (in red) blocks both IL-13-induced release of TGFα, phosphorylation of Akt, and proliferation.
INTERLEUKIN-13 INDUCED PROLIFERATION IS
DEPENDENT ON PRESENTATION OF TGF\(\alpha\),
NOT SHEDDING.
Abstract

The pleiotropic cytokine interleukin (IL)-13 is associated with numerous biological processes including inflammatory diseases such as allergic asthma. Utilizing an in vitro model consisting of primary human bronchial epithelial cells we have demonstrated previously that IL-13 induces proliferation of these cells, an event mediated by the autocrine activity of transforming growth factor-α (TGFα). TGFα is an integral membrane protein requiring proteolytic processing to a mature growth factor with this processing usually accomplished by TNFα converting enzyme (TACE). We demonstrate here that IL-13-induced proliferation is TACE-dependent in NHBE cells, and that IL-13-induced proliferation does not simply require TACE activation. Rather, IL-13 also provokes the redistribution of TGFα from intracellular locations to the apical membrane where it encounters TACE, resulting in growth factor cleavage. This finding provides the first example of a cytokine inducing the cellular redistribution, and subsequent shedding, of a growth factor.
INTRODUCTION

Growth factors and cytokines serve integral functions in physiological processes as diverse as proliferation, differentiation, angiogenesis, immune responses and disease progression. In a process implicating many cell types such as the immune response, the relationship between cytokines and growth factors can influence the response of tissues that become surrounded by an inflammatory milieu, and serve ultimately to resolve any inflammation-induced changes in these structures.

We have demonstrated previously that interleukin (IL)-13 induces the proliferation of differentiating normal human bronchial epithelial (NHBE) cells, with this proliferation to be mediated by autocrine activity of epithelium-derived transforming growth factor-α (TGFα) (Booth, 2001). IL-13, a member of the Th2 class of cytokines and a known contributor to the pathogenesis of allergic asthma (Grunig, 1998; Wills-Karp, 1998; Zhu, 1999) affects the epithelial cells lining airways in numerous ways, provoking the release of factors such as TGFα (Booth, 2001) and eotaxin (Li, 1999). These factors, in turn, affect neighboring epithelial cells as well as additional cell types that form the airway walls including underlying fibroblasts. While it is well documented that epithelial cells, including those lining the airways, produce and release growth factors, the mechanism, or mechanisms, regarding cytokine-induced release of these growth factors has not been elucidated.

TGFα is a growth factor of great significance, being required in essential biological processes such as differentiation, development, proliferation, and, if over expressed, to the development of certain disease states such as mammary, squamous, and renal carcinomas, melanomas, hapatomas and glioblastomas (Appendix II). The release
of TGFα requires proteolytic cleavage of the membrane-associated peptide, termed shedding, a process usually accomplished by the ADAM (a disintegrin and metalloproteinase) family member TNFα converting enzyme (TACE/ADAM17) (Peschon, 1998). TACE is known to be activated by protein kinase C (PKC) (Massague and Pandiella, 1993; Arribas, 1997), nitric oxide (NO) (Zhang, 2000) and the mitogen activated protein (MAP) kinase family member extracellular signal-regulated kinase (Erk) (Diaz-Rodriguez, 2002; Fan, 2003), however no evidence has been presented for cytokine-induced activation of TACE, although cytokines are known to affect PKC, NO and Erk (Refs, L’Allemain, 1994) within cells.

TACE is present on the surface of cells, and it is known also to localize in perinuclear compartments in conjunction with TNF-α (Schlondorff, 2000). TGFα has also been found to be stored in intracellular compartments in monocytes and neutrophils (Calafat, 1997), and to be an integral cell surface protein asking the question are the intracellular stores mobilized in response to a stimulus resulting in growth factor shedding, or are the cell surface localized peptides only affected by such a stimulus? Through the use of an in vitro model employing primary human bronchial epithelial (NHBE) cells grown in an air/liquid interface (ALI) culture, we describe our attempts to address this question. This model provides an excellent system for in vitro study, as NHBE cells grow and differentiate as observed in vivo, creating essentially an in vitro epithelial tissue.

Here, we demonstrate that IL-13-induced proliferation of NHBE cells requires TACE; however, the mechanistic link between IL-13 and TGFα shedding involves more than a simple increase in TACE activity. Rather, we show that IL-13 induces
mobilization of intracellular TGFα to the apical cell surface where it encounters the cleavage enzyme.
Results

TACE induces TGFα-mediated proliferation of NHBE cells.

Previous studies have suggested that numerous pathologies involving epithelial hyperproliferation are mediated by TGFα/EGFR (epidermal growth factor receptor) autocrine/paracrine growth loops. A recent study from our laboratory suggested that IL-13 initiated proliferation of NHBE cells via a similar mechanism (Booth, 2001). TGFα, initially translated as a membrane-tethered protein that requires proteolytic cleavage to become a mature growth factor, is cleaved by TACE in a number of cell types (Peschon, 1998; Borrell-Pages, 2003). In this current study, we wished to determine if the addition of exogenous TACE could cleave existing surface-membrane expressed growth factor resulting in cellular proliferation. The addition of exogenous recombinant human (rh)TACE resulted in an increase of free TGFα in the surrounding medium (Fig 1a) and rhTACE induced cellular proliferation similar to that induced by IL-13 and TGFα (Fig 1b). These results indicate that NHBE cells maintain TGFα on the extracellular membrane in a form that is amenable to proteolytic activity and where the rhTACE interacts with TGFα resulting in its cleavage. We next wanted to determine if the exogenous rhTACE was indeed cleaving surface expressed TGFα and not an additional growth factor that might have the capacity to induce proliferation. The addition of neutralizing anti-TGFα antibody attenuated the proliferative effect induced by exogenous rhTACE (Fig 1c) suggesting that rhTACE cleaves surface-expressed TGFα, that then induces proliferation of the epithelial cells.
**Role of TACE in IL-13-induced proliferation.**

After determining that TACE can induce cellular proliferation mediated by TGFα, we wanted to determine whether endogenous TACE is involved in IL-13-induced proliferation of NHBE cells. We first examined the effects of various inhibitors of TACE on IL-13-induced shedding of TGFα. Tissue inhibitor of metalloproteinase (TIMP)-3 is a documented inhibitor of TACE while its family member TIMP-1 has no effect on TACE (Amour, 1998; Lee, 2001). TIMP-3 attenuated IL-13-induced TGFα shedding (Fig 2a). And, as expected, TIMP-1 had no effect on IL-13-induced growth factor shedding (Fig 2a). Similarly, anti-TACE antibodies also blocked IL-13-induced TGFα shedding (Fig 2b). These data suggest that endogenous TACE is required for IL-13-induced TGFα shedding in NHBE cells.

To confirm this requirement of TACE for IL-13-induced TGFα shedding, antisense oligonucleotides against TACE were utilized. TACE antisense oligonucleotides inhibited IL-13-induced NHBE cell proliferation (Fig 3a) as well as IL-13-induced shedding of TGFα (Fig 3b). TACE antisense oligonucleotides did not inhibit TGFα induced proliferation, only IL-13 induced proliferation (Fig 3a), indicating that TACE is required for IL-13 induced proliferation of NHBE cells. RT-PCR demonstrated no observable suppression of TACE mRNA levels due to the addition of the antisense oligonucleotides (data not shown). We have demonstrated previously that IL-13-induced proliferation of NHBE cells requires TGFα, and having now demonstrated that TACE antisense oligonucleotides inhibit both IL-13-induced proliferation and TGFα shedding, and that neutralizing anti-TGFα antibodies block rhTACE-induced proliferation, we
conclude that TACE cleavage of TGFα is required for IL-13-induced proliferation of NHBE cells.

After determining that addition of rhTACE is sufficient to induce TGFα shedding, we examined the effect IL-13 had on TACE. We wished to determine whether or not IL-13 simply activated TACE resulting in increased TGFα shedding. TACE exists in two forms, an inactive latent form and an active form. Activation of TACE requires proteolytic cleavage of the enzyme resulting in the removal of a 20-kDa section of the protein. Over a time course of IL-13 stimulation, total active TACE protein levels were only slightly elevated at longer IL-13 exposure timepoints (Fig 4a). Similarly, 4 and 24-hour stimulation with IL-13 had no effect on TACE mRNA levels within the epithelial cells as determined by RT-PCR (Fig 4b). These data suggest that IL-13 does not dramatically affect overall TACE protein or mRNA levels within the cells.

In many cases TACE-mediated shedding is induced by the activation of PKC (Massague and Pandiella, 1993, Arribas, 1997), we therefore wished to determine if PKC activation alone was sufficient to induce proliferation. Exposure of cells to the known PKC activator phorbol-12-myristate 13-acetate (PMA) did not result in and increase in TGFα shedding (Fig 4c) or cellular proliferation (Fig 4d) as both TGFα shedding and proliferation remained at control levels. Interestingly, PMA did induce the secretion of IL-8, a process known to be PKC-dependent (Zhang, 2000), while IL-13 had no effect on IL-8 secretion (Fig 4e). These data suggest that in our system TGFα shedding is not dependent solely on PKC-activation.
**IL-13 induces intracellular movement of TGFα.**

One of the simplest ways to increase levels of mature TGFα in the surrounding media following IL-13 exposure would be proteolytic cleavage and release of preexisting growth factor. NHBE cells are known to release other proteins upon stimulation, the most notable of which are the mucin proteins, the glycoprotein component of airway mucus (Li, 2001). In mucin release from NHBE cells, constitutive levels of protein within the cells are rapidly mobilized to the cell surface for secretion in response to external stimuli.

Intracellular stores of TGFα have been noted in other cell types, specifically neutrophils and monocytes (Calafat, 1997) and intracellular stores of EGF have been reported in human submandibular and parotid glands (Cossu, 2000; Lantini, 2001). If NHBE cells also possess such stores then these growth factor reserves could potentially be mobilized to the cell surface following an extracellular stimulus, such as IL-13. Once at the cell surface constitutively active, or moderately induced, TACE could cleave and release TGFα. Such an intracellular redistribution could explain the rapid release of TGFα following IL-13 exposure and also explain why activation of PKC alone is not sufficient to induce TGFα shedding. The growth factor needs to be physically moved to the active enzyme in order to facilitate shedding; merely activating the enzyme is not enough.

Confocal microscopy was used to determine whether or not the proposed scenario occurs in NHBE cells. Following IL-13 exposure, intracellular TGFα does indeed move to the surface of NHBE cells in response to IL-13. As demonstrated in Figure 5a, untreated NHBE cells express TGFα constitutively throughout the cytoplasm with some extracellular membrane expression, while TACE expression is localized to the
extracellular membrane, mainly on the apical surface. After a 15 min exposure to IL-13, the TGFα expression pattern is not appreciably altered (data not shown) with the growth factor distributed throughout the cytoplasm. However, after 30 minutes of IL-13 stimulation the expression pattern of TGFα changes dramatically (Fig 5c) with the TGFα condensing into specific regions within the cytoplasm, accompanied by an increased apical presence of the growth factor. Simultaneously, TACE expression patterns are altered, with the enzyme seemingly localizing in greater concentration to the apical surface of the cells to distinct regions of the membrane.

Following a 60-minute exposure to IL-13 the expression patterns of both TGFα and TACE have changed further compared to untreated cells (Figure 5e). TGFα expression is not detectable in the basal areas of the cells with the majority of the growth factor expressed on the apical surface or just below the membrane surface. Meanwhile TACE appears to have been internalized by the cells, very little remains on the apical surface with most now within the cytosol. As a form of feedback control, TACE is known to be internalized following PMA-stimulation (Doedens, 2000). This internalization appears to be occurring in NHBE cells following IL-13 stimulation.

