

Integrin $\alpha v\beta 8$ -Mediated Activation of Transforming Growth Factor- β Inhibits Human Airway Epithelial Proliferation in Intact Bronchial Tissue

Lars Fjellbirkeland,* Stephanie Cambier,*
V. Courtney Broaddus,[†] Arthur Hill,[‡]
Paul Brunetta,[†] Gregory Dolganov,[†]
David Jablons,[‡] and Stephen L. Nishimura*

From the Department of Anatomic Pathology and Lung Biology Center,* San Francisco General Hospital, University of California at San Francisco/Mt. Zion Cancer Center and the Department of Medicine,[†] Pulmonary Division, and the Department of Surgery,[‡] University of California at San Francisco, San Francisco, California

Transforming growth factor (TGF)- β is a potent multifunctional cytokine that is an essential regulator of epithelial proliferation. Because TGF- β is expressed almost entirely in a latent state *in vivo*, a major source of regulation of TGF- β function is its activation. A subset of integrins, $\alpha v\beta 8$ and $\alpha v\beta 6$, which are expressed in the human airway, has recently been shown to activate latent TGF- β *in vitro*, suggesting a regulatory role for integrins in TGF- β function *in vivo*. Here we have developed a novel, biologically relevant experimental model of human airway epithelium using intact human bronchial tissue. We have used this model to determine the function of integrin-mediated activation of TGF- β in the airway. In human bronchial fragments cultured *in vitro*, authentic epithelial-stromal interactions were maintained and integrin and TGF- β expression profiles correlated with profiles found in normal lung. In addition, in this model, we found that either the integrin $\alpha v\beta 8$ or TGF- β could inhibit airway epithelial cell proliferation. Furthermore, we found that one mechanism of integrin- $\alpha v\beta 8$ -dependent inhibition of cell proliferation was through activation of TGF- β because anti- $\beta 8$ antibody blocked the majority (76%) of active TGF- β released from bronchial fragments. These data provide compelling evidence for a functional role for integrin-mediated activation of TGF- β in control of human airway epithelial proliferation *in vivo*. (Am J Pathol 2003, 163:533–542)

Homeostatic programs tightly regulate the proliferation of airway cell types and disturbances in this regulation can contribute to airway diseases such as lung cancer or asthma.¹ The mechanisms of airway homeostasis are

incompletely understood but involve the interaction of cells with the extracellular matrix and with soluble cytokines/growth factors.^{2–4} Integrins are the major family of molecules that regulate cell-extracellular matrix interactions whereas TGF- β constitutes a major family of cytokines/growth factors that regulates airway epithelial cell and subepithelial myofibroblast proliferation, pathological hallmarks of lung cancer and asthma, respectively.^{2,5}

Abundant evidence indicates a role for integrins in epithelial cell proliferation *in vitro*, whereas there is little known on the role of integrins in cell proliferation *in vivo*.⁶ Integrin effects on cell proliferation have been attributed to integrin-ligand interactions resulting in the initiation of intracellular signaling pathways mediated through integrin cytoplasmic domains.^{3,7} However, direct interactions of integrins with TGF- β have recently been described, raising the possibility that integrins might also function as regulators of extracellular signals.^{8,9} Such cell surface regulation of cytokine activity could provide a mechanism for autocrine and paracrine regulation of homeostasis.

TGF- β is secreted by almost every cell in the body in a latent form that must be activated for it to bind to its receptors and initiate signaling through the TGF- β -signaling mediators.¹⁰ Latency of TGF- β is maintained through the noncovalent association of the active TGF- β peptide with its propeptide, the latency-associated peptide (LAP).¹¹ The mechanisms regulating activation of TGF- β are incompletely understood but involve either conformational alterations or proteolysis of the LAP.^{8,11–14} Of note, the LAPs of TGF- $\beta 1$ and - $\beta 3$ contain integrin recognition motifs, Arg-Gly-Asp (RGD), that serve as high-affinity ligand-binding sites for the airway integrin $\alpha v\beta 8$.⁸ We have recently shown in lung cancer cell lines that $\alpha v\beta 8$ binds to the RGD sequence of the LAP of latent TGF- β and mediates activation of the latent TGF- β complex through a mechanism that is dependent on the transmembrane protease, MT1-MMP.⁸

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Address reprint requests to Stephen Nishimura, M.D., Department of Pathology, Bldg. 3, Rm. 207, San Francisco General Hospital, 1001 Potrero Ave., San Francisco, CA 94110. E-mail: cdog@itsa.ucsf.edu.

Table 1. Characteristics of Subjects Used in the Study

Subject no.	Sex	Age	Diagnosis	Smoker	Neoadjuvant therapy
1	M	55	NSCLC*	Former	None
2	M	56	NSCLC	Former	Chemo
3	F	71	Carcinoid	Former	None
4	F	69	NSCLC	Former	None
5	M	66	NSCLC	Former	None
6	M	61	Metastasis	Former	None
7	F	42	NSCLC	Former	Chemo
8	M	73	Lymphoma	Former	None
9	M	63	NSCLC	Former	None
10	M	85	NSCLC	Former	None

*Non-small-cell lung cancer.

In the airway, integrin-mediated activation of TGF- β is likely to regulate both cell proliferation and extracellular-matrix production. For instance, in two-dimensional culture, $\alpha v\beta 8$ -mediated activation of TGF- β inhibited cell growth.¹⁵ In lung cancer xenografts, heterologous expression of $\alpha v\beta 8$ was associated with an increase in soluble active TGF- β , tumor growth inhibition, and excess extracellular matrix production.^{8,15} However, no studies have addressed the role of integrin-mediated activation of TGF- β in cell proliferation and extracellular matrix production in the airway *in vivo*.

The integrin $\alpha v\beta 8$ is expressed in the basal cells of the human airway.¹⁵ Basal cells are the major proliferative compartment of the proximal airway and are the predominant cell type in direct contact with the basement membrane.¹⁶ Autocrine and paracrine effects of TGF- β on basal cells could influence airway epithelial and subepithelial myofibroblast proliferation and basement membrane thickness and, thus, could be important in understanding both the pathogenesis of lung cancer and of airway remodeling in asthma.¹ However, airway basal cells are difficult to study for several reasons. For one, they resist isolation and growth *in vitro* cultures. For another, in the bronchial tree of rodents, they are essentially absent and their function may be served by other cell types.^{17,18} In this regard, rodent models do not accurately reflect the biology of human airways. Therefore, to examine human airway proliferation in a biologically relevant context, a new system was required.

