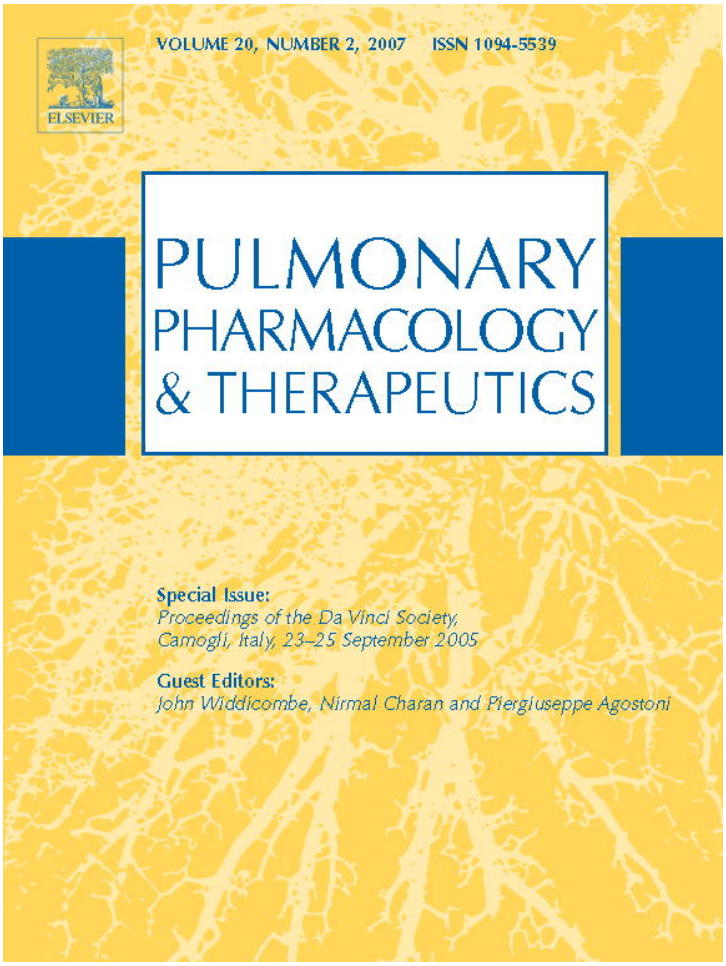

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A three-dimensional in vitro model of angiogenesis in the airway mucosa

H. Garrett Thompson, David T. Truong, Craig K. Griffith, Steven C. George*

Department of Biomedical Engineering, 204 Rockwell Engineering Center, University of California, Irvine, Irvine, CA 92697, USA

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Abstract

Bronchial asthma is an inflammatory disease characterized by chronic intermittent bronchoconstriction. A key feature of the disease is structural changes in the airway wall (airway remodeling) consistent with tissue growth and chronic wound healing including angiogenesis. The epithelium directs mesenchymal processes during both embryogenesis and wound healing, and thus we hypothesized that the bronchial epithelium plays a critical role in directing angiogenesis. To study angiogenesis in the airways, we have developed a three-dimensional (3-D) in vitro model of the airway mucosa that consists of normal differentiated human bronchial epithelial cells (NHBE), normal human lung fibroblasts (NHLF), and human umbilical vein endothelial cells (HUVEC). The HUVEC are coated on dextran beads and suspended in a fibrin gel approximately 2 mm beneath a confluent monolayer of NHLF which are just beneath the confluent monolayer of differentiated NHBE. In the presence of fibroblasts, visible capillaries reaching lengths of up to 1 mm sprout from the HUVEC-coated beads. Over 11 days in culture, the bronchial epithelium produces transforming growth factor-β2 (TGFβ2, 60 pg/ml), significantly increases vascular endothelial growth factor (VEGF) more than 6-fold to a concentration of 1.85 ng/ml, but does not significantly impact total network formation. Exogenous TGFβ2 stimulates VEGF production in a dose-dependent fashion (0–400 pg/ml) through a MAPK-dependent pathway, but also inhibits capillary network formation. We conclude that the bronchial epithelium produces biologically relevant concentrations of VEGF and TGFβ2 in a 3-D model of the airway mucosa that may be useful in probing mechanisms of angiogenesis in asthma.