Additionally, following 4-hour exposure to IL-13 very little TGFα remains within the NHBE cells either intracellular nor on the extracellular membrane. The relatively small amount of growth factor that is present appears to be expressed in the area of the cell where the TGFα was maintained prior to stimulation, suggesting that new constitutive protein is being synthesized (Fig 5g). TACE expression once again appears intracellular, with expression primarily in the basal region of the cells. This is the first
demonstration that a cytokine, in this case IL-13, can induce the mobilization of stored growth factor, TGFα, from the cytoplasm to a cell surface, where shedding is triggered.
Discussion

Our observations reveal a mechanism by which a cytokine induces the movement of an intracellular, stored growth factor to the cell surface, where the protease required for growth factor processing is expressed, resulting in shedding of the growth factor. Specifically, IL-13 induces the intracellular movement of TGFα to the cell surface of human airway epithelial cells where the growth factor interacts with the already present and constitutively active TACE. The implications of this novel IL-13-induced mechanism are vast. By maintaining intracellular reserves of growth factors, and perhaps other molecules, epithelial cells can be considered effector cells. The maintenance of these intracellular reserves prevents the unnecessary or unintended cleavage of surface tethered molecules otherwise expressed on the surface of the cell. These cleavage events could occur in response to neutrophil elastase or other proteases. Without such a storage mechanism everyday exposure to airway irritants could lead to cleavage events mediated by neutrophil elastase or other proteases responding to airway insults. Such proteases might liberate ligands such as TGFα resulting in unwarranted consequences such as upregulation of mucin gene expression and unnecessary proliferation (Kohri, 2002).

Our results indicate that initially, prior to treatment with IL-13, TGFα is sorted to the basal portion of NHBE cells. This observation is in agreement with previous reports analyzing epithelial cells of the kidney (Dempsey, 2003). However, following IL-13 stimulation of NHBE cells, TGFα moves from the basolateral region of the cells towards the apical surface. It is on the apical surface where the majority of TACE is expressed in unstimulated cells. Perhaps this movement to the apical surface is a mechanism designed to narrow the scope of the effects of TGFα on other cell types such as underlying
fibroblasts or smooth muscle cells, or by keeping the ligand and sheddase physically separated, the cells are preventing unwanted cleavage of the growth factor. In either case, by shedding TGFα on the apical surface, NHBE cells direct TGFα towards neighboring epithelial cells or resident and infiltrating inflammatory cells rather than towards fibroblasts or other connective tissue. In the case of an airway injury where IL-13 is present, epithelial regeneration is a key step in the healing process.

Previous studies present conflicting reports regarding the requirement of TGFα cleavage and effective binding of EGFR. Wong et al. (1989) reported initially that TGFα cleavage is not required and that surface expression alone was sufficient to facilitate autocrine/paracrine functional activation of EGFR. Recently, Borrell-Paiges et al. (2003) demonstrated that cleavage of TGFα is required for activation of EGFR in tumor progression. Additionally, Hinkle et al. (2003) reports that multiple metalloproteinases process proTGFα, specifically, TGFα requires two distinct cleavage events, N-terminal and C-terminal cleavages, both of which TACE is capable of performing. Additionally, an unknown protease, perhaps ADAM10, is capable of performing the C-terminal cleavage. The data presented herein suggests that inhibition of TGFα shedding through the use of inhibitors of TACE and antisense oligonucleotides is sufficient to decrease IL-13-induced shedding of TGFα. It is possible that in both cases, inhibitors and antisense oligonucleotide, that TACE function is not completely abolished. Indeed, we still observe some, although markedly depressed, TGFα shedding in both cases. By inhibiting TGFα shedding we are directing the TGFα towards an autocrine loop instead of promoting migration of the mature growth factor to nearby cells. TGFα knockout mice are phenotypically similar to mice that are unable to proteolytically shed TGFα.
indicating that in some cases cleaved TGFα is required for normal development and that unprocessed growth factor does not adequately initiate cellular proliferation, especially in vivo. Such would also be the case where a tissue does not produce TGFα, a tissue not of ectodermal origin, which requires the growth factor for normal growth and development. Furthermore, cells that are deficient in TACE still are able to shed TGFα, albeit at extremely retarded levels as compared to cells with functional TACE, suggesting that an additional protease or proteases are capable of cleaving TGFα. Overall it seems that surface protein shedding is an event that is controlled at multiple levels with many forms of compensation. This intricate design suggests that protein shedding is an indispensable cellular function.

Recently, heregulin-α, another member of the EGF family of growth factors, was shown to be present exclusively in the apical membrane of human airway epithelia (Vermeer, 2003) while its receptors, erbB2-4, are present only on the basolateral membrane. This arrangement allows for ligand-receptor interaction only after epithelial integrity is disrupted, or when the tight junctions between cells are opened. Interestingly, IL-13 is known to affect epithelial tight junctions and membrane permeability. IL-13 affects the intracellular signaling intermediary phosphatidylinositol 3-kinase (PI3K) (Wright, 1997; BWB and LDM submitted manuscript). PI3K regulates epithelial adhesion (Ceponis, 2000), allowing for the migration of epithelial cells following an injury to facilitate the healing process. By altering the tight junctions amongst the epithelium and inducing the movement and subsequent shedding of TGFα from the apical surface of the cells, IL-13 potentially not only effects epithelial cells but also additional surrounding cell types such as the underlying fibroblast and smooth muscle
cells of the lungs. TGFα does in fact migrate into the basal compartment in our model following IL-13 exposure. Media samples analyzed for TGFα following IL-13 exposure indicate a sharp increase in TGFα levels not only on the apical surface (Figs 5b and 6b), but also in the basal compartment (Fig 2b and d, 3a, and 4a). Several airway diseases, such as asthma and bronchitis, are characterized by chronic elevated levels of IL-13, chronic increase in epithelial permeability, and airway remodeling (Kennedy, 1984; Godfrey, 1997; Goto, 2000).

The data presented here indicate the presence of TACE not only on the apical surface of NHBE cells but also TACE expression along the sides of the cells. TACE cleaves not only TNFα and TGFα, but cleaves many other membrane-tethered molecules including additional members of the EGF-family (HB-EGF (Sunnarborg, 2002), neuregulin (Montero, 2000) and amphiregulin (Lemjabbar, 2003; Gschwind, 2003) and their receptors (HER4) (Rio, 2000), as well as p75 TNFα receptors, and L-selectin adhesion molecules (Black, 1997; Peschon, 1998). The presence of TACE along the junctions between cells suggests a function of cell/cell interactions involving TACE, perhaps as a sheddase or involvement in regulating cell adhesion.

The IL-13-induced rapid release of preformed molecules also minimizes the reaction time in response to induced stress and injuries, and initiates healing processes along with providing potential preemptive protection as growth factors migrate to cells and tissues at a distance from the effected region of epithelium. Migrating growth factors are essential molecules in many different physiological processes. In the case of airway remodeling, TGFα is involved in epithelial cell proliferation resulting in the overall thickening of the epithelial layer. TGFα has also been implicated as a factor in the
progression of pulmonary fibrosis, an essential injury response and characteristic of airway remodeling (Korfagen, 1994; Madtes, 1998).

The exact mechanism by which the cytokine induces the movement of the growth factor remains unclear but will likely provide potential drug targets including those aimed at blocking the negative impact of asthmatic airway remodeling and those targeting the enhancement of epithelial repair following lung injury. The release of stored growth factors by cells in response to cytokines is a concept of global importance. Growth factors exhibit their functions during many stages of development, cellular differentiation and dedifferentiation, the healing process, and inflammation. If the normal levels of growth factor present are repeatedly altered in response to a cytokine during these events, it is feasible that inappropriate outcomes will be unavoidable.
METHODS

Cell Culture and ELISA

NHBE cells (Cambrex, Walkersville, MD) were grown on transwell membranes as described previously (Krunkosky, 2000). Medium was changed every other day until the cells reached confluence, at which time the apical medium was removed to establish an air/liquid interface (ALI), while the basolateral medium continued to be changed daily. Experimentation was carried out when mature secretory cells are prominent, approximately day 7-9 after ALI establishment. Following treatments, media samples were collected and analyzed with commercially available TGFα or IL-8 ELISA kits according to manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Antisense assays

Antisense oligonucleotides were utilized following a protocol modified from Li et al, 2001. Briefly, cells were treated for 3 days with varying concentrations of phosphorothioate-modified antisense oligonucleotides, scrambled oligonucleotides or vehicle only (FuGene6, Roche, Indianapolis, IN). FuGene6 was added only on the first day at manufacturer’s suggested concentration. On the third day the cells were treated with IL-13 for the times indicated and media samples collected, cells counted, and mRNA collected using TRI Reagent (Sigma, St. Louis, MO) according to manufacturer’s guidelines for further analysis. Oligonucleotides were synthesized by Invitrogen (Rockville, MD).
**Proliferation assays**

Approximately 7-9 days following ALI establishment, NHBE cells were grown without EGF for 24 hrs prior to exposure to IL-13 (10 ng ml⁻¹) and [³H]-thymidine. Cultures were then incubated with 1 μCi ml⁻¹ [³H]-thymidine (specific activity = 87.1 Ci mmol⁻¹; Perkin Elmer Life Sciences, Inc., Boston, MA) together with specific reagents described. Following 24 hrs of incubation, medium was removed and the cultures washed with cold PBS. Cultures were then washed twice with 10% TCA for 15 minutes on ice. Transwell insert membranes were then removed and placed in 1 ml scintillation fluid and kept overnight to allow for cell lysis. Incorporated radioactivity was analyzed in a LKB 1209 RACKBETA liquid scintillation counter. To perform manual cell counts, NHBE cells were liberated from the Transwell membranes with warm Versene (Invitrogen, Grand Island, NY) for 5-10 min at 37°C and counted using a hemacytometer.

**RT-PCR**

Total RNA was extracted from NHBE cells with TRI Reagent and reverse transcribed with First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Indianapolis, IN) in accordance with manufacturer’s guidelines using specific oligonucleotides. PCR reactions were carried out using Taq polymerase (Boeringher Mannheim, Mannheim, Ger), and all reactions employed a Perkin Elmer GenAmp PCR System 2400. PCR products were separated by gel electrophoresis in 2% agarose and visualized by ethidium bromide staining.
Cell lysis and Western analysis

Total protein was extracted from NHBE cells using RIPA buffer containing Roche Complete protease inhibitor cocktail (1 mM EDTA; 1% NP-40; 0.5% sodium deoxycholate, 0.1% SDS, 30 µg ml⁻¹ pancreas extract, 3 µg ml⁻¹ pronase, 0.8 µg ml⁻¹ thermolysin, 1.5 µg ml⁻¹ chymotrypsin, 0.2 µg ml⁻¹ trypsin, and 1.0 mg ml⁻¹ papain). Protein concentration was determined using the Bradford dye-binding procedure (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA). Protein samples were mixed 1:1 with gel loading buffer and boiled for 5 min. Samples were separated via SDS-PAGE on 10-20% precast gradient gels (Bio-Rad). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) that was then blocked in 5% nonfat milk/PBS for 1 hr at room temperature. Membranes were probed with primary anti-TACE antibody (R&D Systems, Minneapolis, MN) at a concentration of 1:1000 in 5% nonfat milk/PBS overnight at 4°C. The membranes were then washed twice with 0.01% Tween-20/PBS for 30 min at room temperature. After the second wash, the membrane was exposed to HRP-conjugated secondary antibody diluted 1:5000 in 5% nonfat milk/PBS for 45 min at room temperature. Washes were repeated and bands visualized with ECL (Amersham, Buckinghamshire, UK). The blots were stripped using a commercially available kit (Chemicon Int., Temecula, CA) and the process repeated with anti-actin (Santa Cruz Biotech, Santa Cruz, CA) as the primary antibody.