In this study, we have developed a novel, biologically relevant human airway model and used it to determine that $\alpha v\beta 8$ -mediated activation of TGF- β is a major homeostatic mechanism during human airway epithelial repopulation. We demonstrate that $\alpha v\beta 8$ is the predominant TGF- β -activating integrin expressed in the bronchial fragment model. Treatment of bronchial fragments with either neutralizing anti- $\alpha v\beta 8$ or anti-TGF- β antibodies (Abs) increases airway epithelial proliferation. Finally, we have determined that one mechanism of $\alpha v\beta 8$ -mediated regulation of proliferation involves the activation of TGF- β because treatment with neutralizing anti- $\beta 8$ Abs significantly decreases TGF- β activation.

Materials and Methods

Tissues

Fresh lung tissue was obtained in the operating room from patients undergoing pneumonectomies or lobectomies for primary or metastatic lung tumors. Exclusion criteria were active lung infection, present smoking history, and neoadjuvant radiation therapy. Superficial endobronchial tissue from tumor-free, first to fifth generation bronchi was harvested using biopsy cup forceps. The clinical characteristics of each subject is given in Table 1. Human tissues were obtained from consenting patients using a protocol approved by the Committee on Human Research at the University of California, San Francisco.

Bronchial Fragment Culture

The biopsies were immediately put into ice-cold bronchial epithelial growth medium (Clonetics, San Diego, CA). Within 4 hours, under a dissecting microscope, the biopsies were cut into fragments ~0.5 mm in diameter. Each fragment was then placed in an individual well of an agar-coated 24-well plate and incubated overnight at 37°C in a humidified incubator in 7% CO₂ using the liquid overlay culture technique as described.¹⁹

Bronchial Fragment Studies

After recovering overnight, fragments with beating cilia, as evidence of a viable epithelium, were randomly transferred to individual wells on a 96-well agar-coated plate. Each well contained 50 μ l of fresh bronchial epithelial growth medium with either neutralizing or control monoclonal Abs. The Abs were either 100 μ g/ml anti- $\beta 8$, clone 37E1;⁸ 10 μ g/ml anti-TGF- β , clone 1D11 that blocks TGF- β 1-3 (R & D Systems, Minneapolis, MN); or 100 μ g/ml of the isotype-matched control Ab (HLA-A, B, C, clone W6/32; America Type Culture Collection, Rockville, MD). The fragments were transferred to new agar-coated plates with fresh medium and Ab after 1 day and thereafter every other day. At various time points, fragments were fixed for 30 minutes in 10% formalin and embedded in paraffin.

TGF- β Bioassay

The TGF- β bioassay was performed as previously described,⁸ with the following modifications. Ciliated airway fragments at day 1 of culture were randomized into groups and were treated with the neutralizing anti- β 8, anti-pan-TGF- β , or control Abs as above. After culture overnight, fragment groups (four per group) were co-cultured for 16 to 20 hours with the TGF- β reporter cell line, TMLC²⁰ in individual wells of a 96-well plate containing neutralizing Abs at the above concentrations. The samples were analyzed as previously described.⁸ Relative luciferase units are defined as arbitrary luminometer units of experimental minus the TMLC background values.

Immunohistochemistry

Deparaffinized 4- μ m sections of bronchial fragments were processed for immunohistochemistry using the primary Abs polyclonal anti- β 8,¹⁵ monoclonal anti-keratin 34 β E12 (DAKO Corporation, Carpinteria, CA), monoclonal Ki-67 clone MIB 1 (Immunotech, Westbrook, ME), polyclonal anti-TGF- β 1 and anti-TGF- β 3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by the avidin-biotin-peroxidase detection systems LSAB2 or Envision+ (DAKO). Hematoxylin was used as a counterstain. The anti-keratin Ab 34 β E12 recognizes the high molecular weight (HMW) cytokeratin types 1, 5, 10, and 14.¹⁶

Determination of Cell Proliferation

Three slides with random sections (4 μ m) from each treatment group were immunolabeled with the proliferation marker anti-Ki-67, as above. Before counting, the slides were blinded by an independent investigator. The epithelial cells occupied the most superficial cell layers covering the fragments and were easily identified by their epithelioid morphology. The total number of epithelial cells covering the surface of each fragment and the number of epithelial cells with Ki-67-positive nuclei were counted manually under a standard light microscope using a \times 40 objective. After unblinding of the slides, the sum of cells from each fragment was calculated and the percentage of Ki-67-positive epithelial cells determined, henceforth referred to as the Ki-67 labeling index. Alternatively, to determine the concentration of Ki-67-positive cells in either the original intact epithelium, the transition zone, or the outgrowing epithelium, the number of Ki-67-positive epithelial cells found per \times 40 field ($n = 15$) in each epithelial zone was recorded and expressed as labeled cells/high-power field (HPF).

Differential Gene Expression Analysis Using Two-Step Reverse Transcriptase-Polymerase Chain Reaction (PCR)

The method used has been described in detail elsewhere.²¹ Briefly, eight fragments cultured for 3 days were snap-frozen separately in liquid nitrogen and individually

sonicated for 1 minute in 0.7 ml of fresh RLT buffer (Qiagen, Valencia, CA) with 10 μ l/ml of β -mercaptoethanol (Sigma, St. Louis, MO) and 20 μ g/ml of linear acrylamide (Ambion Inc., Austin, TX). Total cellular RNA was isolated using a Qiagen RNeasy mini kit as specified by the manufacturer. After DNase treatment (RQ1 RNase-free DNase I; Promega, Madison, WI), RNA was further purified on a second RNeasy mini column and stored frozen at -70°C . Total RNA was reverse-transcribed using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). To control for genomic DNA contamination, RT+ and reverse transcriptase reactions were performed. PCR amplification of resulting cDNA was performed using Advantage 2 polymerase (Clontech, Palo Alto, CA) and a mixture of gene-specific primers for the integrin subunits β 1, β 6, and β 8, and the housekeeping gene GAPDH. Primer sequences are available at <http://astmagenomics.ucsf.edu>. Commercial RNA (human total RNA panel 1, Clontech) was used as a reference pool for transcript quantification. Approximately 0.1 μ l of the PCR product was used in a real time PCR reaction run in 10 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) as described.²¹ Plasmid template controls for the integrin subunits β 1 (a gift from Y. Takata, Scripps Research Institute, La Jolla, Ca), β 5 (American Type Culture Collection, Rockville, MD), and β 8²² were used as standards for calibration curves. Integrin log copy numbers were plotted against the raw cycle threshold (Ct) values and the resulting calibration curves used to determine the relative transcript copy numbers. For determination of differential gene expression levels, raw Ct values were normalized to Ct values for the housekeeping gene GAPDH.