Keywords: 3-dimensional; Airway mucosa; Angiogenesis; Asthma; Epithelium; Fibrin gel; In vitro model; TGFbeta; U0126; VEGF

1. Introduction

Bronchial asthma afflicts 5–10% of the population, and, despite significant understanding of the pathogenesis, the prevalence of this disease remains high [1,2]. The disease is characterized broadly by chronic inflammation and repetitive, yet reversible, bronchoconstriction. In addition, the pathophysiology is characterized by progressive structural changes in the airway termed airway remodeling that include phenotypic alterations in the airway epithelium (e.g. goblet cell hyperplasia), subepithelial fibrosis, smooth muscle hyperplasia, and angiogenesis [3]. The latter is thought to contribute to airway oedema and the enhanced delivery of inflammatory cells and mediators, and may be modulated by the altered phenotype of the epithelium and subepithelial fibroblast. Due to the relative inaccessibility of the bronchial circulation, human studies have been limited to bronchial biopsies or uptake of soluble gases to understand angiogenesis in asthma. However, advances in primary cell culture techniques provide new opportunities to develop in vitro tissue models of cell–cell communication and angiogenesis relevant to asthma.

Angiogenesis is a multi-step process critical to wound healing and other pathophysiological conditions involving matrix degradation and endothelial cell sprouting, followed by migration, proliferation, alignment, tube formation, and anastomosis to other vessels. Many factors are known to influence angiogenesis and wound healing but teasing out the effects of each factor in a biologically relevant context has proven challenging. Thus, relatively little is known about the absolute importance of each factor [4–8]. In an

*Corresponding author. Tel.: +1 949 824 3941; fax: +1 949 824 2541.
E-mail address: scgeorge@uci.edu (S.C. George).

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attempt to clarify and identify the factors involved, many in vitro models of neovascularization have been developed [9–14]. Under conditions specific to each model, the endothelial cells undergo a morphological change resulting in the formation of a network of capillary-like tubes with lumina. Endothelial cells of different vascular origins have been used to develop these models, such as human umbilical vein endothelial cells (HUVEC) or microvascular endothelial cells, which display organ specificity and morphological heterogeneity.

Among the cytokines identified to date, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGFβ) are the most extensively characterized [15–18]. TGFβ family members are pluripotent regulators of cell growth and differentiation that are secreted as latent complexes that need to be converted into active forms before interacting with their ubiquitous receptors [19]. The roles of TGFβ isoforms are not fully understood, as both stimulatory and inhibitory effects have been reported in vitro and in vivo [20,21]. However, TGFβ2 has been shown to be of particular importance in modulating endothelial cell behavior [16,22,23].

This study presents a new three-dimensional (3-D) tissue construct comprised of three distinct primary human cells. First, primary HUVEC are seeded onto cytodex microcarrier beads suspended in fibrin. Next, primary normal human lung fibroblasts (NHLF) are seeded atop the fibrin gel at a distance supporting diffusion of soluble mediators. Finally, a mucocilliary differentiated monolayer of primary normal human bronchial epithelial cells (NHBE) on a permeable membrane cultured at an air–liquid interface is positioned over the fibroblasts. The aims of this study were to characterize and validate this new biologically relevant 3-D model of the airway mucosa, and demonstrate its usefulness in probing mechanisms of angiogenesis in asthma.

2. Materials and methods

2.1. Chemicals and reagents

Recombinant human TGFβ2 and anti-TGFβ2 neutralizing antibody were purchased from R&D Systems (Minneapolis, MN). Human VEGF and TGFβ2 were measured from the conditioned media (CM) by ELISA in 96-well plate format per the manufacturer's instructions (R&D Systems). All other chemicals were from Sigma (St. Louis, MO) unless specified otherwise. Gelatin polyacrylamide gels were purchased from BioRad (Hercules, CA).

2.2. Cell culture

Primary NHBE and primary NHLF were purchased from Cambrex (Walkerville, MD) and cultured in bronchial epithelial growth medium (BEGM; Cambrex Bio Science Walkersville) and fibroblast growth medium (FGM-2; Cambrex Bio Science Walkersville), respectively. HUVEC were freshly isolated from umbilical cords following a technique previously described in detail [13]. The HUVEC used in these experiments were all previously frozen after one passage.

