The Role of TGF-β2 on Endothelial Cell Angiogenesis in an *in vitro* Model of the Respiratory Mucosa

Introduction

Recent figures from the Centers for Disease Control and Prevention estimate the prevalence of asthma among Americans to be 20.3 million persons. Despite greater efforts to better understand and prevent this disease, incidence has steadily increased over the past quarter century. Asthma is a chronic inflammatory disease of the bronchial airway resulting in airway constriction. Symptoms of this airway constriction include wheezing, breathlessness, chest tightness, and cough (Guerra, 2005). One current model of the disease mechanism proposes that inflammation first leads to tissue injury. Local factors then repair this injury resulting in the structural changes associated with asthma including airway wall thickening, increased airway fibrosis, increased smooth muscle mass, and increased vascularization (Homer & Elias, 2004). Another model of the disease proposes that environmental factors such as pollution or allergens first cause injury which then leads to the inflammation characteristic of asthma (Walker et al., 2003).

Increased vascularization is a common feature of many inflammatory processes. Increased vascularization can be the product of both angiogenesis and microvascular remodeling. Specifically, angiogenesis refers to the growth of new blood vessels (Fig. 1) while microvascular remodeling refers to changes in existing blood vessels (McDonald, 2001). Both processes are highly correlated with asthma in particular (Dunnill, 1960, Salvato, 2001). Many factors are known to influence angiogenesis. These include members of the fibroblast growth factor (FGF) family, vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), angiogenin, platelet-derived growth factor (PDGF), tumour necrosis factor alpha (TNF-α), interleukins, chemokines, angiopoietins, and transforming growth factors alpha and beta (TGF-α and –β) (Demenico, 2004). There are three different isoforms of TGF-β (TGF-β1, -β2, and –β3), a multifunctional cytokine present in mammals.

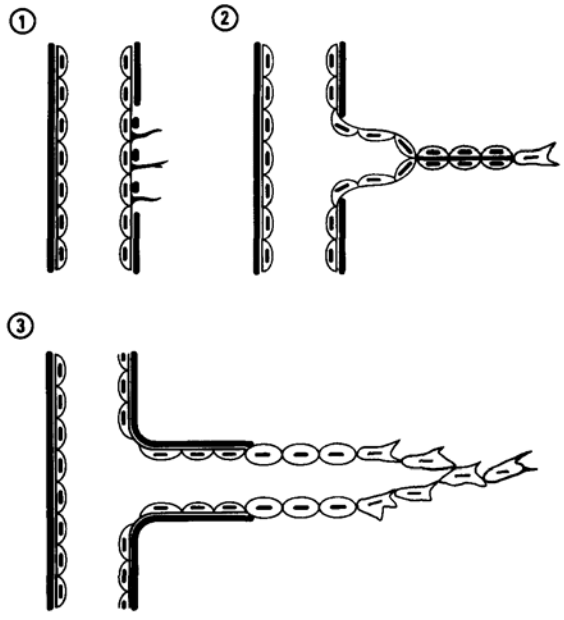

Figure 1 Diagram of an angiogenic process. (1) Endothelial cell activation is followed by basement membrane degradation and extension of cytoplasmic processes towards the stimulus. (2) This is followed by cell migration into the surrounding matrix and the formation of capillary sprouts. (3) Sprout maturation occurs by reconstitution of the basement membrane. (Pepper, M.S., 1997).

Preliminary findings in our lab indicate TGF-β2 secretion is increased when bronchial epithelial cells are injured (Fig. 2). TGF-β family members are generally involved in the regulation, proliferation, differentiation, migration, and survival of many different cell types (Lebrin et al., 2004, Roberts, 1993). The exact nature of this involvement is still under debate with conflicting results. In a developing quail heart explant model, TGF-β2 in concert with TGF-β1 and TGF-β3 enhanced angiogenesis while TGF-β1 or TGF-β2 alone inhibited it (Hofifield, 2004). A transgenic mouse model suggested TGF-β1 and its receptors are positive regulators of endothelial cell differentiation and vascularization (Pepper, M.S., 1997). A studying using Hepatocyte Growth Factor (HGF) showed TGF-β2 inhibited HGF dependent processes in endothelial cells and with it angiogenesis (Manganini & Maier, 2000).