Statistics

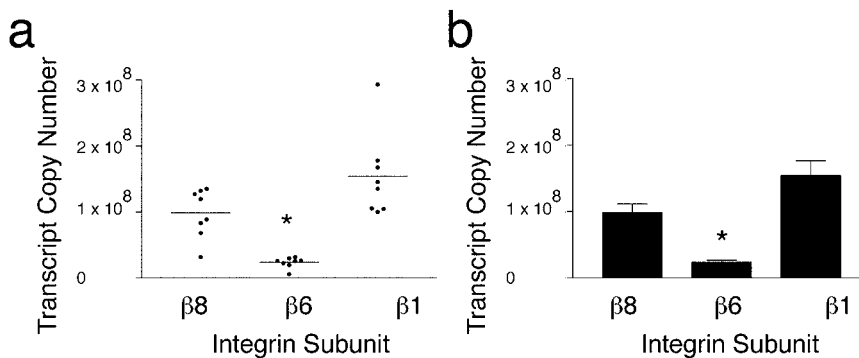
Student's *t*-test was used for comparison of two data sets; analysis of variance (analysis of variance for parametric and the Mann-Whitney test for nonparametric data sets) or linear regression for more than two data sets. Tukey's or Dunn's test were used for parametric and nonparametric data, respectively, to find where the differences lay. Significance was defined as $P < 0.05$. Statistical software was Prism v.3, and InStat v.3 (GraphPad Software, Inc., San Diego, CA).

Results

Integrin α v β 8 Is the Predominant TGF- β -Activating Integrin Present in Airway Epithelium in Human Bronchial Fragments

We used real-time PCR to assess the expression of β 8 transcript in bronchial fragments²¹ (Figure 1, a and b). The β 1 integrin subunit was used as a comparison because the β 1 integrins are known to be highly expressed in the airway.^{23,24} We expected the transcript levels of β 8 to be far less than β 1 in bronchial fragments because β 8 expression in the airway is confined to the basal cell layer of the epithelium and β 1 integrins (ie, α 2 β 1, α 3 β 1, α 5 β 1,

Figure 1. The $\beta 8$ integrin subunit mRNA is highly abundant in airway fragments in culture. **a:** The relative abundance of the $\beta 8$, $\beta 6$, and $\beta 1$ integrin subunit transcripts was compared using nested primers to amplify preamplified oligo-dt-primed cDNA from airway fragments after 3 days in culture ($n = 8$). Shown are the relative transcript numbers for each integrin subunit from each fragment. Bars indicate the median value. Each circle, square, or diamond represents a single fragment. **b:** The same data shown in **a** is depicted in bar graph form to show SE. *, $P < 0.001$.



and $\alpha 9\beta 1$) are highly expressed throughout the epithelium and in the stroma.^{15,23,24} Surprisingly, we found that the $\beta 8$ mRNA was expressed at only slightly lower levels (0.7-fold difference) than the $\beta 1$ integrin subunit (Figure 1, a and b). Therefore, we conclude that the $\beta 8$ transcript is highly expressed by the airway epithelium in bronchial fragments.

We also compared the expression of the $\beta 8$ subunit relative to the $\beta 6$ integrin subunit transcript because $\beta 6$ is the only other integrin β subunit known to participate in the activation of TGF- β .^{8,9,13,25} Like $\beta 8$, the expression of the $\beta 6$ integrin subunit in the airway is confined to the epithelium.²⁶ The $\beta 8$ transcript was expressed at higher levels in each of eight fragments than the $\beta 6$ transcript (4.5-fold difference, $P < 0.001$) (Figure 1, a and b) supporting previous reports that $\beta 6$ is not highly expressed in noninflamed airway epithelium.^{26–28} Because $\beta 8$ and $\beta 6$ mRNA in tissues correlates with the presence of $\alpha \nu \beta 8$ and $\alpha \nu \beta 6$ protein,^{27,29,30} these data suggest that the integrin $\alpha \nu \beta 8$ is more highly expressed than the integrin $\alpha \nu \beta 6$ in bronchial fragments. We therefore focused our subsequent investigations on determining the function of $\alpha \nu \beta 8$ in bronchial fragments.

The real-time PCR methodology was validated for determining relative integrin subunit expression levels by demonstrating that preamplification of small amounts of control RNA (10 ng) did not change the relative representations of the $\beta 8$, $\beta 6$, or $\beta 1$ integrin subunit and by showing essentially no differences in amplification efficiency between the same subunits throughout a large range (10^8) of input template concentration (data not shown).

The TGF- β -Activating Integrin $\alpha \nu \beta 8$ Is Expressed by Airway Basal Cells during Airway Epithelial Repopulation

After harvest, bronchial fragments were partially covered by an intact normal airway epithelium (Figure 2, a and b). From this intact epithelium, cells radiated outwards to cover the whole surface of the fragments by 7 days of culture (Figure 2c) creating a new squamoid epithelium (Figure 2b). This new epithelium gradually became stratified with no obvious sign of goblet or ciliated cell differentiation during the 1-week culture period used in this study.