Passage 3 NHBE were seeded atop 24 mm diameter 0.4 μm pore size transwell polyester membranes (Costar, Cambridge, MA) at a density of 150,000 cells/transwell. These seeded transwells were then submerged in media for 6 days to allow the epithelium to attach and reach confluence. For the first day, the media consisted of BEGM. For days 2–6, the media was switched to a 50:50 mixture of BEGM and DMEM:F12 with a retinoic acid concentration of 50 nM. At day 7, an air–liquid interface was established by aspirating off the media atop the transwells allowing the epithelium to differentiate for approximately 2 weeks [24]. All NHBE cultures were immunofluorescently stained for tublin IV and Muc5AC to demonstrate adequate mucociliary differentiation. NHLF (passages 7–10) were grown to approximately 75% confluence in 10 cm tissue culture dishes (Costar, Cambridge, MA) before use. Low passage HUVEC (passages 1 and 2) were grown to approximately 75% confluence in tissue culture flasks (Fischer Scientific, Pittsburgh, PA) with EGM-2 without VEGF before use.

2.3. Tissue construct

The model that we used to study the effects of TGFβ2 on angiogenesis was based on a previously described tissue construct [13]. HUVEC were seeded onto cytodex microcarrier beads (Sigma) by gentle agitation of 4 million HUVEC and 10,000 beads in 5 ml of EGM-2 without VEGF in a T-25 tissue culture flask (Corning). The mixture was shaken for 1 min at 20 min intervals for 4 h. Afterwards, the bead mixture was transferred to a new flask with an additional 5 ml of EGM-2 and then incubated at 37 °C overnight to allow the unseeded HUVEC to attach to the flask. The HUVEC-coated beads were washed in serum-free EGM-2 without VEGF and added to a 2.5 mg/ml fibrinogen solution. Eight hundred microliters of cells were plated in the presence of thrombin and allowed to polymerize at room temperature. This gel was further incubated at 37 °C for 30 min after which 50,000 NHLF were plated atop the fibrin gel at a distance demonstrated to support diffusion of soluble mediators from the NHLF layer to the HUVEC layer.

Where applicable, differentiated NHBE were suspended over the fibroblasts in the transwells, and a 1 mm separation distance was established by using spacers constructed from PDMS poured onto the inside of 12-well plate covers. Fig. 1 is a schematic depiction of the tissue constructs. In all cases, CM was collected 1 day after the tissue was constructed to serve as baseline control. Media were collected at 48 h intervals and replaced with appropriate media. Images were taken at days 6 and 11.

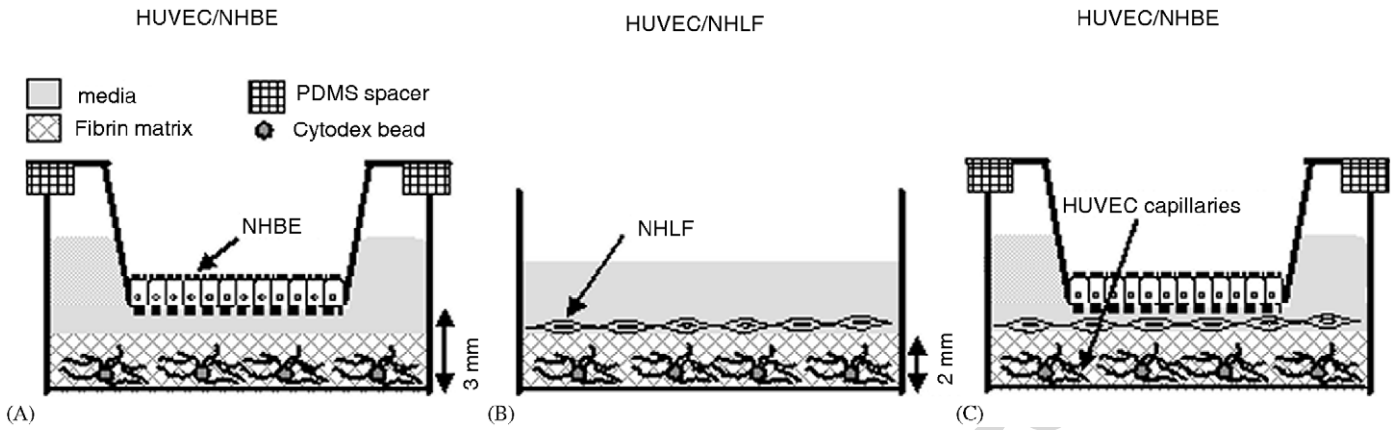

Fig. 1. Schematic of tissue co-culture models of the respiratory mucosa. Human umbilical vein endothelial cells (HUVEC), normal human bronchial epithelial cells (NHBE), and normal human lung fibroblasts (NHLF) are co-cultured together in different combinations to mimic the respiratory mucosa. (A) HUVEC are grown with NHBE to form the HUVEC/NHBE model. (B) HUVEC are grown with NHLF to form the HUVEC/NHLF model. (C) HUVEC, NHBE, and NHLF are grown together to form the HUVEC/NHBE/NHLF model.