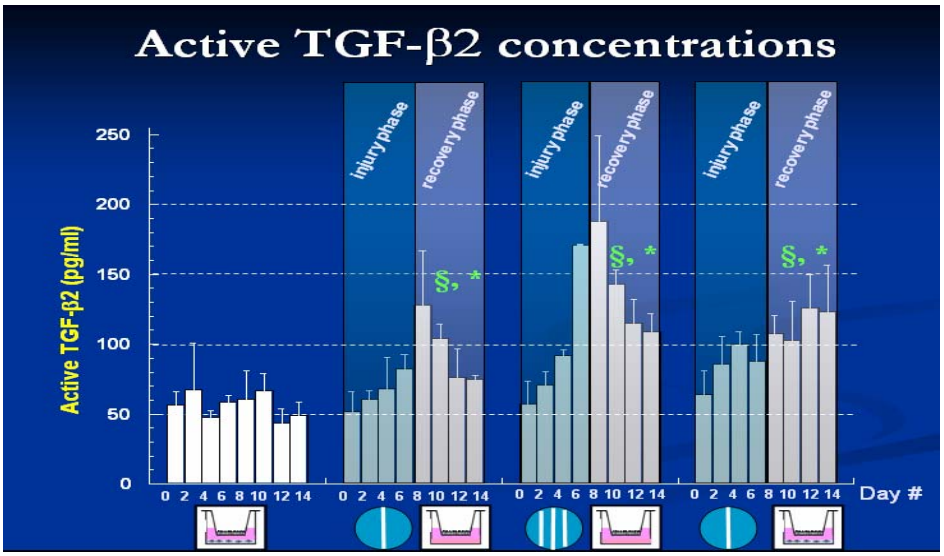

Figure 2. TGF-β2 concentrations increase after epithelial wounding in an epithelial / fibroblast construct. Basal concentrations of approximately 50 pg/ml increase up to almost 200 pg/ml upon wounding. A single vertical line indicates one denudation while three lines indicate three denudations. Courtesy of Dr. H. Garrett Thompson.

Vascular endothelial growth factor (VEGF) is a primary factor involved in angiogenesis. VEGF has been found to play a role in physiological angiogenesis (Carmeliet et al., 1996, Holifield et al., 2004), proliferative wound healing (Nissen et al, 1998), angiogenesis in asthma (Homer & Elias, 2004), and rapid angiogenesis in engineered tissue (Elcin et al., 2001). Preliminary studies in our lab using an engineered tissue construct (Griffith et al., 2005) demonstrate the requirement of VEGF for angiogenesis.

Primary Objectives

The primary objectives of this project are four-fold. To utilize a novel *in vitro* three dimensional model of the respiratory mucosa consisting of a differentiated epithelium, a pluripotent fibroblast layer, and endothelial cells to

1. Study the effects of exogenous TGF-β2 on angiogenesis
2. Study the effects of epithelial wounding on angiogenesis

3. Study the effects of exogenous TGF-β2 on VEGF secretion
4. Study the effects of epithelial wounding on VEGF secretion

We believe this *in vitro* tissue construct adequately models many of the cell-cell interactions of lung tissue *in vivo*. Past studies by researchers in our lab suggest the tissue construct is viable for studying cell-cell communication. Endothelial cells sprout within several days of exposure to fibroblasts and bronchial epithelial cells (Fig. 3). Because TGF-β2 has been shown to inhibit angiogenesis (Manganini & Maier, 2000), we predict it will likewise inhibit sprouting in our model. We believe it may also have an autocrine inhibitory effect on VEGF secretion.