Confocal Imaging

Following IL-13 (10 ng ml⁻¹) treatment, cells were fixed with 4% formalin. All staining was carried out in Transwell inserts. Cells were washed with PBS, permeablized with
0.2% Triton X-100 in PBS, and stained with primary antibodies, either anti-TGFα (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-TACE, followed by a 45 min treatment in the dark with appropriate secondary antibodies tagged with Alexa 488 or Alexa 647 (Molecular Probes, Eugene, OR), respectively. The Transwell membrane inserts were then removed and mounted on glass slides in Vectasheild mounting media (Vector Laboratories, Burlingame, CA). Cells were visualized with a Nikon Eclipse TE2000-E confocal microscope equipped with a Plan Apo 60X water immersion objective. Entire experiments were repeated 3-times resulting in 6 samples per timepoint. Each sample was divided into quadrants and images were collected from each quadrant allowing analysis of 1000-1400 cells per sample.

**GenBank accession number**

TACE  NM 003183

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References


**Figure 1:** TACE-induced proliferation mediated by TGFα.  

**a.** NHBE cells were treated with rhTACE (10 ng ml⁻¹) for 1 hr after which surrounding medium was analyzed for the presence of TGFα by ELISA (* p<0.05, mean ± SEM, n = 3).  

**b.** NHBE cells were treated with rhTACE (10 ng ml⁻¹), IL-13 (10 ng ml⁻¹), or TGFα (5 ng ml⁻¹) for 24 hrs. Proliferation was determined via [³H]-thymidine incorporation (* p<0.05, mean ± SEM, n = 6).  

**c.** NHBE cells were treated with IL-13 (10 ng ml⁻¹), TACE (10 ng ml⁻¹) or TACE plus neutralizing anti-TGFα (0.2 µg ml⁻¹)(n=6, mean± SEM, * p<0.05 vs CON, † p<0.05 vs IL-13).
Figure 2: TACE involvement in IL-13-induced proliferation. NHBE cells were exposed to IL-13 (10 ng ml⁻¹) ± inhibitors of TACE. Cells were exposed to either TIMP-1 or TIMP-3 (both at 2 µg ml⁻¹) for 30 min prior to IL-13 exposure. a, TGFα shedding determined via ELISA after 1 hr (n=4, mean± SEM, * p<0.05 vs corresponding control, †p<0.05 vs IL-13 alone). Hatched bars-TIMP-1, Dotted bars-TIMP-3. b, Cells were exposed to IL-13 ± anti-TACE and TGFα shedding determined after 1 hr (n=6, mean± SEM, * p<0.05 vs untreated, †p<0.05 vs IL-13 alone).
Figure 3: Blocking TACE inhibits IL-13-induced effects. TACE antisense oligonucleotides or corresponding scrambled oligonucleotides were added to NHBE cell cultures for 3 days with transfection agent (FuGene6) added only on the first day. On third day of treatment IL-13 or TGFα was added for 24 hrs and a, cell number determined (* p<0.05 vs Con without oligo, †p<0.05 vs IL-13 alone, ‡p<0.05 vs TGFα alone, # p<0.05 vs IL-13 within treatment group, mean ± SEM, n=6). b, media collected and analyzed via ELISA (* p<0.05 vs untreated, †p<0.05 vs IL-13 alone, mean ± SEM, n = 4).
Figure 4: PKC activation alone not sufficient to induce IL-13 effects. a, TACE protein levels were analyzed via Western following IL-13 (10 ng ml⁻¹) treatment for 5, 15, 30, 60, 360 and 1440 min. Membranes were chemically stripped and probed for β-actin. b, mRNA levels of TACE and β-actin transcripts were analyzed following IL-13 treatment for 4 and 24 hrs. Cells were treated with IL-13, or PMA (10 nM) and c) TGFα shedding determined after 1 hr (n=4, mean±SEM, * p<0.05) and (d) proliferation determined by [³H]-thymidine incorporation after 24 hrs (n=6, mean±SEM, * p<0.05). e, The secretion of IL-8 was examined by ELISA following 1 hr exposure of IL-13 or PMA (n=6, mean±SEM, * p<0.05).
Figure 5. Immunostaining of TGFα and TACE intracellular locations. The cellular distribution of TGFα and TACE in NHBE cells following stimulation with IL-13 for various periods of time was determined using confocal microscopy. Images presented as x-y planes or y-z planes from apical surface, midline and basolateral surface: TGFα (red) and TACE (green). (a) Untreated control images. 30 min samples (b) untreated and (c) treated with IL-13. 60 min samples (d) untreated and (e) with IL-13. 4 hrs samples (f) untreated and (g) with IL-13. Scale bars represent 10 μM.

Figure 6. Movement of TGFα following IL-13 stimulation. Confocal images of NHBE cells presented in x-y plane. (a) Control cells, (b) NHBE cells treated for 60 min with IL-13. TGFα (red) and TACE (green), scale bar represents 10 mm. (c) Illustration demonstrating expression patterns of TGFα and TACE in response to IL-13 at the times indicated. Colors represent TGFα (red) and TACE (green).
CHAPTER V
SUMMARY AND FUTURE DIRECTIONS

The studies presented in this dissertation investigated the interaction between a cytokine, IL-13, and NHBE cells in vitro. These studies were the first to demonstrate that IL-13 induces the proliferation of these epithelial cells in culture and were among the earliest studies to investigate the mechanisms involved in IL-13-induced proliferation that include autocrine action of growth factors, growth factor receptors, intracellular signal transduction pathways, and growth factor cleaving enzymes. Three findings described here have not been reported previously. These findings are:

1) IL-13-induced proliferation of NHBE cells in vitro is mediated by epithelium-derived TGFα binding to EGFR;

2) IL-13-induced proliferation of NHBE cells requires both the MAP kinase and PI3K signaling pathways, and

3) IL-13 induces the intracellular redistribution of TGFα to the apical surface of NHBE cells where it is cleaved by TACE.

The studies reported here utilized primary human airway epithelial cells grown in an air/liquid interface (ALI) culture system as an in vitro model. When NHBE cells are grown in a submerged manner, cellular differentiation is retarded. However, as reported previously, ALI cultures develop to mimic the structure and function of the in vivo airway epithelium (Krunkosky, 2000) by abandoning conventional methods wherein cells remain submerged throughout experimentation. Thus, the ALI culture system was utilized in these studies to allow use of differentiated epithelial cells and to have cultures absent of non-epithelial cell types such as inflammatory cells and airway fibroblasts. The lack of additional cell types allows the data to be interpreted as “epithelial-only” meaning
that no effects were generated or compromised by the interactions of any cells other than epithelial cells. Additionally, this ALI culture system uses primary cells, not immortalized cell lines. Immortalized cell lines are often virally transformed or genetically altered in some other respect. Such genetic alteration inherently changes the phenotype of any given cell as well as the relationship of signaling pathways within that cell. These alterations may result in gene expression and repression that is different when compared to that regularly observed in normal cells.

Using primary airway epithelial cell cultures, I demonstrated initially that IL-13-induced proliferation of NHBE cells is mediated by TGFα. It was shown previously that IL-13 is essential for the development of numerous pathogenic characteristics of asthma. Specifically, chronic exposure of epithelial cells in the in vivo airway to IL-13 results in the thickening of the epithelial layer, airway hyperresponsiveness and goblet cell hyperplasia (Grunig, 1998; Wills-Karp, 1998; Zhu, 1999; Venkayva, 2002). While previous studies clearly indicated a role for IL-13 in these processes, the exact mechanisms by which this cytokine provokes these changes have not been elucidated, and thus, the exact intracellular signal transduction pathways governing these IL-13-induced processes are still being investigated.

Others have also demonstrated previously that interactions between inflammatory cells (such as neutrophils and eosinophils) and airway epithelial cells do occur (Burgel, 2001; Kohri, 2002). Results from these studies indicated that products from inflammatory cells, such as neutrophil elastase, can cleave TGFα from the surface of the epithelial cells. The free TGFα could migrate and activate the EGFR in turn upregulating mucin gene expression and protein production.
In contrast, the results I present here demonstrate clearly that IL-13 alone is capable of inducing the rapid release of TGFα from airway epithelial cells, as the ALI cell system is devoid of additional cell types such as immune cells. Therefore, the use of ALI cell cultures exposed to purified IL-13 precludes any interaction of varying cell types and eliminates the possibility that any additional inducing-agents are originating from such other cell types. In addition, my results show that this IL-13-induced proliferation is attenuated by inhibiting the intrinsic tyrosine kinase activity of the EGFR and by neutralizing the epithelial-derived TGFα. Taken together, these data indicate that IL-13-induced proliferation is mediated by an autocrine TGFα/EGFR interaction in NHBE cells in vitro, without the aid of mediators derived from other cell types.

Secondly, my findings indicate that TGFα-mediated, IL-13-induced proliferation of NHBE cells is a result of activation of both the MAP kinase and PI3K-signaling pathways. Through the use of chemical inhibitors to target members of these kinase-mediated pathways, specifically to MEK and PI3K, the activation of the MAP kinase and PI3K pathways following IL-13 stimulation was demonstrated in my studies. This approach was chosen due to a desire to examine affected signal transduction pathways in an unaltered state in primary cells that are not amenable to transfection at a sufficiently high rate to allow use of dominant-negative strategies. Western analyses revealed that IL-13 induces the phosphorylation of Erk and EGFR. Even though in many cell types tyrosine phosphorylation of EGFR is a known activator of the MAP kinase pathway (Campbell, 1995), in NHBE cells phosphorylation of Erk in response to IL-13 is not inhibited by blocking the EGFR tyrosine kinase with the inhibitor AG1478 or by application of neutralizing TGFα antibody; yet application of AG1478 blocks NHBE cell
proliferation. Similarly, inhibiting MEK blocks IL-13 induced proliferation. Taken
together, these data demonstrate that activation of the MAP kinase pathway by IL-13 is
not TGFα and EGFR mediated, even though IL-13 induced proliferation of NHBE cells
occurs via the MAP kinase pathway and requires TGFα.

The involvement of the PI3K pathway in IL-13-induced proliferation was
demonstrated by using the specific inhibitor LY294002. Addition of this compound not
only blocked IL-13 and TGFα-induced proliferation in a concentration-dependent
manner but LY294002 also blocked IL-13-induced phosphorylation of Akt/PKB a
downstream target of PI3K. Additionally, PI3K was discovered to be involved in IL-13-
induced release of TGFα from NHBE cells as LY294002 also inhibits IL-13-induced
shedding of TGFα. These data suggest that IL-13 induces proliferation of NHBE cells
via an autocrine TGFα interaction with EGFR, and that activation of both MAP kinase
and PI3K signal transduction pathways is required for proliferation. It also appears likely
that this dual activation by IL-13 involves both EGFR-dependent and EGFR-independent
pathways.

Crosstalk between the MAP kinase and PI3K signaling pathways has been
demonstrated previously, specifically regulated via the small guanine nucleotide binding
protein Raf (Zimmerman, 1999). The cellular outcomes of this crosstalk depend upon the
differentiation stage of a given cell (Rommel, 1999). Thus, it is logical to suggest that
IL-13 may provoke different responses in NHBE cells during different stages of cellular
differentiation, an observation confirmed by additional members of the laboratory
(Apparo, KBC and Martin, LD; unpublished results).
Having acknowledged that IL-13 activates EGFR-dependent and EGFR-independent pathways and the probability that crosstalk is occurring between the MAP kinase and PI3K signal transduction pathways, it was important to determine whether or not IL-13 and TGF\(\alpha\) maintain independent functions. Both IL-13 and TGF\(\alpha\) are known to induce the activation of various transcription factors, most notably STAT6 by IL-13 and AP-1 by TGF\(\alpha\). The functional genomic screen reported in the appendix indicates that IL-13 and TGF\(\alpha\) independently activate transcription factors as well as activate an overlapping subset of transcription factors in NHBE cells. Thus, it is likely that IL-13 activates transcription factors through at least two different intracellular signaling pathways, perhaps the MAP kinase and PI3K pathways.