2.4. Quantitation of tubulogenesis

Images were taken at the indicated day at 4× or 10× magnification. Criteria for viable beads included: (1) beads had to be covered with cells; (2) cells could not have anastomosed with neighboring capillary networks, and (3) cells could not have touched the sides of the well. Five beads with these criteria were randomly selected from each well and analyzed using Scion Image (NIH, Bethesda, MD). Three parameters of tubulogenesis were characterized by two or three experimenters including (1) total capillary network length, (2) total number of vessel segments, and (3) number of sprouts, as previously described [13]. Capillaries had to have clearly defined lumens and penetrate at least 100 μm into the surrounding gel.

3. Results

Fig. 2A shows representative tissue constructs in the presence or absence or exogenous VEGF. Those cultured in the absence of NHLF failed to organize stable capillaries. Although NHBE are the major source of VEGF in the CM, they cannot support capillary formation in the absence of fibroblasts (Fig. 2B,C). Exogenous VEGF (1000 pg/ml) significantly increases total network length only in the presence of NHBE (Fig. 2B).

Because NHBE secrete active TGFβ2 (Fig. 2D), we examined TGFβ2-dependent VEGF expression in the HUVEC/NHLF model system. In the absence or presence of exogenous VEGF, TGFβ2 induces an increase in expression of VEGF in a dose-dependent and time-dependent manner (Fig. 3A). In order to determine the source of TGFβ2-induced VEGF, HUVEC, NHLF, or both were cultured in the absence or presence of 400 pg/ml TGFβ2. Individually, HUVEC and NHLF treated with TGFβ2 increased VEGF expression approximately 50%, but a 300% increase in VEGF was observed with the combined tissue construct (Fig. 3B). The addition of activated TGFβ2 significantly decreased tubulogenesis in terms of total network length (Fig. 3C), number of sprouts and number of segments (data not shown) in a dose-dependent manner in the presence of exogenous VEGF. In the absence of exogenous VEGF, TGFβ2 had no effect on tubulogenesis, even as late as day 11.

Addition of TGFβ2-neutralizing antibody did not affect VEGF concentrations in the CM (Fig. 3A), but resulted in increased total network length (Fig. 3C), increased number of sprouts and increased number of segments (data not shown), at the day 6 time point relative to the media control. The overall network length of vessels remained static between days 6 and 11 in antibody-treated tissues, but continued to increase over the same time period in untreated tissues. Active TGFβ2 levels were measured from the CM from all control tissues throughout the experimental time course and were found to be not detectable within the sensitivity limits of the assay ($\leqslant$7 pg/ml).

TGFβ2 exerts opposite effects on VEGF production and endothelial cell sprouting in the presence of exogenous VEGF. While TGFβ2 inhibits endothelial cell sprouting, branching and tube elongation, it induces VEGF expression. Fig. 4B demonstrates that TGFβ2 induces VEGF secretion through the MAPK pathway, as TGFβ2-dependent VEGF production is inhibited by p44/p42 MAPK inhibition. Interestingly, addition of U0126 MAPK inhibitor increases basal secreted VEGF concentrations relative to the vehicle and media controls. However, addition of the inhibitor prevents TGFβ2-mediated VEGF expression. Importantly, endothelial cell sprouting also progresses through the MAPK pathway, as p44/p42 inhibition ablates basal tube formation (Fig. 4A).