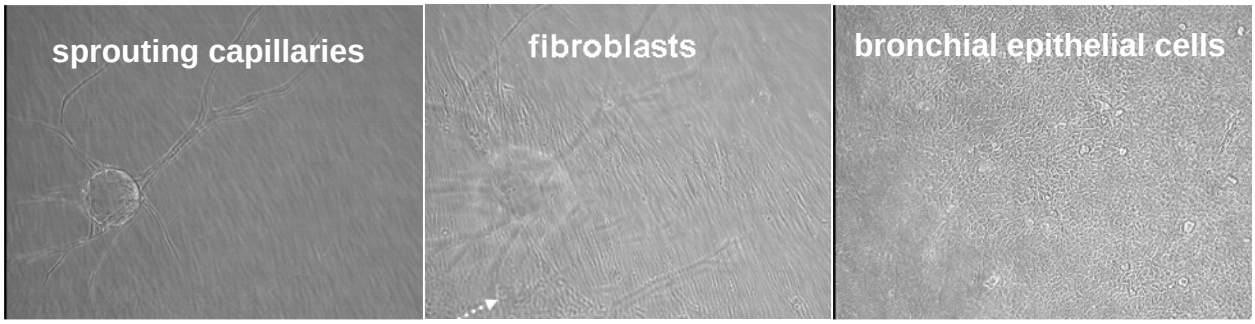

Figure 3: Previous photographs of the three cell types in the three dimensional tissue construct. Capillary sprouting is enhanced in the presence of the apical epithelium. Courtesy of Dr. Steven C. George.

Materials and Methods

Tissue construct

The model that we are going to use to study the effects of TGF-β2 on angiogenesis is a modification of a previous tissue construct (Griffith et al., 2005). In that construct, primary human umbilical cord vein endothelial cells (HUVECS) were seeded onto cytodex microcarrier beads suspended within a fibrin gel. After incubation, fibroblasts were plated atop the fibrin gel at a distance demonstrated to support diffusion of soluble mediators from the fibroblast layer to the HUVEC layer (Figure 4).

We will modify this model by plating an apical epithelial cell layer on a permeable membrane suspended over the fibroblasts. Prior to addition of the epithelial layer, primary human bronchial epithelial cells are seeded atop a transwell polyester membrane. These cells are then submerged in media for 5 days allowing the epithelium to attach and reach confluence. For the first 48 hours, the media consists of basal epithelial growth medium (BEGM) and low retinoic acid concentration. For days 3-5, the media is switched to a 50:25:25 mixture of BEGM:DMEM:Hams F12 with a high reitinoic acid concentration. At day 11, an air-liquid interface is established allowing the epithelium to differentiate for approximately 2 weeks after which the cells are ready for experimentation.