We confirmed that the integrin subunit $\beta 8$ was expressed on the cell membrane and in the cytoplasm of basally located cells in the normal conducting airways, as earlier reported (Figure 2d).¹⁵ The same staining pattern was observed in the intact epithelium on the fragments during the culture period (Figure 2e). In the transition zone between the original epithelium and the new outgrowing epithelium, $\beta 8$ immunolabeling was found throughout the thickness of the epithelium but was more intense in the basally located cells (Figure 2e). After 7 days of culture, membrane staining for $\beta 8$ was seen in all layers of the newly outgrown stratified epithelium without a preference for basally located cells (Figure 2f). An anti- $\beta 6$ Ab that stains formalin-fixed paraffin-embedded tissue was not available to make direct comparisons of $\beta 6$ and $\beta 8$ immunostaining.

To characterize the basally located cell type that stained with the $\beta 8$ Ab, we used an anti-HMW cytokeratin Ab that recognizes basal cells of the normal human bronchial epithelium (Figure 2, g to i).¹⁶ HMW cytokeratin staining was found in the cytoplasm of the basally located cells in the intact epithelium confirming, that in the normal human airway, the cells that express $\beta 8$ are basal cells. The epithelial cells that had migrated out to cover the exposed stroma of the cut surfaces of the fragments were also positive for HMW keratin. Therefore, these cells were also basal cells. This is consistent with other studies reporting that migrating cells initially filling a mechanical wound space are derived from the basal cell population.³¹ After 7 days of culture, when the migrating cells had begun to differentiate to form a multilayered new epithelium, only the basally located cells were immunolabeled with the anti-HMW cytokeratin Ab (Figure 2i), whereas all layers were immunolabeled with the anti- $\beta 8$ Ab (Figure 2f). This indicates that $\beta 8$ is expressed in nonbasal cell types during early airway differentiation events whereas in the normal airway it is confined to basal cells.

In the normal human airway there is a low turnover, with fewer than 1% of cells labeled with Abs to the Ki-67 antigen (Figure 2j), a nuclear protein expressed in late G₁ through M phase.^{16,32} The predominant proliferating airway epithelial cell type is the basal cell, suggesting that it functions as a progenitor cell.¹⁶ In cultured fragments, Ki-67-labeled airway basal cells could be easily detected (Figure 2, k and l). During the first 2 days of culture, a