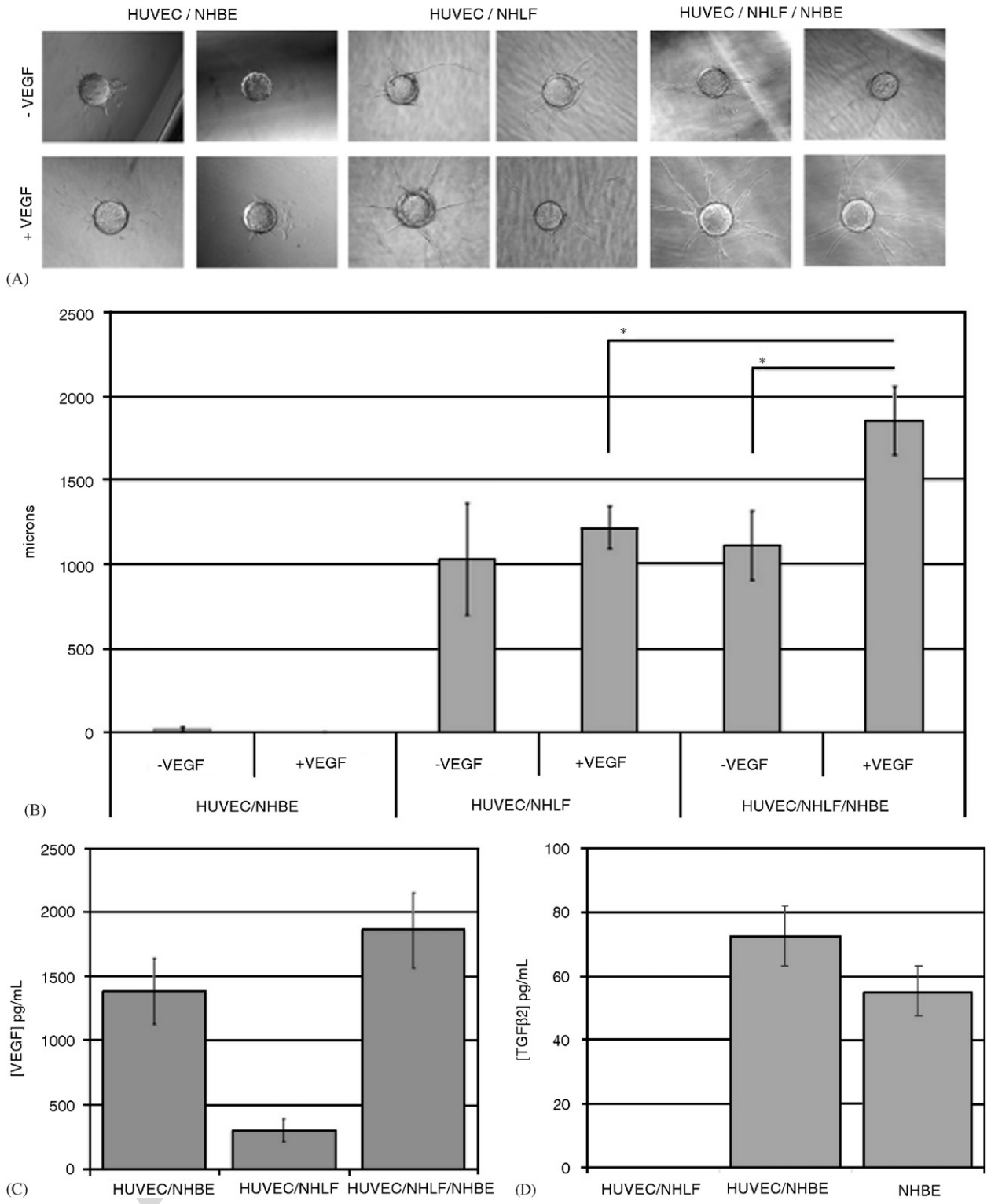

Fig. 2. VEGF-induced endothelial cell tubulogenesis in 3-D requires fibroblasts and epithelium. Human umbilical vein endothelial cells (HUVEC) seeded on cytodex beads in fibrin were cultured with primary human bronchial epithelial cells (NHBE), primary normal human lung fibroblasts (NHLF), or both. (A) Brightfield microscopy ($10\times$) of HUVEC sprouting in the absence or presence of VEGF. (B) Mean $\pm$ SEM of total network length of five to eight beads from three separate tissues. $p<0.05$ (*). (C) VEGF protein levels in the CM in the three tissue constructs cultured in the absence of exogenous VEGF. (D) Active TGFβ2 levels in the CM from the indicated tissue constructs. Mean + SD for eight tissues.