The overlapping set of transcription factors activated by these two stimuli is intriguing. My data demonstrate that in addition to those factors activated by TGF\(\alpha\), IL-13 activates an additional set, even though autocrine production of TGF\(\alpha\) in response to IL-13 is known to occur in these cells and is a prominent feature of their IL-13-induced proliferation. It is most logical then that some transcription factor activation must be accomplished via a signal transduction pathway that is not TGF\(\alpha\) mediated, possibly via pathways activated from the Jak/STAT6 or Jak/IRS-2/MAP kinase pathway interactions. Further experimentation is required in this area to fully characterize this overlap in transcription factor activation. Such experimentation should include determination of which signal transduction pathways are involved in the IL-13 vs. TGF\(\alpha\)-mediated activation. It is anticipated that use of specific inhibitors, antisense oligonucleotides, or siRNAs, will lead to elucidation of the exact pathways involved in the activation of each of the factors as well as identification of pivotal “switch-like” signaling proteins.
The third new finding presented in this dissertation is that IL-13 induces the movement of intracellular TGFα to the apical cell surface where it is released into the surrounding environment. TGFα has been shown to be located in intracellular stores in monocytes and neutrophils (Calafat, 1997) but such storage in epithelial cells, or the more general movement and subsequent release of the growth factor following cytokine stimulation, has not been reported previously. Activation of proteases by protein kinase C (PKC) and nitric oxide (Arribas, 1996; Zhang, 2000) has resulted in the release of various growth factors, cytokines, adhesion molecules and receptors. In the studies presented here, within resting NHBE cells, intracellular quantities of TGFα are present with some cell surface expression of the growth factor. Following exposure to IL-13, NHBE cells rapidly release TGFα. This rapid release is accompanied by a change in cellular organization in relation to TGFα protein expression. TGFα, which is expressed normally throughout the cytoplasm of NHBE cells, is moved rapidly in the cells to areas near the apical cell surface and, after a period of time, TGFα protein expression is no longer observed within the cells. Interestingly, mRNA levels of the TGFα transcript do not change following IL-13 exposure at the time points analyzed. This finding would suggest that the TGFα gene is constitutively expressed in NHBE cells and that the translated protein is stored as a reserve to be mobilized for use when needed. Such a mechanism would also limit uncontrolled proliferation as the stored and released growth factor pool could be used during an acute repair event. This proliferative event would be self-limiting, as no more growth factor would be available until the slow, constitutive expression of TGFα could refill the intracellular stores.
In a variety of cells, including the NHBE cells used in this study, TGFα is processed from its initial transmembrane form to its mature soluble form by the ADAM (a disintegrin and metalloproteinase) family member TNFα converting enzyme (TACE or ADAM17), a process known as shedding (Peschon, 1998). Elimination of either TGFα or TACE through the use of antisense oligonucleotides demonstrates the necessity for both molecules in IL-13-induced proliferation of NHBE cells in vitro.

My studies indicate that in NHBE cells, TGFα is shed due to cleavage by TACE, but IL-13 does not greatly affect TACE activation; however, IL-13 does alter TACE expression patterns within the NHBE cells. Unstimulated NHBE cells express TACE near the apical cell surface. Following IL-13 treatment, the TACE expression pattern changes with the enzyme condensing into punctate regions, with some internalization of the enzyme observed. After a period of time, 30-60 minutes of IL-13 exposure, the condensed regions of surface expressed TACE are merged with areas of expressed TGFα. After 4-hour exposure of NHBE cells to IL-13, the surface expressed TGFα is no longer observable while the TACE expression pattern is reverting to its “unstimulated” state. These findings suggest that after TACE and TGFα merge, TGFα is shed due to cleavage by TACE resulting in an observable decrease in surface expression of the growth factor. These studies form the foundation for further investigation into the ability of IL-13 to induce the intracellular movement of other growth factors along with its potential to activate other ADAMs or MMPs.

Overall, these studies have shed light on the mechanism of IL-13 induced NHBE cell proliferation. However, there are many aspects of this mechanism that are still unknown. For example, following translation, TGFα is modified, glycosylated and
cleaved, and directed throughout cells via the Golgi apparatus. TGFα is moved throughout epithelial cells via the Golgi apparatus and, more specifically, TGFα associates with Golgi associated proteins, such as Golgi reassembly stacking protein of 55kDa (GRASP55) (Kuo, 2000) and syntennin (Fernandez-Larrea, 1999). In both cases TGFα associates with the PDZ domains of each protein. The PDZ domains interact with the C-terminus of proteins including transmembrane TGFα. Protein PDZ-domains are required for correct insertion of TGFα and other proteins into cellular membranes. These protein domains provide potential targets for future research. To further define this mechanism, it will be necessary to investigate the signaling steps between the IL-13 receptor and the intracellular stores of TGFα. Specifically, the signaling molecules involved in this process need to be identified and the order in which they are activated needs to be deciphered.

PDZ proteins described previously interact with transmembrane proteins and localize to submembranous areas just within the plasma membrane (Fanning, 1998). Interestingly, both FAS (CD95) and the neurotrophin receptor TrkB are rapidly transported to the cell surface following p53 and cAMP activation (Bennett, 1998; Meyer-Franke, 1998). These proteins are mobilized from intracellular compartments. I showed in the screen of activated transcription factors that p53 is activated in NHBE cells within 15 minutes of exposure to IL-13 or TGFα. This activation of p53 by IL-13 or TGFα could play a role in the movement of TGFα as it does in FAS and TrkB intracellular movement.

It is also possible that activation of more than one signaling pathway is required for the movement of TGFα. In one of my studies, I activated PKC alone with no
observable effect on TGFα release; others have activated both PKC and PKG in NHBE cells and witnessed secretion of mucin granules, a process requiring their movement to an apical location in these same cells (Li, 2001). This is but one combination of kinases that requires investigation for its potential role in directing intracellular movement of TGFα.

This research also forms the foundation for future studies examining the pathways that encompass the overlapping transcription factor activation observed between IL-13 and TGFα. TGFα is known to signal via the MAP kinase pathway in numerous cell types (Ezeh, 1998), but in my studies it was also shown that the PI3K pathway is involved in TGFα signaling in NHBE cells. IL-13 also acts through PI3K in addition to STAT6 and IRS-2 in various cell types including airway smooth muscle cells (Laporte, 2001, Hershey, 2003). IL-13-induced phosphorylation of IRS-2 in colonic epithelial cells (Wright, 1997), in turn leads to binding of IRS-2 to the p85 regulatory subunit of PI3K or the adapter protein Grb2, that is constitutively associated with Sos. These interactions then activate Ras leading to Raf activation and subsequent activation of the MAP kinase family member Erk. Raf is known to be involved in crosstalk between the MEK-Erk and PI3K pathways in myoblasts (Rommel, 1999; Zimmerman, 1999). Examination of these signal transduction intermediaries (IRS-2, Ras, Raf and Sos) could potentially enhance understanding of IL-13 induced effects in NHBE cells, as well as suggest targets and approaches for therapeutic intervention.

Finally, a third potential direction for extending this research is to investigate whether or not IL-13 induces the release of additional growth factors, cytokines or other molecules from NHBE cells or from other non-epithelial or non-respiratory-related tissues. Organs other than the lungs, such as the kidneys, the digestive tract and skin are
also prone to allergic immune responses, and, therefore, may well have evolved into IL-13 responders. For example, it is quite possible that the colon, kidney and esophagus may employ IL-13-induced TGFα proliferative loops in the course of injury, repair and remodeling that occur in these tissues. Or, alternatively, IL-13 might provoke similar loops involving different growth factor release and/or production, thus providing some tissue specificity.

Both IL-13 and TGFα are involved in a variety of cancers, with TGFα being shed by TACE in response to numerous stimuli including PKC-activation, activation of MAP kinases, and in response to nitric oxide. While these studies have only examined shedding of TGFα, further experimentation might reveal an intracellular redistribution of other growth factors in a manner similar to the redistribution of TGFα observed in NHBE cells. Thus, such studies might point the way to understanding the conserved mechanism of IL-13/growth factor responses that may function in a wide variety of cell types.

Based on knowledge that the IL-13Rα2 receptor acts as a decoy receptor with the ability to actively diminish IL-13-induced responses, efforts are already underway to explore the IL-13-receptor as a target for anti-cancer and asthma therapies. Thus, by building on the findings set forth in this document to extend our knowledge of the effects of IL-13 in a variety of tissues or pathogenic states, it should be possible to apply these novel anti-receptor therapies already in development to a wide array of disease conditions, while finding a way to preserve essential repair aspects of the pathogenic remodeling mechanisms.
References:


APPENDIX I

INTERLEUKIN-13 AND TRANSFORMING GROWTH FACTOR-α ACTIVATE OVERLAPPING SETS OF TRANSCRIPTION FACTORS IN HUMAN BRONCHIAL EPITHELIAL CELLS
Abstract

Signal transduction pathways are activated by extracellular stimuli to mediate intracellular events that result in activation of multiple transcription factors (TFs) to regulate gene expression. Often times the same signaling cascade is activated by multiple stimuli. Previous studies have suggested that the TH2-type cytokine, interleukin (IL)-13 mediates many biological functions in mammalian airway epithelial cells. These functions include proliferation mediated by an autocrine/paracrine loop involving transforming growth factor α (TGFα) and activation of MAPK and PI3K signaling pathways. To further understand the signaling pathways, we sought to profile the TFs that are activated by IL-13 and TGFα in normal human bronchial epithelial (NHBE) cells using a protein/DNA array system containing a total of 54 TF consensus binding sequences as targets. In addition to TFs activated by only one of the stimuli, both IL-13 and TGFα induce the activation of a set of overlapping transcription factors suggesting that IL-13 and TGFα initiate at least one intersecting signal transduction pathway. In summary, these results provide the basis for further studies in NHBE cells to identify and validate these TFs and their effects on the expression of genes involved in cellular proliferation/differentiation.
Introduction

Activation of signal transduction pathways occurs upon stimulation of cells by various extracellular agents. Signaling molecules in the cascade relay, amplify and integrate diverse signals, allowing the cells to coordinate a wide variety of cellular functions. These functions can include proliferation, differentiation, development, inflammatory responses, apoptosis, and cell migration (Marshall, 1995). Different extracellular stimuli may induce distinct intracellular signaling cascades that ultimately lead to activation of transcription factors (TFs) that control gene expression. Signaling pathways can function independently of one another, but often time there is overlap, or crosstalk, involving one or more of the protein signaling intermediates (Moelling, 2002). This crosstalk can result in the same TF being activated in response to different extracellular stimuli allowing a cell to respond efficiently in a variety of external environments.

Interleukin (IL)-13, secreted by Th2 lymphocytes, has emerged as a major cytokine involved in many biological processes including bronchial epithelial cell proliferation (Booth, 2001), B cell class switching, and production of growth factors and chemokines (Izuhara, 2000). In addition, IL-13 is recognized as a central mediator during the progression of inflammatory airway diseases such as allergic asthma (Grunig, 1998; Wills-Karp, 1998). IL-13 mediates these functions through interaction with its cognate receptor, and transmits signals from the cell surface to the nucleus through signaling pathways including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Kuperman, 2002), the phosphatidylinositol 3-kinase (PI3K) pathway (Wright, 1997; Ceponis, 2000; Kelley-Welch, 2003), and the
extracellular-signal regulated kinase (Erk) branch of the mitogen activated protein (MAP) kinase signaling pathway (Booth, submitted).

We have demonstrated previously that IL-13-induced proliferation of normal human bronchial epithelial (NHBE) cells in vitro is mediated by the autocrine/paracrine action of TGFα and its receptor, the epidermal growth factor receptor (EGFR) (Booth, 2001). Growth factors, such as transforming growth factor-α (TGFα), are known to activate a wide spectrum of intracellular signal transduction pathways including the PI3K and MAPK pathways, although we have shown that IL-13-induced activation of the MAPK signaling pathway is not mediated by TGFα or EGFR (Booth, submitted).