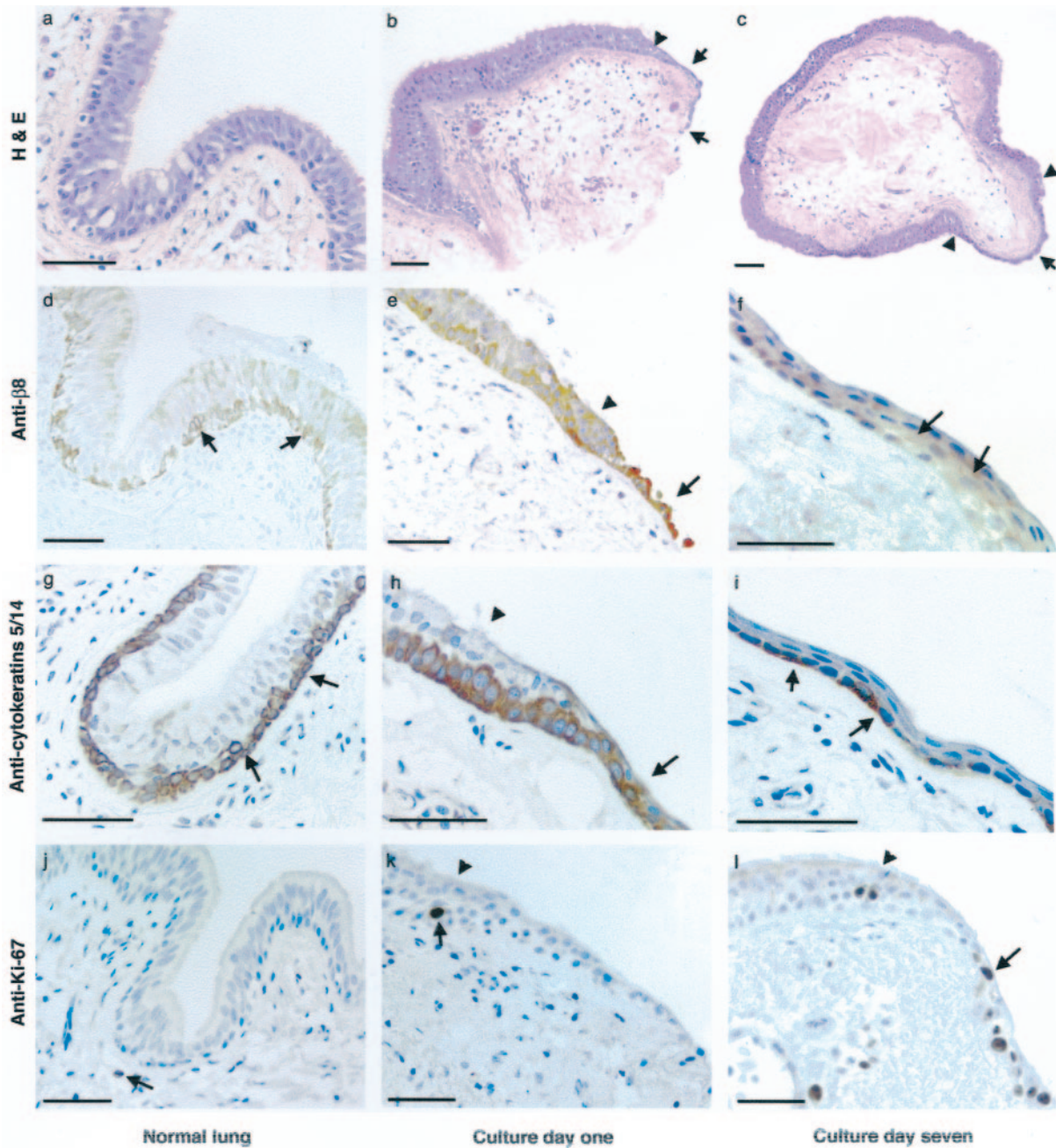


Figure 2. The airway integrin subunit $\beta 8$ is expressed in the proliferative and nonproliferative basal cell compartment in the bronchial fragment model. **a–c:** H&E staining. **a:** Normal human bronchus. **b:** Bronchial fragment cultured for 1 day. The fragment is only partially covered by an epithelium. Outgrowing epithelial cells (**arrows**) migrate from the intact epithelium at a transition zone (**arrowhead**) to cover the exposed subepithelial stroma. **c:** Bronchial fragment cultured for 7 days. The exposed stroma (area between **arrowheads**) has been completely covered with outgrowing epithelium (**arrow**). **d–f:** Immunostaining using polyclonal anti- $\beta 8$ Ab. **d:** The integrin subunit $\beta 8$ is mostly restricted to basally located cells in normal bronchial epithelium (**arrows**). **e:** After 1 day of culture, the integrin subunit $\beta 8$ is expressed by outgrowing epithelial cells (**arrow**), in all cells in the tapering transition zone (**arrowhead**), and in the basal cells of the original epithelium. **f:** After 7 days of culture, membrane staining for $\beta 8$ in all layers of the outgrowing epithelium is seen (**arrows**). **g–i:** Immunostaining using the anti-HMW keratin Ab 34 β E12. **g:** In normal bronchial epithelium, 34 β E12 stains basal cells (**arrows**). **h:** After 1 day of culture, 34 β E12 staining is seen in the outgrowing epithelium (**arrow**) and in the basal compartment of the intact ciliated epithelium (**arrowhead**). **i:** After 1 week of culture, only basally located cells in the outgrowing epithelium stain with 34 β E12. **j–l:** Immunolabeling using an anti-Ki-67 Ab that labels proliferating cells. **j:** Ki-67-positive cells are rare in normal adult airway. A single immunopositive stromal cell (**arrow**) is shown. **k:** After 2 days of culture a few proliferating cells (**arrow**) could be found in the transition zone (**arrowhead**). **l:** After 1 week of culture, numerous immunopositive cells are seen in the transition zone (**arrowhead**) and in the outgrowing epithelium (**arrow**). Shown are representative fields (**a–l**). Scale bar, 50 μ m.

significantly higher concentration of Ki-67-positive cells (2.5 ± 0.4 cells/HPF, $P = 0.02$) were located in the immediate vicinity of the transition zone between the original intact epithelium and the outgrowing epithelial cells than in the original intact epithelium (1.3 ± 0.3

cells/HPF) (Figure 2k). At this time point, the outgrowing epithelium had little Ki-67 staining (0.6 cells/HPF), indicating that cell migration accounted for the initial outgrowth, not proliferation. By 1 week of culture, a significantly higher concentration of Ki-67-positive cells was

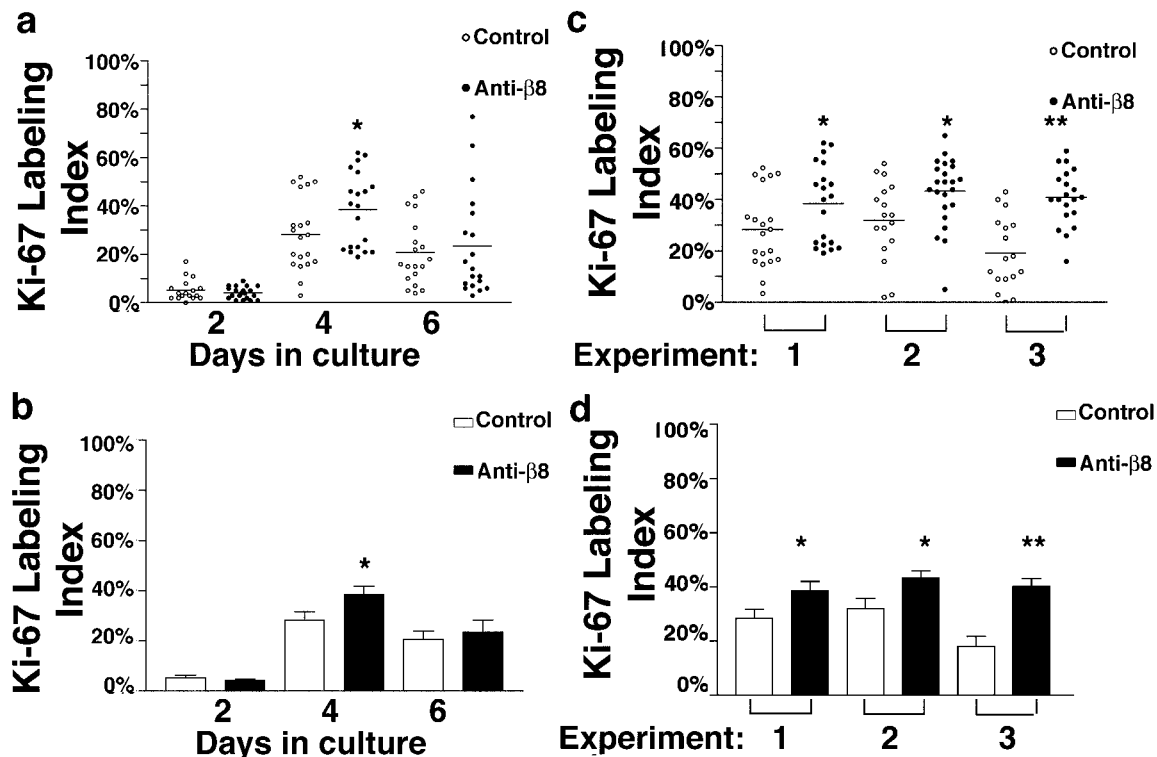


Figure 3. The integrin $\alpha\beta 8$ inhibits airway epithelial proliferation in bronchial fragments. Airway fragments were cultured in individual wells for 2, 4, and 6 days in the presence or absence of a neutralizing anti- $\beta 8$ Ab. Cell proliferation was assessed using Ki-67 immunostaining as a surrogate proliferation marker. Ki-67-labeling index was defined as the percentage of Ki-67-positive airway epithelial cells present in three random sections of each fragment. Each circle represents one fragment. **a:** Time course demonstrating that proliferation is significantly increased at days 4 and 6 of culture. The maximal increase is seen at day 4. The number of proliferating cells is significantly higher in anti- $\beta 8$ -treated (closed circles) relative to nontreated fragments (open circles) at day 4 of culture. The bar indicates the mean value. Each circle represents a single fragment. **b:** The experiment shown in **a** is shown in bar graph form to show SE. **c:** The experiment shown at day 4 in **a** is compared to two sequential experiments, each performed with fragments obtained from a different patient, using anti- $\beta 8$ Abs (closed circles) compared to an isotype-matched control Ab (open circles). **d:** The experiment shown in **c** is shown in bar graph form to show SE. All three experiments show significant differences in cell proliferation between anti- $\beta 8$ and control treated groups. *, $P < 0.05$; **, $P < 0.001$.