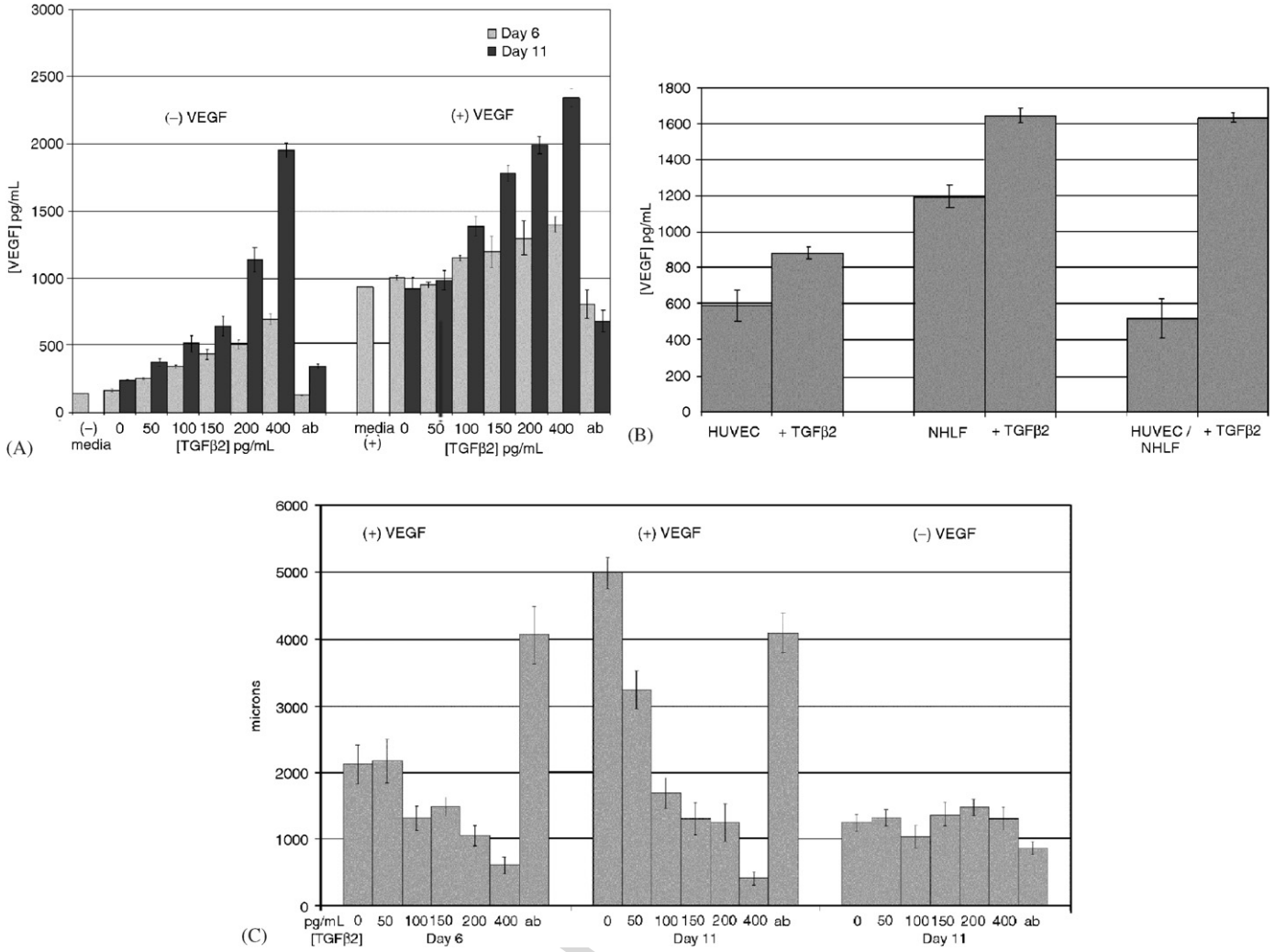

Fig. 3. TGFβ2 stimulates VEGF secretion, but inhibits tube formation only in the presence of exogenous VEGF. (A) In the absence or presence of exogenous VEGF in the media, TGFβ2 increases VEGF secretion in a dose-dependent manner. CM from three tissues, assayed in duplicate by ELISA from two separate experiments, were assessed for VEGF concentrations at two time points. 'Ab' represents TGFβ2 neutralizing antibody. (B) HUVEC, NHLF, or both were cultured in the presence of VEGF for 11 days in the absence or presence of 400 pg/ml TGFβ2. Six samples were assayed for VEGF in duplicate from two separate experiments. Mean $\pm$ SEM for each condition. (C) Mean $\pm$ SEM of total network length for each condition.