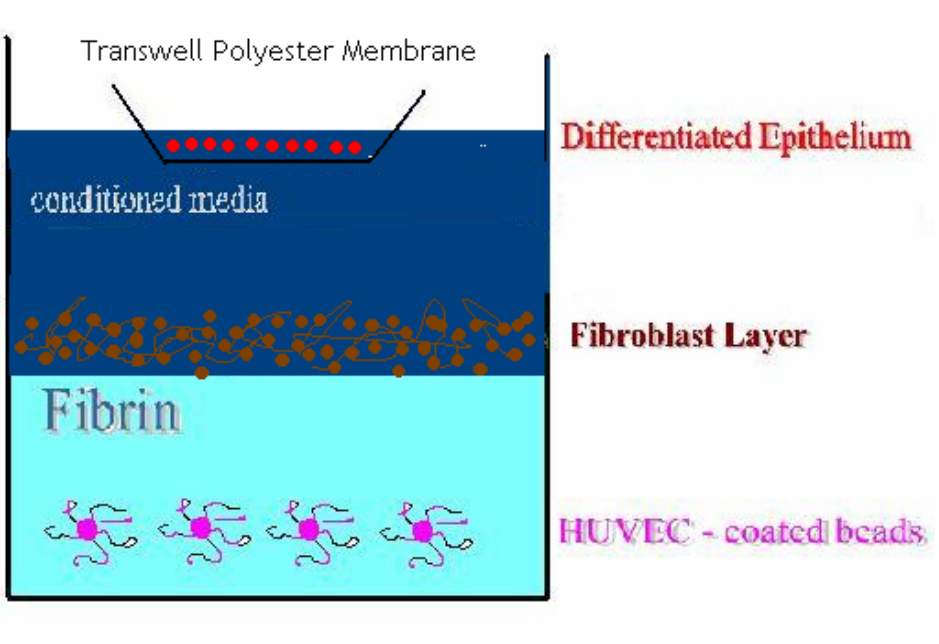

Figure 4. *In vitro* Epithelial / Fibroblast / Endothelial tissue construct. A differentiated epithelium monolayer on a porous transwell polyester membrane lies in a liquid-air interface. The porous membrane allows epithelial soluble mediators to diffuse to the lower layers.

Experimental Design

The first round of experimentation will be a pilot study to observe the effects of solely TGF-β2 on angiogenesis. These experiments will not include the epithelial cell layer and will only be a dose response of exogenous TGF-β2 (Table 1). The negative control consists of just the endothelial cells and the fibroblasts without any exogenous TGF-β2 added. The proposed concentrations of exogenous TGF-β2 up to 200 pg/ml to be added are typical of those found *in vivo*. 400 pg/ml is an extreme dose meant to elicit the acute response.

Study 1

Exogenous TGF-B2 (pg/ml)	Endothelial Cells	Fibroblasts	Epithelial Cells
0 (- control)	Yes	Yes	No
50	Yes	Yes	No
100	Yes	Yes	No
150	Yes	Yes	No
200	Yes	Yes	No
400	Yes	Yes	No

Table 1: The experimental design of the pilot study showing [TGF-β2] and tissue construct cell types.

Subsequent experiments will include the epithelial cell layer to examine the effects epithelium injury and TGF-β2 on angiogenesis as well as VEGF concentration in the media. It has been

demonstrated that wounding the epithelium results in the release of TGF-β2 (Fig. 2). To assess the TGF-β2 specific effects on angiogenesis and VEGF secretion, studies are planned using a monoclonal TGF-β2 neutralizing antibody (Table 2). In all cases, exogenous TGF-β2 will be added at 24 hour intervals.

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Study 2

Exogenous TGF-B2 (pg/ml)	Endothelial Cells	Fibroblasts	Epithelial Cells
0 (- control)	Yes	Yes	Yes
50	Yes	Yes	Yes
100	Yes	Yes	Yes
150	Yes	Yes	Yes
200	Yes	Yes	Yes
400	Yes	Yes	Yes
0	Yes	Yes	Yes / Wounded
Anti-TGF-β2	Yes	Yes	Yes / Wounded

Table 2: The experimental design of the second study showing [TGF-β2], tissue construct cell types, and epithelial wounding.

Data analysis

Characterization of angiogenesis will be done using the three indices described in Griffith et al., 2005: total capillary network length, total number of vessel segments per bead, and number of vessel sprouts whose lengths are over 100 μm.

VEGF concentration will be assessed using the Enzyme Linked Immunosorbent Assay (ELISA) protocol, in the absence and in the presence of exogenous TGF-β2.

Timeline

The first study will take approximately three weeks to complete. Production of the endothelial / fibroblast models will take approximately one week to complete. This length of time should be sufficient to proliferate enough cells to build the model. Because angiogenesis will be monitored over a 7 day period, experimentation will take one week to complete as well. Another one week is reserved for data analysis.

The second study will take significantly longer due to the extra time required for the epithelial cells to differentiate. Following two weeks of cell proliferation, five to six days are required for the harvested epithelial cells to attach to the transwells and become confluent. At that point an air-liquid interface is established, and the epithelium is induced to differentiate, lasting approximately another two weeks.

Responsibilities

My responsibilities will include

1. Learning about the biology of the
    a. Disease
    b. Lung tissue
2. Gaining hands-on experience in a number of laboratory techniques including
    a. Tissue culture
    b. Experimental design
    c. Trouble shooting
    d. Data analysis
    e. Classic biochemical assays (e.g. ELISA)
    f. Familiarity with the laboratory equipment present
3. Doing the experiments

References

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