Both IL-13 and TGFα are known to activate various TFs. The most studied IL-13-activated TFs are the STAT proteins. STAT6 is a well documented target of IL-13-induced signal transduction pathways in numerous cell types (Chomarat, 1998; Lee, 2001; Kelley-Welch, 2003) while more recently, additional members of the STAT family, STAT1α, STAT3, STAT5A and STAT5B, were identified as IL-13 targets in human monocytes (Roy, 2002). Likewise, TGFα is a known activator of STAT3, as well as other TFs including AP-1, AP-2, NFκB and p53 (Gille, 1997; Factor, 2001; Xiao, 2003).

The purpose of the study presented here was to investigate the activation of TFs following IL-13 or TGFα exposure in normal human bronchial epithelial (NHBE) cells cultured in vitro using an air/liquid interface (ALI) system where in NHBE cells mimic the functional and morphological characteristics of the in vivo airway epithelium (Krunkosky, 2000). To gain insights into the biological effects of IL-13 and TGFα on NHBE cells we used an array technology that allows for the analysis of multiple TFs, 54 in all, in a single experiment. Conventionally, TF activity has been analyzed in vitro by
gel shift or electrophoretic mobility shift assays (EMSA), or in vivo with reporter assays. In both conventional in vitro and in vivo assays, only one TF can be analyzed per experiment. The array technology we employed allows for high throughput analysis and its reliability has been confirmed previously using conventional EMSA (Jiang, 2003).

Using this new array technology we demonstrate that IL-13 and TGFα induce the activation of TFs in NHBE cells within 15 minutes of exposure. Furthermore each stimulus induces the activation of its own unique set of TFs, with an additional set of TFs activated by exposure to either IL-13 or TGFα, suggesting activation of parallel signal transduction pathways by these stimuli.
Methods

Reagents

NHBE cells and bronchial epithelial growth media (BEGM) and supplements were purchased from Cambrex (Walkersville, MD). Transwell membranes were purchased from Costar Corporation (Cambridge, MA). IL-13 and TGFα were purchased from R&D Diagnostics (Minneapolis, MN). Nuclear Extraction Kits and TranSignal Protein/DNA Arrays were purchased from Panomics, Inc. (Redwood City, CA). All other reagents, except those needed for cell culture as referenced below, were purchased from Sigma (St. Louis, MO).

NHBE Cell Culture

NHBE cells (Cambrex, Walkersville, MD) were grown on transwell membranes as described previously (Krunkosky, 2000). Briefly, following expansion, NHBE cells (passage 2) were plated at a density of approximately 35,000 cells per cm² on transwell membranes coated for 1 hour with 50 µg/ml rat-tail collagen, type I (Collaborative Research, Bedford, MA). Initially, the cells were kept submerged in a 50:50 mix (volume: volume) of bronchial epithelial growth media (BEGM): Dulbecco’s modified Eagle medium with high glucose (DMEM-H) supplemented with 0.13 mg/ml bovine pituitary extract (Pel-Freeze, Rogers, AR), 5 x 10⁻⁶ M all-trans retinoic acid (Sigma), 1 µg/ml bovine serum albumin (Chemicon, Temecula, CA), 0.5 ng/ml EGF, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin, 0.5 µg/ml epinephrine, 6.5 ng/ml triiodothyronine (Cambrex), and 44.0 IU/ml nystatin (Amresco, Solon, OH). Medium was changed every other day until the cells reached confluence between days 6 to 8 in...
culture, at which time the apical medium was removed to establish an air/liquid interface (ALI) after which time the basolateral medium was changed daily.

**Treatments of samples**

NHBE cells were grown in ALI culture for 9 days; at this time appearance of mature secretory cells is prominent. NHBE cells were treated for 15 or 60 minutes with IL-13 (10 ng/ml) or TGFα (5 ng/ml) from the basal lateral surface only or in the case of control samples a media change only. These concentrations were previously determined to yield the greatest proliferation results and are concentrations that are not toxic to cells as determined by LDH release cytotoxicity assay (Data not shown). Following treatment, nuclear extraction was carried out as outlined below. Each exposure, with subsequent nuclear extraction, was repeated at least twice.

**Nuclear Extraction**

Nuclear extraction was performed using a commercially available kit (Nuclear Extraction Kit obtained from Panomics, Inc, Redwood City, CA), according to manufacturer’s instructions. Briefly, cells were washed twice with PBS followed by lysis in extraction buffer A (10 mM HEPES; pH 7.9, 10 mM KCl, 10mM EDTA, protease inhibitor cocktail (Sigma) and 4% IGEPAL) on ice for 10 min with gentle shaking. Cells were harvested from transwell inserts by scraping in extraction buffer and then pipetting several times to break up any clumps of cells. Nuclei were collected by centrifugation at 15,000 x g for 3 min at 4°C. The pellet was resuspended in extraction buffer B (20 mM HEPES; pH 7.9, 400 mM NaCl, 1 mM EDTA, 10% glycerol, protease inhibitor, 10 mM DTT) and mixed
for 2 hrs at 4°C with gentle rocking. The mixture was then centrifuged at 15,000 x g for
20 min at 4°C and the supernatant containing the extracted protein collected.
Concentration of protein in the extracts was determined using the Bradford dye-binding
procedure (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA).

**Transcription Factor Activation Assay**

Activation of transcription factors was determined using a commercially available assay,
TranSignal Protein/DNA Array, from Panomics according to manufacturer’s protocol.
Briefly, 5 µg of nuclear extract from each sample was combined with TranSignal Probe
Mix and separated in 2% agarose gel in 0.5X TBE. The separated protein/DNA
complexes from the nuclear extracts were excised from the gel and extracted at 55°C.
The extracted complexes were resuspended in deionized H₂O and hybridized to array
membranes. Following hybridization each blot was washed, treated with Streptavidin-
HRP conjugate, washed again, and stained with the included Substrate solution. Each
array was visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech,
Piscataway, NJ).

**Analysis of Array Data**

Exposure time was adjusted until the majority of the spots intended for alignment that
consist of biotinylated DNA were of equal signal intensity. The exposed autoradiographs
were electronically scanned and analyzed using Labworks imaging and analysis software.
The alignment spots of each array were set to an equivalent intensity ensuring consistent
exposure between arrays. The analysis files generated by Labworks software were
exported to Microsoft Excel for further analysis. Fold changes were determined by dividing the average differences of each sample by the average differences of the additional sample, (ex. Average of replicates on a single blot of IL-13 treated sample/Average of replicates on a single blot of untreated sample=Fold change induced by IL-13). Any change of two-fold increase or decrease was considered significant.
Results

Activation of transcription factors by IL-13

To determine whether IL-13 is capable of inducing the activation of TFs in NHBE cells following 15 and 60 minutes of cytokine exposure, nuclear extracts prepared after IL-13 treatment were hybridized to a TranSignal Protein/DNA array, which contains 54 consensus-binding sequences for specific TFs. Figure 1 demonstrates the relative levels of activation of TFs following exposure of NHBE cells to IL-13 for 15 min compared to activation in untreated cells. TFs that were activated by IL-13 (10 ng/ml) at 15 minutes or 60 minutes of exposure are shown in Table 1. The quantitative analyses of the image results indicated in the table were induced two-or more fold over control levels. We observed that a greater number of TFs were activated at the shorter time point (15 min) than at the longer exposure point (60 min). Further, no overlap in TF activation between these two points was observed. These data suggest that IL-13 activates TFs in NHBE cells at varying times to varying degrees.

TABLE 1

<table>
<thead>
<tr>
<th>15 min exposure</th>
<th>Fold Increase</th>
<th>60 min exposure</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-E2</td>
<td>2.1</td>
<td>USF-1</td>
<td>2.0</td>
</tr>
<tr>
<td>PRE</td>
<td>2.6</td>
<td>TR(DR-4)</td>
<td>2.1</td>
</tr>
<tr>
<td>NFκB</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAR(DR5)</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEF-1</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RXR(DR1)</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT4</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ets/PEA3</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Activation of transcription factors by TGFα

We have shown previously that IL-13-induced proliferation of NHBE cells *in vitro* is mediated by the autocrine/paracrine action of TGFα (Booth, 2001). We next wanted to determine if TGFα activated a similar set of TFs as IL-13, with the hypothesis being that any activation of proliferation-related transcription factors by IL-13 would result from the autocrine activity of TGFα. Table 2 demonstrates the set of TFs that were activated by TGFα (5 ng/ml) at 15 minutes and 60 minutes of growth factor exposure. TGFα was found to activate a subset of TFs that were also activated by IL-13 in addition to activating its own unique set of factors (Figure 2A). The TFs that are activated by both IL-13 and TGFα include NFκB, MEF-1, RXR(DR1), STAT4, and NF-E2. Of the group of TFs that is activated by both IL-13 and TGFα, the fold increase of activation over control levels is similar between the two stimuli except for NFκB and STAT4. In the case of NFκB, the level of activation following IL-13 stimulation is almost double that induced by TGFα, 4.4 to 2.8; while STAT4 activation is higher following TGFα exposure than IL-13 exposure, 5.4 to 3.3. Induction of the overlapping subset of activated TFs could be regulated by the IL-13-induced autocrine/paracrine activity of TGFα, yet the differences in levels of activation observed for certain TFs suggest that each stimulus can exert its own unique degree of activation of the upstream events leading to TF activation. This difference argues for a role of differential signaling pathways induced by IL-13 or TGFα in the activation of at least some of the overlapping TF subset.
TABLE 2

<table>
<thead>
<tr>
<th>Transcription factors induced by TGFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min exposure</td>
</tr>
<tr>
<td>CREB(1)</td>
</tr>
<tr>
<td>USF-1</td>
</tr>
<tr>
<td>Bm-3</td>
</tr>
<tr>
<td>NFκB</td>
</tr>
<tr>
<td>STAT4</td>
</tr>
<tr>
<td>MEF-1</td>
</tr>
<tr>
<td>p53</td>
</tr>
<tr>
<td>RXR(DR1)</td>
</tr>
<tr>
<td>RAR(DR5)</td>
</tr>
<tr>
<td>CBF</td>
</tr>
<tr>
<td>c-Myb</td>
</tr>
<tr>
<td>Pbx1</td>
</tr>
<tr>
<td>AP-1</td>
</tr>
<tr>
<td>NF-E2</td>
</tr>
</tbody>
</table>

IL-13 downregulates CREB

The only TF included in the array to be down regulated by IL-13 at either time point examined was CREB(1). It was downregulated following a 15 min IL-13 stimulation (Table 3). CREB(1) was activated by TGFα at the same time point (Table 1). This could be an example of IL-13 inducing alternate signal transduction pathways than those activated by TGFα in NHBE cells. Of the TFs analyzed, there was no significant downregulation following TGFα exposure at either timepoint investigated.

TABLE 3

<table>
<thead>
<tr>
<th>Transcription factors decreased by IL-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min exposure</td>
</tr>
<tr>
<td>CREB(1)</td>
</tr>
</tbody>
</table>
**IL-13 and TGFα activate different sets of transcription factors**

To further analyze the differences in TFs activated by IL-13 and TGFα, the arrays were analyzed for changes in TF activation between the two stimuli. In addition to comparing TF activation in response to both stimuli compared to control levels, the results of the two stimuli were compared to each other. Tables 4 and 5 demonstrate that in addition to activating a common set of factors as described above, IL-13 and TGFα each activate a unique set of transcription factors. IL-13 activated EGR(1) at 15 minutes and STAT4 at 60 minutes over TGFα (level of IL-13 activation divided by level of TGFα activation), while TGFα activated CREB(1), AP-1, AP-2, and E2F1 over IL-13 (level of TGFα activation divided by level of IL-13 activation). These data demonstrate that IL-13 and TGFα activate different sets of transcription factors at the time points analyzed.