4. Discussion

Asthma is a chronic disease of the airways resulting in repetitive airway constriction and injury. Local factors repair wounded tissue, resulting in airway remodeling including new vascularization. The current study presents a new in vitro tissue construct that mimics the 3-D structure of the airway mucosa. The model utilizes three different primary human cells including bronchial epithelial cells, lung fibroblasts, and HUVEC, and is particularly useful in demonstrating how intercellular interactions can modulate angiogenesis. In particular, this study demonstrates how the impact of VEGF and TGFβ2 on angiogenesis depends critically on the presence of neighboring cells.

When suspended in a fibrin matrix (the normal matrix for healing wounds), HUVEC seeded on microcarrier beads form sprouts within 2 to 3 days post-assembly. In the presence of NHLF, fully formed tubes form from multiple endothelial cells with patent lumens within 6 days (Fig. 2A, B). When cultured for 11 days, the capillary network and matrix continues to remodel, developing an extensive anastomosing network among neighboring beads (data not shown). The addition of NHLF to the model is critical as endothelial cells in gels made without fibroblasts migrated off the beads, and failed to organize into stable capillaries. Similar results were obtained previously in our laboratory using human dermal fibroblasts [13]. The addition of NHBE to the model substantially enhances VEGF production (Fig. 2C) in a manner that appears additive to the VEGF produced by the NHLF, but this addition does not enhance vessel network formation (Fig. 2B). This finding may be due to basal TGFβ2 production by the NHBE (Fig. 2D).

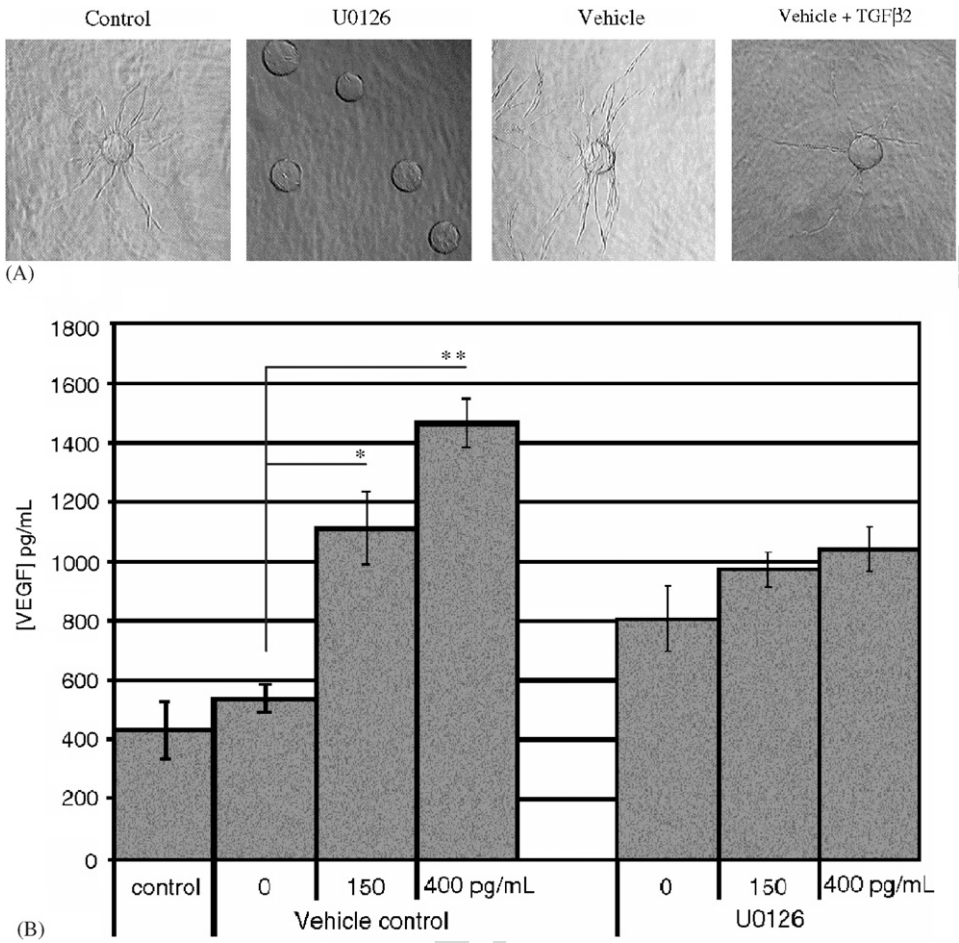

Fig. 4. U0126, an inhibitor of p42/p44 MAPK, inhibits both VEGF secretion and tubulogenesis. (A) Representative brightfield (10 ×) images of HUVEC sprouting in the presence of 10 μM the MAPK inhibitor U0126. 'Control' represents media control; 'vehicle' is DMSO vehicle control. (B) Quantitation of TGFβ2-dependent VEGF secretion in the presence of p42/p44 MAPK inhibitor or vehicle control. $p<0.01$ (*), $p<0.001$ (**).