found in both the transition zone (7.6 ± 1.5 cells/HPF, $P = 0.002$) and outgrowing epithelium (3.3 ± 0.8 cells/HPF, $P = 0.005$) (Figure 2) consistent with the repair sequence found *in vivo* involving flattening of cells, migration, and then proliferation.³³

Bronchial Fragments as a Model to Assess Airway Epithelial Proliferation in a Complex Biological Environment

To examine airway proliferation in intact bronchial fragments, we quantified Ki-67 immunostaining using manual counting. We found that the Ki-67-labeling indices of untreated fragments, at each time point, varied considerably (Figure 3a, open circles) presumably because individual fragments, although obtained from a single patient, represented a heterogeneous population varying in size and in the amount of intact original epithelium and stroma. Despite the heterogeneity of the fragments, statistically significant differences could be seen in the proliferation indices between the populations of fragments at different time points (Figure 3, a and b; open circles, open bars). Thus, significant increases in proliferation were seen at days 4 and 6 of culture (day 2 versus day 4,

$P < 0.001$; day 2 versus day 6, $P < 0.01$) (Figure 3, a and b; open circles, open bars).

The Integrin $\alpha\beta 8$ Inhibits Epithelial Cell Proliferation during Epithelial Repopulation in Bronchial Fragments

To determine whether $\alpha\beta 8$ affects airway proliferation in complex biological environments, we treated bronchial fragments with a neutralizing anti- $\beta 8$ Ab (Figure 3, a and b; closed circles, filled bars). After 2 days in culture, we found a slight increase in the percentage of labeled airway epithelial cells, when compared to the normal airway *in vivo* ($<1\%$), but no difference between the Ab treated (4.2%) and control (media alone) groups (5.3%) (Figure 3, a and b). In contrast, after 4 days of culture, we found a statistically significant increase in the number of proliferating cells in the anti- $\beta 8$ -treated group (38.5%) compared to the control group (28.0%, $P < 0.05$) (Figure 3, a and b). After 6 days in culture, the rates of proliferation of both groups declined and no significant differences were seen between the groups (23% and 21%). Based on these results, subsequent proliferation studies were done only at day 4 of culture.

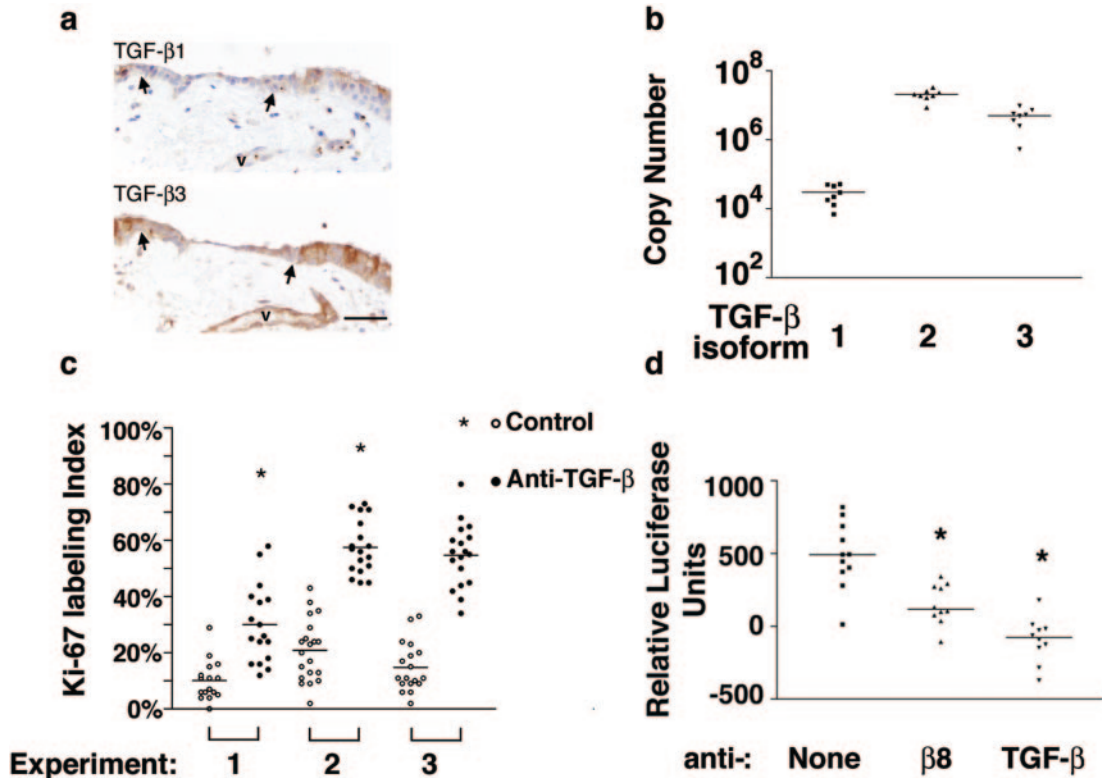


Figure 4. TGF- β -signaling pathways are involved in the inhibition of airway epithelial cell proliferation in the airway fragment model. **a:** Immunostaining of TGF- β 1 and TGF- β 3 of bronchial fragments at day 1 of culture. TGF- β 3 staining (**bottom**) is intense compared to TGF- β 1 (**top**). The area between the **arrows** is the new epithelium. **v** indicates a vessel. **b:** Real-time PCR of bronchial fragments for TGF- β 1, - β 2, and - β 3. Shown is the approximate transcript copy number. Bar indicates mean values. Each **square** or **diamond** represents a single fragment, $n = 8$. All fragments were obtained from the same patient. **c:** Neutralizing anti-TGF- β Abs significantly increase cell proliferation in three sequential experiments. Each experiment represents fragments obtained from a different patient. Each **circle** represents a single fragment. The bar indicates the mean value. *, $P < 0.001$. **d:** Antibodies to β 8 block release of active TGF- β by bronchial fragments. Bronchial fragments were treated with or without anti- β 8 or anti-TGF- β and co-cultured overnight with mink lung epithelial cells stably expressing firefly luciferase cDNA under the transcriptional control of the TGF- β -responsive, plasminogen-activator inhibitor promoter.²⁰ Each data point represents four fragments. Data are pooled from fragments obtained from two patients. Shown are relative luminescence units. *, No treatment *versus* anti- β 8 or anti-TGF- β , $P < 0.001$. Scale bar, 50 μ m.