**TABLE 4**

<table>
<thead>
<tr>
<th>Transcription factors induced by IL-13 over TGFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min exposure</td>
</tr>
<tr>
<td>EGR(1)</td>
</tr>
</tbody>
</table>

**TABLE 5**

<table>
<thead>
<tr>
<th>Transcription factors induced by TGFα over IL-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min exposure</td>
</tr>
<tr>
<td>CREB(1)</td>
</tr>
<tr>
<td>AP-1</td>
</tr>
<tr>
<td>AP-2</td>
</tr>
<tr>
<td>E2F1</td>
</tr>
</tbody>
</table>
Discussion

This study demonstrates that both IL-13 and TGFα activate a number of TFs within 15 minutes of exposure, and that the majority of those factors active at 15 minutes post-exposure are no longer active 60 minutes after exogenous cytokine or growth factor stimulation. Furthermore, IL-13 and TGFα each activated their own set of TFs with a number of factors overlapping between the two stimuli at the 15 min time point (Figure 2A). The overlap of activated TFs ceased by 60 minutes of stimulation (Figure 2B).

Interestingly, when comparisons are made between the groups of TFs activated by the two stimuli, additional significance emerges. By this method of analysis, IL-13 induced the activation of EGR(1) at 15 minutes and STAT4 at 60 minutes at a greater level than TGFα induced activation of these factors. Similarly, TGFα activated CREB(1), AP-1, AP-2, and E2F1 to a greater extent than IL-13 at the 15-minute timepoint.

We demonstrated recently that both IL-13 and TGFα activate the mitogen activated protein (MAP) kinase and the phosphatidylinositol 3-kinase (PI3K) signal transduction pathways in NHBE cells in vitro (Booth, submitted). The initiation of these proliferative signaling pathways by both stimuli may explain the overlapping of transcription factors activated by these two stimuli. In addition to the above-mentioned MAP kinase and PI3K signaling pathways, IL-13 is known to activate the Jak/STAT signal transduction pathway (Kelley-Welch, 2003). Specifically, IL-13 induces the phosphorylation, dimerization, and nuclear translocation of STAT6 (Lee, 2001). The additional involvement of Janus kinases in IL-13 induced activation of transcription factors can explain, at least partially, the existence of activated transcription factors that are not TGFα-mediated following IL-13 exposure since TGFα is not known to activate
these kinases. Other possible explanations regarding the disparity in the sets of activated transcription factors include the possibility that regulatory protein phosphatases are activated in response to IL-13 (Jiang, 2000) or that IL-13 induces alterations in EGFR expression, both of which would impact IL-13-induced autocrine/paracrine TGFα-induced signal transduction pathways in a way that exogenous addition of TGFα would not.

Previous studies have demonstrated the ability of IL-13 to induce the activation of transcription factors in multiple pulmonary tissues, including the epithelium (Lee, 2001). Interestingly, our results vary from those published previously. Different model systems and concentrations of IL-13 used in this study could explain this difference. While both groups utilized NHBE cells, we adopted an air/liquid interface culture system that promotes differentiation of the epithelial cells and used IL-13 at a concentration of 10 ng/ml, while Lee et al. (2001) cultured NHBE cells in a traditional submerged system and utilized IL-13 at a concentration of 100 ng/ml, a ten-fold difference in cytokine levels. Differing concentrations of IL-13 are known to have distinct effects on NHBE cells grown in an ALI system. A low concentration of IL-13 (1 ng/ml) significantly increases goblet cell differentiation while an IL-13 concentration of 10 ng/ml markedly decreased goblet cell differentiation (Atherton, 2003).

To summarize, we observed that both IL-13 and TGFα induce the activation of a battery of transcription factors in NHBE cells in vitro. Each stimulus activates its own group of TFs and, interestingly, there is a subset activated by both IL-13 and TGFα. This overlap only exists at the earliest time point investigated (15 minutes). The activation pattern of TFs induced by both IL-13 and TGFα is dramatically different at the longer
time point investigated (60 minutes), suggesting that cytokine and growth factor mediated activation of transcription factors is tightly regulated in a temporal manner. Activation of multiple parallel pathways appears to be controlling the biological effects of IL-13 and TGFα such as proliferation and differentiation in NHBE cells. Therefore, further investigations are needed to understand other signal transduction pathways initiated by both IL-13 and TGFα. While our present findings provide some clues to the TFs that are being induced by cytokines and growth factors, additional studies would enable us to decipher more completely how these multiple pathways interact with TFs to effect the biological function of epithelium and other cell types in the event of airway-remodeling.
References


12. Izuhara, K. et al. Recent advances in understanding how interleukin 13 signals are involved in the pathogenesis of bronchial asthma. Archivum Immunologiae et Therapiae Experimentalis 48, 505-512 (2000).


Figure 1. Representative TranSignal arrays. Top panel depicts a membrane hybridized with nuclear protein samples from untreated cells. The bottom panel depicts a membrane hybridized with nuclear protein samples from cells treated with IL-13 (10 ng/ml) for 15 min. The circles are used to highlight TFs and their differences in activation. Red circles indicate a 2-fold or higher change in activation, while yellow circles indicate a 2-fold or greater decrease in activation.
Figure 2. Illustration depicting the transcription factors activated by IL-13 (10 ng/ml) and TGFα (5 ng/ml) following (A) 15-minute exposure and (B) 60-minute exposure to the appropriate stimulus. Each stimulus activates its own set of TFs with an overlap of these observed at 15 minutes of exposure. No overlap is present following a 60-minute stimulation.
APPENDIX II

Diverse Roles of Transforming Growth Factor-α Throughout the Mammalian Life Span

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Running Title: Roles of TGFα

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ABSTRACT

Transforming growth factor-alpha (TGFα) is a member of the epidermal growth factor (EGF) family that was discovered in 1978 in conditioned medium from Rous sarcoma virus transformed fibroblasts. Expression of the TGFα gene is highly regulated in response to exogenous cellular signals including cytokines and other growth factors. Although the transmembrane form of TGFα can occasionally serve as a biologically active autocrine/paracrine growth factor, further proteolytic cleavage of this membrane-anchored precursor generates soluble, mature TGFα which serves as a potent mitogen of many cell types. Such mitogenic activity originates as TGFα activates EGF-receptor subtypes, initiating intracellular signal transduction pathways. As a mitogen, TGFα has been implicated as a participant in physiological processes as diverse as blastocyst implantation, wound healing, and lung and eyelid development. It further plays a role in pathological processes such as tumor growth and progression. Thus, TGFα can be considered a growth factor essential to the execution of a variety of biological processes that occur throughout the mammalian lifespan.
INTRODUCTION

Transforming growth factor-α (TGFα) is a molecule of profound importance throughout the course of life. It plays roles in the initial implantation of the fertilized egg in the uterine wall, regulates fetal development and puberty, and then, ultimately, death, either through disease, apoptosis, or senescence. The objective of this review is to solidify the overall importance of TGFα throughout the mammalian lifespan by offering a comprehensive understanding of the varied roles and activities of this growth factor in mammalian cellular and organismal life.

Transforming growth factors were first identified in 1978 in conditioned medium from Rous sarcoma virus-transformed fibroblasts (DeLarco and Todaro, 1978). It was observed that addition of this partially purified material to normal fibroblasts caused the reversible appearance of a malignant phenotype. Later it was shown that this transforming activity was caused by the actions of two proteins, now designated transforming growth factor-alpha (TGFα) and transforming growth factor-beta (TGFβ). It was observed subsequently that TGFα is an embryonic growth factor inappropriately expressed in neoplasia (Coffey et al, 1992), and, hence, its role in cellular events is far broader than simply the regulation of pathological conditions that result in tumor initiation.

The binding of TGFα to the epidermal growth factor receptor (EGFR) initiates cellular proliferation and migration events involved in embryogenesis, organ development, wound healing, and bone reabsorption. Furthermore, while this mitogenic peptide is involved in the transformation of normal cells into malignant growths, it also promotes accompanying normal development such as angiogenesis. Recent studies
utilizing TGFα knockout mice have demonstrated that the mice, while born viable, develop unnaturally in that their lungs do not branch correctly, their hair is unusually coarse and wavy, and their eyelids do not fuse properly (Mann et al, 1993; Berkowitz et al, 1996). These findings suggest a role for the growth factor in very diverse developmental activities, albeit activities requiring cellular proliferation.

**STRUCTURE OF TGFα GENE AND PROTEIN**

The human TGFα gene spans 70-100 kb on chromosome 2p13 and contains six exons that are transcribed into a 4.5-4.8 kb mRNA encoding a 160 amino acid polypeptide (Coffey et al, 1992). The promoters of the rat and human TGFα gene have been cloned and their most obvious characteristics include the apparent lack of TATA and CCAAT sequences, and high GC content. The human core promoter contains a nonconsensus TATA box that is recognized by a TATA-binding protein and an initiator element that accurately orient the transcription complex. Additionally, p53 plays a role as a transcriptional enhancer in humans. DNase I footprinting reveals a 43-base pair element protected by nuclear proteins, one of which is the transcription factor AP-2. AP-2 controls transcription by binding one of the cis-acting elements, the distal regulatory element, in the core promoter (Wang et al, 1997). The TGFα promoter regulates expression of the gene throughout development and mediates changes induced by DNA methylation, hormones, cytokines (Hallbeck et al, 2001), and high glucose concentrations (Sayeski and Kudlow, 1996).

The translated precursor protein (proTGFα) contains an extracellular NH2-terminal sequence of 23 uncharged, apolar amino acids that functions as a signaling
peptide (Wong et al, 1989). The subsequent 74 amino acid sequence contains glycosylation sites (Briely et al, 1997) as well as the mature peptide sequence. A hydrophobic domain of 23 amino acids bordered by basic amino acids suggests proTGF\(\alpha\) to be an integral membrane protein. A cysteine-rich 39 amino acid sequence modified by palmitoylation composes the COOH terminal cytoplasmic tail (Figure 1).

**TGF\(\alpha\) TRANSLATION AND PROCESSING**

Following transcription of the *TGF\(\alpha\)* gene, the protein is translated in the rough endoplasmic reticulum and moved to the Golgi for further post-translational modifications. The proTGF\(\alpha\) remains in the Golgi until it is moved into small cytoplasmic membrane bound vesicles (Ezzat et al, 1995; Kuo et al, 2000). These vesicles either remain within the cytoplasm of the cell or fuse with the outer cell membrane. It is in these small vesicles or on the cell surface where the final cleavage and modifications, including removal of glycosylated sidechains, take place resulting in the mature form of TGF\(\alpha\).

It has been demonstrated that cleavage of proTGF\(\alpha\) into its mature form is at least, in part, due to the activity of tumor necrosis factor-alpha converting enzyme (TACE) (Peschon et al, 1998). TACE is a member of the ADAM (A Disintegrin And Metalloproteinase) family of metalloproteinases, enzymes that contain both metalloprotease and disintegrin domains. TACE has also been shown to be responsible for the cleavage of several other transmembrane proteins including TNF\(\alpha\), \(\beta\)-selectin, p75 TNFR and HER4 (Peschon et al, 1998; Rio et al, 2000; Zhao et al, 2001).
Cleavage of the mature peptide from proTGFα depends on cleavage of alanine-valine bonds at the amino and carboxyl termini (Derynck et al, 1984; Briley et al, 1997). The carboxy-terminal valine residues are required for the peptide’s maturation and intracellular routing. The processed protein structure has three loops, A, B and C, created by disulphide bridges extending from cysteine residues (Fig. 1). Residues from the A and C loops and the C-terminal tail are responsible for the formation of the major binding interface with the epidermal growth factor receptor (EGFR) (McInnes et al, 1998), while the B loop provides a structural scaffold for this site (McInnes et al, 1996) (Figure 1). The reported sizes of mature TGFα range from 5 to 20 kD. This variation may be due in part to differential glycosylation and proteolytic cleavage as well as dimerization and the presence of binding proteins.