HUVEC, cultured either alone or with NHLF, metabolize exogenous VEGF from the culture medium (Fig. 2B). Only one-half of the 1 ng/ml VEGF is measurable in the medium after 48 h at day 6 or day 11, whereas the full dose is detectable in CM from fibroblasts. Individually, both HUVEC and NHLF increase VEGF expression in response to TGFβ2. Together, however, there is a synergistic effect in the co-culture model in which a 3-fold increase in VEGF expression is observed with TGFβ2. TGFβ-stimulated release of VEGF via the p44/p42 MAPK pathway has been previously demonstrated in human retinal pigment epithelial cells [25], osteoblasts [26], human cytotrophoblasts [27], peritoneal mesothelial cells [28] and aortic smooth muscle cells [29]. To our knowledge, however, this is the first demonstration of TGFβ2 stimulation of VEGF production using a co-culture model system.

Addition of adjacent differentiated normal human bronchial epithelial cells supported further capillary formation only in the presence of NHLF and additional exogenous VEGF. Although epithelial cells are the primary source of VEGF in the model system, other fibroblast-derived factors are clearly required for sprouting and capillary formation, and this conclusion is consistent with our previous report which demonstrated a diffusion limitation of fibroblast-derived factors through the fibrin matrix [13].

The finding that exogenous VEGF tends to further enhance the numbers of sprouts and of vessels in the 3-cell model (data not shown), and significantly increases total network length only in the presence of epithelial cells, suggests either that VEGF concentration needs to be greater than 2 ng/ml to have an effect, or that the additional VEGF acts on epithelial cells, signaling alternate soluble factors that enhance angiogenesis, or that the additional VEGF stimulates an increase in angiogenesis directly on the HUVEC/NHLF tissue via existing or newly synthesized epithelial-derived factors. The finding that HUVEC/NHLF tissues cultured in the absence of VEGF form sprouts more slowly and are unaffected by TGFβ2 suggests that the effects of TGFβ2 are limited to actively migrating, aligning, and tube-forming endothelial cells.

Inhibition of endogenous TGFβ2 by addition of a neutralizing antibody did not affect VEGF concentration, but resulted in an increase in all measured vasculogenesis parameters at the day 6 time point. Together these data suggest that sprouting can be un-coupled from VEGF production. The seemingly inhibitory effect of basal TGFβ2 levels was overcome by day 11, concomitant with an increase in endothelial cell number, network length and cell–cell contacts. Although active TGFβ2 was not detected in any condition, it is consistent with our results that show low basal expression of TGFβ2 inhibits the maximal angiogenic response, seen with the addition of the antibody.

TGFβ2 exerts opposite effects on VEGF production and endothelial cell sprouting in the presence of VEGF. Addition of U1026, which specifically inhibits the MAPK pathway downstream of the SMAD crosstalk pathway, demonstrates that TGFβ2 induces VEGF secretion through the MAPK pathway. The finding that U0126 MAPK inhibitor increases basal secreted VEGF concentrations relative to the vehicle and media controls suggests that regulation of basal VEGF levels via this pathway. High VEGF levels alone are not sufficient to induce sprouting or tube formation and elongation. Fig. 2C shows that VEGF is required for elongation, but Fig. 3A and B shows that even with elevated VEGF levels, inhibition of the MAPK pathway prevents sprouting. Further downstream targets need to be tested to uncouple the VEGF and angiogenic pathways.

In summary, we have generated a viable 3-D tissue construct of the airway mucosa using three cell types to study cell–cell and synergistic interactions in the angiogenic response. It has been demonstrated that: (1) VEGF is required for endothelial cell elongation, but is not sufficient for endothelial cell sprouting; (2) when exposed to TGFβ2, co-cultures of HUVEC and NHLF express VEGF to a greater degree than either cell type alone; (3) sprouting of endothelial cells occurs via the MAPK pathway, and (4) TGFβ2-induced VEGF production occurs via the MAPK pathway.

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