To confirm the negative effect of α v β 8 on cell proliferation at day 4 of culture, we tested fragments obtained from two additional patients with neutralizing anti- β 8 and isotype-matched control Abs (Figure 3, c and d). Again, a significant increase in mean epithelial cell proliferation was seen in the anti- β 8 treated groups. No differences were seen between control groups treated with control Abs or medium alone indicating that the control Ab had no effect on proliferation (Figure 3, c and d).

TGF- β Inhibits Airway Epithelial Cell Proliferation in Bronchial Fragments

As a first step in determining if the mechanism of α v β 8-mediated growth inhibition in intact bronchial fragments could be mediated through TGF- β , we assessed the presence of the TGF- β isoforms in bronchial fragments using immunolocalization and quantitative PCR strategies. Using Abs to the two isoforms of TGF- β that contain the RGD binding motif, TGF- β 1 and TGF- β 3, we determined that both TGF- β 1 and - β 3 were expressed in airway fragments (Figure 4a). The TGF- β 1 Ab lightly stained the intact and newly formed epithelium, scattered submucosal fibroblasts, macrophages, and endothelium (Figure 4a, top). The TGF- β 3 Ab heavily stained the intact

and newly formed epithelium, the submucosal fibroblasts, macrophages, and blood vessels (Figure 4a, bottom). In the bronchial epithelium of the airways of normal lung samples, the TGF- β 1 and - β 3 Abs stained in an identical pattern as seen in the intact epithelium of the bronchial fragments (data not shown). In bronchial fragments, the TGF- β 3 transcript number, as assessed by real-time PCR, was expressed \sim 200-fold in excess of the TGF- β 1 transcript (Figure 4b). However, the TGF- β 2 transcript was the most highly expressed, \sim 700-fold in excess of the TGF- β 1 transcript (Figure 4b).

We next determined that active TGF- β played a role in regulating airway epithelial cell proliferation in bronchial fragments. In three consecutive experiments, we found a significant increase in Ki-67 labeling in fragments cultured with anti-TGF- β compared with the control Ab (Figure 4c). Although, in two of the three experiments, treatment with the anti-TGF- β Ab increased cell proliferation to a greater extent than treatment with the anti- β 8 Ab, the overall effects were similar (Table 2).

To determine the mechanism of α v β 8-dependent inhibition of airway proliferation, we sought to determine whether α v β 8 and TGF- β might be acting through common pathways. We used bronchial fragments co-cultured with a TGF- β reporter cell line. In this co-culture system,

Table 2. Summary of Data from Proliferation Studies

Experiment*	Fragments (n)		Epithelial cells, [†] mean (SD)		Ki-67 labeling index, [‡] % (SD)		P
	Anti- $\beta 8$	Control	Anti- $\beta 8$	Control	Anti- $\beta 8$	Control	
1	20	20	1638 (844)	1916 (1104)	38.5 (15.4)	28.3 (15.1)	<0.05
2	23	17	973 (483)	1260 (640)	43.3 (13.3)	32.0 (15.4)	<0.05
3	20	17	905 (724)	660 (470)	40.9 (10.9)	19.2 (13.9)	<0.001
	Anti-TGF- β	Control	Anti-TGF- β	Control	Anti-TGF- β	Control	P
1	17	16	1594 (575)	1715 (1077)	30.1 (13.1)	10.1 (7.1)	<0.001
2	18	16	647 (318)	461 (397)	57.6 (9.6)	23.0 (10.6)	<0.001
3	18	18	917 (591)	682 (436)	54.7 (11.3)	14.8 (8.8)	<0.001

*Each experiment represents fragments obtained from a different patient.

[†]Mean number of epithelial cells counted per fragment.

[‡]Percentage of epithelial cells showing nuclear staining with anti-Ki-67.

bronchial fragments remained floating above and did not contact or adhere to the reporter cell monolayer. Using this no-contact model, we confirmed that active TGF- β was indeed produced and released by bronchial fragments in culture and that the majority of the active TGF- β produced was $\alpha v\beta 8$ -dependent (Figure 4d). Thus, treatment with $\beta 8$ Ab inhibited 76% of the TGF- β activation in the co-culture system (Figure 4d). Taken together these data strongly suggest that the $\alpha v\beta 8$ -dependent growth inhibition of airway epithelium in bronchial fragments is because of the $\alpha v\beta 8$ -dependent release of active TGF- β .

Discussion

The major finding of this study is that integrin-mediated activation of TGF- β inhibits airway epithelial cell proliferation in a model of human airway epithelial repopulation. This finding provides the first direct evidence that an integrin in the human airway can promote homeostasis through the regulation of epithelial cell growth. Furthermore, we have evidence that this regulation is achieved through a novel mechanism, the integrin $\alpha v\beta 8$ -dependent activation of TGF- β , a cytokine that is critical for airway homeostasis.²

We established the human airway fragment system to model the human airway *in vivo*. In this model, biologically relevant airway cellular and stromal components are maintained. Indeed, in bronchial fragments, integrin and TGF- β expression profiles correlate well with the immunohistochemical expression profiles seen in the normal airway *in vivo*.^{15,26,27} Thus, this system overcomes the problems of two-dimensional airway epithelial culture systems where authentic stromal interactions, normal airway epithelial differentiation and normal patterns of integrin expression are lost. In addition, this system bypasses questions of relevance when making comparisons between human and rodent species, which differ significantly in cell-type distribution and airway biology. Furthermore, the human bronchial fragment system is easily manipulated using neutralizing antibodies, suggesting a general application to investigation of human airway biology.