The cleavage of proTGFα into its mature form allows the growth factor to migrate from the location of synthesis to other areas of the same tissue or to other nearby tissues. For example, in the gut the majority of the TGFα mRNA is present in the crypt cells while activation of the EGFR by TGFα occurs elsewhere in the intestine, demonstrating the ability of TGFα to migrate following translational and subsequent cleavage (Barnard et al, 1991).

**SOURCES OF TGFα**

TGFα is generally produced by tissues of ectodermal origin (Table 1). TGFα has been found to be produced *in vivo* and *in vitro* by keratinocytes (Coffey et al, 1992) and by airway epithelial cells *in vivo* (Ruocco et al, 1996; Hardie et al, 1999) and *in vitro* (Booth, 2001). Production of TGFα has also been detected in the normal pituitary,
mammary epithelium (Liu et al; Smith et al), gastrointestinal tissues (Coffey, 1992), prostate, brain, and uterine epithelium (Hansard et al, 1997; Bush et al, 1998). Mucosal tissues such as colon mucosa and bronchial mucosa produce significant quantities of the growth factor. Some cells of the immune system are also known to produce TGFα. For example, TGFα has been shown to be stored in subpopulations of cytoplasmic granules in human neutrophils and monocytes (Calafat et al, 1997).

Table 1. Cellular sources of TGFα

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Epithelium</td>
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<tr>
<td>Airway</td>
<td>Booth, Ruocco, Hardie</td>
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<tr>
<td>Endometrial</td>
<td>Bush, Hansard</td>
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<td>Lens</td>
<td>Chen</td>
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<td>Liver</td>
<td>Hufnagl</td>
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<td>Mammary</td>
<td>Liu, Smith</td>
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<tr>
<td>Oesopharygeal</td>
<td>Calabro, Glickman</td>
</tr>
<tr>
<td>Prostate</td>
<td>De Miguel, Tørring</td>
</tr>
<tr>
<td>Renal tubular</td>
<td>Everitt</td>
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<tr>
<td>Intestinal</td>
<td>Habel, Pérez-Tomás, Ziober</td>
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<tr>
<td>Inflammatory cells</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>Calafat, Hallbeck, Madtes</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Calafat</td>
</tr>
<tr>
<td>Gastrointestinal tissues</td>
<td>Xian, Coffey</td>
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<tr>
<td>Lung fibroblasts</td>
<td>Vivekananda</td>
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**THE TGFα RECEPTOR**

TGFα belongs to the epidermal growth factor (EGF) family of growth and differentiation factors. Other members of this family include EGF, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epieregulin (EPR), heregulin (HRG), neuregulin-1 (NRG-1), neuregulin-2 (NRG-2), and epigen (EPI). An amino acid motif of about 40 residues containing six cysteines, known as the EGF-like
motif, is the common feature of these peptides. These six cysteines form three intramolecular disulphide bonds that are essential for mitogenic activity (Raab and Klagsbrun, 1997). All of the growth factor family members are synthesized as transmembrane molecules, which are subsequently cleaved proteolytically to release the soluble mature growth factors. Both the membrane-anchored and the soluble forms can be biologically active (Wong et al, 1989).

TGFα shares a 40% sequence homology with EGF resulting in their ability to compete for binding to the EGFR, which is one member of a four-member family of receptor protein subunits including EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. These receptors are known in humans as the HERs (Human EGF Receptors). TGFα binds to HER1 (the EGFR) promoting the formation of either receptor homodimers (HER1/HER1) or heterodimers (HER1/HER2, HER1/HER3 or HER1/HER4) depending on cell type (Elenius et al, 1997). In rat olfactory mucosa, TGFα activates EGF-R and ErbB-2 but not ErbB3 or ErbB4 (Ezeh and Farbman, 1998), while in some cell lines such as mouse Ba/F3 lines, TGFα also activates ErbB3 (Riese et al, 1996). Following dimerization, tyrosine residues of the receptor intracellular domains are transphosphorylated initiating signal transduction pathways. These pathways include the mitogen activated protein kinase (MAP) pathway (Ezeh and Farbman, 1998), phospholipase C-γ (PLC-γ) and phosphatidylinositol 3-kinase (PI3K) dependent pathways (Wilson and Gibson, 1999) (Figure 2).

Although TGFα and EGF bind the same receptor, and are over 40% homologous, the respective cellular outcomes they initiate are different; TGFα is known almost exclusively as a potent mitogen while EGF is known to act as an initiator of epithelial
migration, an initiator of cellular differentiation and as a mitogen. These outcomes are due to the differences in binding efficiencies of the two growth factors to the EGFR. EGF and TGFα have similar receptor binding efficiencies at the cell surface but these receptor-ligand affinities change once the receptor/ligand complex is internalized in clathrin-coated pits and progresses through the endosomal pathway. As the pH in the lysosomal vesicles drop, TGFα dissociates about four times more rapidly from the EGFR than does EGF, resulting in rapid recycling of receptors formerly bound to TGFα (Lauffenburger et al, 1998). EGF maintains its bond to the EGFR longer retaining its kinase activity throughout early and late lysosomes. These differences in rates of receptor recycling and maintainance of kinase activity result in the differences in cellular activities induced by these different growth factors (Longva et al, 2002).

Another mode of activity displayed by TGFα, and by some other growth factors, is its ability to act in an autocrine/paracrine manner. Autocrine loops are established when soluble factors released by cells bind to and stimulate receptors on their own surface (Shvartsman et al, 2002). Soluble TGFα has been observed in colon carcinoma cells (Ziober et al, 1993; Karnes et al, 1992) and bronchial epithelial cells (Booth et al, 2001). The TGFα precursor, proTGFα, has also been shown to be biologically active and, in studies utilizing amino acid substitution to generate a form of TGFα that cannot be proteolytically processed, it has been demonstrated that TGFα binds the EGFR on adjacent cells leading to signal transduction (Wong et al, 1989). Although it has been demonstrated in a variety of epithelial tissues that TGFα is active in its membrane-anchored form, activity of this form is less than when the growth factor is in its cleaved
But, whether cleaved or tethered, TGFα is a potent stimulator of intracellular signal transduction events leading to proliferation.

**DEVELOPMENTAL ROLES OF TGFα**

TGFα has been implicated in a variety of developmental events beginning with implantation, proceeding through fetal development, influencing organismal changes at puberty, and finally having a role in aging and death. In general, TGFα serves during development as a facilitator of proliferation. However, as is the case in all physiological processes, no one growth factor is responsible for an outcome, but by TGFα acting in concert with other factors, the required effect is promoted.

**Implantation & Mammogenesis**

In the female reproductive system, TGFα is localized primarily to endometrial epithelial cells, and with the most expression observed in the luminal surface epithelium. In the surface epithelium, TGFα expression is high in the proliferative phase, decreased during the early secretory phase, is at its lowest point in the midsecretory phase, and rebounds in the late secretory phase. The observed decline in expression of TGFα in the epithelium coincides with the timing of preimplantation embryo development and implantation (Hansard et al, 1997). Furthermore, the expression patterns observed during the menstrual cycle suggests that TGFα cleavage is most active during this interval in which preimplantation embryos would be in the uterine cavity. In the mouse uterus, TGFα expression is greater in the luminal epithelium of the receptive uterus during the preimplantation period, suggesting a paracrine and/or juxtacrine role for TGFα in implantation.
One theory is that once TGFα is cleaved, it can rendezvous with the embryo prior to implantation, as mammalian embryos are known to express EGFR on their cell surfaces (Chia et al, 1995; Wiley et al, 1992). Preimplantation development is known to depend on multiple interactions between mother and embryo. EGFR and its ligands have been implicated as potential components of this embryo-maternal cross-talk, mediating, among other things, estrogen action (Nelson et al, 1992). Interactions between the mother and embryo achieve an organizational state on the cellular level that promotes proliferation, embryonic cell division, uterine remodeling and extracellular matrix (ECM) remodeling that allows the embryo to interact with the uterine wall and the ECM itself via various adhesion molecules present on cellular surfaces.

During mammalian implantation, both the uterine epithelium and its ECM are remodeled dramatically. In the baboon, TGFα has been proposed to play a role in decidualization and glandular development. When TGFα is down-regulated in the murine uterus following stimulation of a metallothionein-1 promoter, the expression of TGFβ1 receptor is also down-regulated, resulting in delayed attachment and implantation. TGFβ has powerful effects on angiogenesis and ECM modification. This action suggests a role for TGFα in regulation of additional growth factors during implantation (Slater and Murphy, 2000). TGFα is believed to stimulate production of TGFβ1 in rats and mice (Das et al, 1997) resulting in ECM remodeling.

TGFα transcripts and protein have been detected in blastocysts prior to and after elongation and in uterine glandular and luminal epithelium during early bovine (Kliem et al, 1998) and murine (Brison and Schultz, 1996) gestation. TGFα mRNA has been demonstrated in early cleavage stages of sheep embryos, porcine embryos (Chang et al,
and bovine IVP blastocysts. It has been hypothesized that TGFα may be involved in the complex developmental reorganization that occurs at the onset of blastocyst elongation. TGFα protein is localized to the uterus and embryo in the goat suggesting a role in chorioallantoic differentiation and, consequently, in the intense vascular development which occurs (Flores et al, 1998).

TGFα mRNA expression has been detected during different stages of mammary development (Plath et al, 1997). The highest levels were detected during mammogenesis and involution, which may indicate autocrine or paracrine functions of the growth factor during stages of proliferation and reorganization of the mammary tissue. TGFα protein has also been detected in the ductal and lobuloaveolar stages suggesting a continued role of the growth factor at later stages of mammary development. Expression of TGFα is witnessed in the proliferative cap cells and the stromal fibroblasts around the terminal endbud (Humphreys and Hennighausen, 2000). Levels of TGFα increase 2-3 fold in lactating mice over virgin levels.

Embryonic & Fetal Development

TGFα knockout mice display significant developmental defects. The mice are viable and fertile but demonstrate such developmental abnormalities as impaired lung branching, eye abnormalities including open eyes at birth, reduced eyelid size and impairment of lid fusion, and disoriented hair follicle formation and malformation of the hair shafts resulting in wavy hair and whiskers (Berkowitz et al, 1996). Translation of the growth factor alone is not sufficient on its own to promote these developmental progressions. Supportive evidence of this fact comes from studies with TACE knockout mice. These mice exhibit the same developmental oddities, including lung branching
deficiencies (Zhao et al, 2001) as TGFα knockout mice, suggesting that TACE is an essential component of these TGFα mediated processes. The exogenous addition of either TGFα or TACE rescues the defect and restores proper lung branching of TACE knockout mice in *ex vivo* experiments (Zhao et al, 2001). In the TACE knockout mouse, TGFα gene expression is not altered and thus the addition of exogenous TACE likely cleaves the proTGFα expressed allowing TGFα mediated processes to proceed.

TGFα, as well as EGFR, has been localized via immunohistochemistry to human airway epithelial cells from the trachea to distal airspaces throughout the entire period of fetal development (Ruocco et al, 1996). Transgenic mice overexpressing TGFα displayed morphologic alterations of several organs. The lungs develop abnormally with large alveolar spaces and pulmonary fibrosis (Hardie et al, 1997) as well as hysteresivity (Pillow et al, 2001). Chronic lung injury that develops in some neonates is characterized by simple, evenly distributed terminal air spaces. Since the lungs are one of the last organs to fully develop and TGFα is involved in the injury/repair process (See below), the injuries developed by some neonates may in part be due to an overabundance of TGFα.

There are many additional, non-pulmonary developmental events affected by TGFα that are demonstrated by knockout mice. The livers of these animals exhibit an increase in the amount of normal parenchyma; while the colon displays mucosal hyperplasia with deeper, more cellular glands (Sandgren et al, 1990). The magnitude of these responses was proportional to the level of local, tissue-specific TGFα expression.
Thus, only is the presence of TGFα required during mammalian development but a threshold level of the growth factor is also necessary to ensure proper formation and function of many tissues and organs.

**TGFα IN EPITHELIAL INJURY AND REPAIR**

Increased levels of TGFα correlate with a variety of epithelial injuries including wounded colonic epithelium (Wilson and Gibson, 1999), asbestos-induced and (Liu et al, 1996) oxidant injury of the airway epithelium (Vivekananda et al, 1994), and injury of corneal epithelial cells (Goke at al, 2001).

Some of the increases in growth factor levels observed in response to an epithelial injury are attributable to the influx of inflammatory cells such as neutrophils and macrophages, which produce and secrete these factors. Additional growth factors can also be generated by the affected tissues. For example, fibroblasts do not express TGFα constitutively, but in response to oxidative stress fibroblasts from normal lungs synthesize and release TGFα (Vivekananda et al, 1994), in turn affecting the surrounding epithelium and additional fibroblasts through the production and release of growth factors. Inflammatory cytokines can also induce TGFα production from affected tissues. Using an airway epithelial model, we have shown that normal human bronchial epithelial (NHBE) cells similarly release TGFα in response to the inflammatory cytokine interleukin-13 (IL-13). Addition of IL-13 to these cells in vitro, yields a three-fold increase in soluble TGFα is observed in the surrounding medium by 1-hr of exposure (Booth et al, 2001). Interestingly interleukin-1β (IL-1β) has been shown to stimulate migration and proliferation of alveolar type II epithelial cells in vitro, two steps that are
critical to the repair process. The IL-1β repair effect is EGFR dependant suggesting an EGF or TGFα dependent mechanism (Geiser et al, 2000). If TGFα is neutralized in these cultures proliferation is blocked. Thus, it is believed that IL-1β enhances in vitro epithelial repair by a TGFα-dependant mechanism (Geiser et al, 2000). Interleukin-6 has also been shown to stimulate the production and release of TGFα (Hallbeck et al, 2001).

A general understanding of the epithelial repair process indicates that it is divided into two phases, migration and proliferation. First, epithelial cells adjacent or just beneath the injured surface migrate into the wound to cover the denuded area. This process does not require proliferation, only migration and the deposition of ECM. Epithelial proliferation and differentiation then occur to replenish the decreased cell pool (Dignass at al, 1996).

The mitogenic qualities of TGFα are associated with the increased expression of cell cycle associated proteins. Following TGFα exposure, several early cell-cycle-associated proteins including the growth factor sensor cyclin D1, retinoblastoma protein, the transcription factor E2F-1, and cyclin E are upregulated. This upregulation indicates a progression of cells in the G1 phase towards the G1/S restriction point (Yan et al, 1997; Getchell et al, 2000; Liao et al, 2000).

Although TGFα is typically classified as a mitogen of epithelial cells, it can also act in some cases as an inducer of migration. Following colonic mucosal wounds, TGFα has a role in the initial migration step in the repair of the wound (Wilson and Gibson, 1999). A range of cytoplasmic signaling effectors that are either directly or indirectly activated following phosphorylation of the EGFR mediate this epithelial cell migration. These factors include phospholipase C-γ (PLC-γ), extracellular signal-regulated kinase
(ERK), phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC). The resulting actions of these signaling molecules include activation of transcription factors, regulation of cytoskeletal contractility and effects on cell adhesion.

TGFα also influences differentiation in a variety of cell types. TGFα caused a significant increase in the expression of beaded filament proteins, the unique cytoskeletal elements restricted to developing lens fibers, in an epithelial cell population undergoing initial stages of terminal differentiation into lens fibers derived from chick annular pads (Chen et al, 2001). In these cells, TGFα induced activation of ERK2, a member of the mitogen-activated protein kinase (MAPK) signaling cascade family, and increased phosphorylation of the cyclic AMP response element protein (CREB) transcription factor. When the TGFα/receptor interaction was inhibited, proliferation was attenuated but the cells continued to develop characteristics of a differentiated phenotype. The resulting differentiation is an indication that in addition to inducing proliferation, TGFα is capable of inducing differentiation via the MAPK pathway, albeit independent of a TGFα-induced mitogenic effect. Activation of EGFR, the receptor for TGFα, has also been linked to the regulation of mucin production and the upregulation of mucin genes in airway epithelium (Takeyama et al, 1999; Perrais et al, 2002). Thus, activation of the EGFR has been shown to be a step in the differentiation of airway goblet cells during the development of goblet cell hyperplasia (Martin et al, 2000; Booth et al, 2001).

**TGFα IN DISEASE STATES**

Perhaps the most studied characteristic of TGFα is its prevalence in disease states, particularly various forms of cancer. TGFα is associated with tumor types as diverse as
mammary, squamous, and renal carcinomas, melanomas, hapatomas and glioblastomas. In most forms of cancer where elevated TGFα levels are noted it is believed that TGFα promotes tumor growth and progression via an autocrine/paracrine loop involving the EGFR (Ziober et al, 1993; Torring et al, 2000; Humphreys and Hennighausen, 2000).

Transgenic mice expressing TGFα in the mammary epithelium develop spontaneous focal mammary tumors (Muller et al, 1996). These mice expressed both TGFα and the neu proto-oncogene, suggesting a synergistic interaction between these two members of the EGF family. Interestingly, TGFα has been shown to inhibit the secretions of MMP-2 and MMP-9 in the mammary epithelium of normal mice (Lee et al, 2001). Since MMPs are responsible for the breakdown of the ECM, and this breakdown is required for the progression of tumors, this inhibition can be perceived as a control against tumor growth. A mutation in this control function may play a part in the progression of mammary tumors.

Additionally, TGFα transgenic mice develop epithelial hyperplasia, pancreatic metaplasia and mammary carcinoma (Sandgren et al, 1990; Humphreys and Hennighausen, 2000) and lung fibrosis (Korfhagen et al, 1994). In numerous colon cancer derived cell lines, TGFα is produced and acts in an autocrine manner sustaining the proliferation and survival of these cell lines even in serum free media. This autocrine mechanism of induced proliferation is believed to mimic in vivo conditions of colon carcinoma. The over expression of TGFα is also thought to be one step in the progression of renal cell carcinoma (Everitt, 1997), and neoplastic prostates (De Miguel, 1998; Torring, 2000). Thus, all of these findings point toward a casual link between increased TGFα expression and tumor development.
Immunohistochemical studies have also demonstrated increased expression of TGFα in a variety of non-malignant disease states. These include cystic fibrosis (Hardie et al, 1999), oral leukoplakia and submucosal fibrosis (Srinivasan and Jewell, 2001). TGFα has also been implicated in the development of Barrett’s esophagus, a syndrome in which the normal squamous epithelium is replaced by columnar epithelium (Glickman et al, 2001). In each of these cases, TGFα is thought to participate in the induction of proliferation resulting in an altered tissue condition.

TGFα is also thought to govern pathological aspects of inflammatory airway diseases such as asthma, chronic bronchitis, as well as pulmonary fibrosis. Historically, examination of such a role for TGFα began when EGFR was shown to be integral to the development of airway mucus cell hyperplasia (MCH), a characteristic of the asthmatic airway. Exploration of potential mechanisms regulating MCH development led to the findings in an in vitro model mimicking mucous cell hyperplasia, that indicate bronchial epithelial cells proliferate in response to IL-13. Proliferation in this model is mediated by interaction of TGFα produced by the epithelial cells, with EGFR on these same cells (Booth et al, 2001). In addition, human eosinophils, the most abundant inflammatory cell in the asthmatic airway, have also been shown to induce the release of epithelium derived TGFα that then acts in an autocrine manner to induce mucin production (Burgel et al, 2001). In asthmatic patients the levels of EGFR are elevated, but not the other three members of the receptor family (HER2, 3 and 4) suggesting that growth factors, such as TGFα, that bind to the EGFR, and not to its counterparts, help sustain the disease state (Polosa et al, 2002). In contrast, TGFα levels are also elevated in the airway epithelium,
peribronchial regions and inflammatory cells of patients with cystic fibrosis even though EGFR levels are not elevated (Hardie et al., 1999).

In studies regarding the development of pulmonary fibrosis, transgenic mice overexpressing TGFα in pulmonary epithelial cells developed lung fibrosis (Korfhagen et al., 1994) while mice deficient in TGFα expression developed pulmonary fibrosis at much lower levels than transgenic mice following bleomycin-induced injury (Madtes et al., 1999).

The disease states mentioned above are just a few of the numerous conditions with which TGFα is associated. TGFα, while first described in relation to transformed cell, has become well associated with the development and sustaining of any disease state involving irregular proliferation and growth of cells or tissues, as well as with diseases requiring vast amounts of tissue repair.

**TGFα AND APOPTOSIS**

Balance, between the processes of growth and regression of tissue, is controlled by modulating the rate of proliferation and/or death of cells. In normal, terminally differentiated cells, programmed cell death is a natural means of controlling cell number. This process of apoptosis is under the control of an intricate network of endocrine, paracrine and autocrine interactions, and is regulated by steroids, growth factors, gonadotrophins, and cytokines, factors known to regulate proliferation.

Since TGFα is normally perceived as a growth factor, a stimulator of proliferation, it comes as no surprise that the presence of TGFα and apoptosis are inversely correlated (Habel et al., 2002). TGFα appears to play an active role in limiting
apoptosis by stimulating proliferation, rather than by simply opposing or halting apoptosis. For example, TGFα has been shown to protect ovarian cells from apoptosis alone and in synergy with TGFβ by inducing proliferation and DNA synthesis (Pehlivan et al, 2001). These effects are believed to be important in physiologic maintenance and homeostasis as well as in pathophysiologic conditions associated with excessive growth. During tumor development both proliferation and apoptosis are deregulated in the earliest stages resulting in unchecked proliferation.

A mechanism by which TGFα affects apoptosis is via activation of the GTP-binding protein ras, which can be located downstream of the EGFR (Zushi et al, 1997) in a proliferative signaling pathway. Frequent mutation of the ras protein has been reported in both precancerous adenomas and carcinomas of the colon; thus, a ras mutation may play a role in the development and progression of colonic neoplasia. Mutant ras protein exerts its tumor promoting effect through the loss of GTPase activity resulting in the continuous activation of the ras signaling cascade. If TGFα is continually present and binding to the EGFR and activating the ras pathway in a similar fashion to a ras mutation, the apoptosis-mediating signals are affected. Specifically, overexpression of ras results in elevated levels of bcl-2, a major apoptosis-inhibiting protein (Tsujimoto et al, 1985). This observation suggests that TGFα be perceived as a survival factor or anti-apoptotic agent.

**SUMMARY**

Transforming growth factor-α has been implicated in a variety of physiological functions throughout the course of mammalian life. Even though its initial discovery was from transformed sarcoma fibroblasts and its high profile actions are disease related, this
growth factor is indispensable in normal development and the everyday responses of an organism to environmental change. Loss of TGFα results in gross developmental abnormalities including malformation of the lungs, eyes and other organs. Epithelial cell repair following a variety of injuries is also mediated by TGFα. Even though TGFα is regarded as a marker for and plays a role in the progression of many types of disease states, the loss of this growth factor also severely retards the healing processes.

Continuing discovery of the diverse roles of TGFα is solidifying the importance of this factors in regulating cellular processes essential for the beginning, middle and end of mammalian cellular and organismal life.
ACKNOWLEDGMENT

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LITERATURE CITED


Figure 1. Pro-TGFα. Schematic illustrating the full-length translated pro-TGFα, with arrows pointing towards sites that are cleaved during processing to mature, soluble growth factor and O- and N-glycosylation sites are also indicated. The disulphide bonds are represented as gray lines connecting the cysteine residues, and the resulting loops labeled as A, B, and C.
Figure 2. Intracellular signal transduction pathways initiated by TGFα. Abbreviations: TACE-TNFα converting enzyme, PLC-phospholipase C, PKC-protein kinase C, ERK-extracellular signal regulated kinase, PI3K-phosphatidylinositol 3-kinase, MEK-MAP kinase kinase.