In the bronchial fragment model, the $\beta 8$ subunit transcript was expressed at levels approaching that of the $\beta 1$

subunit, a highly promiscuous and abundant subunit in the human airway.²⁴ Robust $\alpha v\beta 8$ immunostaining can be demonstrated in the normal airway epithelium *in vivo* whereas, in airway epithelial cells in culture, only low levels of $\beta 8$ can be detected.¹⁵ This suggests that $\alpha v\beta 8$ expression is rapidly lost in two-dimensional culture, probably because of loss of the basal cell phenotype. The mRNA level for the $\beta 6$ integrin subunit and TGF- $\beta 1$ was low relative to other integrin subunits or TGF- β isoforms. This may be because of the absence of a systemic inflammatory response in bronchial fragments because inflammation may increase both $\beta 6$ and TGF- $\beta 1$ message levels.^{5,26-28} Therefore, based on integrin and cytokine expression profiles, the bronchial fragment model may be a closer representation of the normal noninflamed airway than the inflamed airway.

The TGF- $\beta 1$ isoform has been extensively studied in the airway where it is thought to play a role in airway remodeling, airway epithelial differentiation and growth regulation, and tumor suppression.^{1,34} In the normal mouse airway, TGF- $\beta 1$ may be the predominant TGF- β isoform expressed.³⁵ However, in the human airway there is little quantitative data investigating the relative expression of the various TGF- β isoforms. Interestingly, we found that the TGF- $\beta 2$ and - $\beta 3$ isoform transcripts were expressed at levels greater than 100-fold higher than the TGF- $\beta 1$ isoform in bronchial fragments. Transcript profiling correlated with the relative intensities of immunostaining of the TGF- $\beta 1$ and - $\beta 3$ isoforms suggesting that the expression of these isoforms is transcriptionally regulated in bronchial fragments. The TGF- $\beta 3$ isoform was co-expressed with $\beta 8$ in the airway parabasal cells, whereas the TGF- $\beta 1$ isoform was barely detected in the airway epithelium. These data demonstrate that TGF- $\beta 2$ and TGF- $\beta 3$ are more highly expressed than TGF- $\beta 1$ in bronchial fragments. Because the LAP of latent TGF- $\beta 2$, unlike the LAP of latent TGF- $\beta 1$ and - $\beta 3$, does not possess an RGD sequence, latent TGF- $\beta 2$ is highly unlikely to interact with αv -integrins.^{8,9,13} Thus, in this system, the integrin $\alpha v\beta 8$ may interact primarily with latent TGF- $\beta 3$. In support of this, we have used peptides modeled on the RGD binding sites of latent TGF- $\beta 1$ and TGF- $\beta 3$ and found that both peptides could effectively inhibit $\alpha v\beta 8$ -

mediated latent TGF- β activation in cultured cell lines (data not shown).

TGF- β 2 was the most highly expressed TGF- β isoform transcript expressed in bronchial fragments. The significance of this finding is unclear because very little of the total TGF- β activation in bronchial fragments could be attributed to TGF- β 2. This could be an artifact of the bronchial fragment system because latent-TGF- β 2 is almost certainly activated in the bronchus *in vivo*, at least during development.¹⁵ Thus, in bronchial fragments TGF- β 2 is likely to be sequestered in a latent state and mechanisms that have been defined for latent-TGF- β 2 activation in other models, do not play a major role in the bronchial fragment system.³⁶

Multiple members of the α v-subfamily of integrins can act as cell surface receptors for the RGD sequence found in latent-TGF- β .^{8,9,13} However, only two of the α v-integrins, α v β 8 and α v β 6, have been shown to mediate activation of latent TGF- β . The selective ability among α v-integrins to activate latent TGF- β is not understood but appears to involve α v-integrin-specific differences. Thus, there is evidence for two distinct α v-integrin-specific mechanisms of activation of latent TGF- β , one used exclusively by α v β 8 and one by α v β 6.^{8,13} These two mechanisms may have evolved to serve separate functions because α v β 8 appears to be expressed most highly in the normal airway, suggesting a role in epithelial homeostasis, and α v β 6 is expressed most highly in inflamed airways, suggesting a role in epithelial repair.^{15,26–28}

Integrin α v β 8 has been shown to mediate activation of latent TGF- β through a proteolytic mechanism involving the transmembrane-type metalloprotease, MT1-MMP.⁸ In this mechanism, the latent domain of TGF- β is cleaved and active TGF- β is released from the cell surface into the cell culture supernatant. Indeed, consistent with a proteolytic release, we were able to show that active TGF- β was released by bronchial fragments into the culture supernatant and that this release was primarily α v β 8-dependent. Furthermore, we found that in bronchial fragments MT1-MMP transcripts were highly expressed because the levels approached or exceeded those of the abundant MMP-2 and MMP-9 (data not shown).

We demonstrated that α v β 8-dependent activation of latent TGF- β accounted for the majority of active TGF- β released by bronchial fragments. Thus, treatment of bronchial fragments with a neutralizing anti- β 8 Ab blocked 76% of the total active TGF- β released. The small amount of active TGF- β released that was not blocked by β 8 Abs may be because of lower efficacy of the anti- β 8 Ab when compared to the pan-TGF- β Ab. Alternatively, there may be other mechanisms of latent TGF- β activation in bronchial fragments.⁹ For instance, we have not ruled out a contribution of the integrin α v β 6 to latent TGF- β activation, although α v β 6 is not highly expressed in either bronchial fragments or normal airway.^{26–28} Finally, there may be some nonintegrin-mediated activation of the latent TGF- β 2 isoform by bronchial fragments in culture. Interestingly, latent TGF- β 2 can also be activated through a metalloproteolytic mechanism.³⁶

To study integrin function in the airway in intact human tissues, we developed a human model of airway proliferation that is faithful to the epithelial-stromal interactions found *in vivo*. The bronchial fragment model is technically easy to establish and to manipulate and the use of intact human tissues offers the opportunity to study airway cell behavior in a complex environment allowing direct extrapolation to human biology and pathology. In summary, we have demonstrated that the integrin α v β 8 regulates airway epithelial cell proliferation through the activation of TGF- β using a human model of the airway. This model provides quantitative data in a system that closely approximates the human airway *in vivo*.

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