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Two-Photon Laser Scanning Microscopy of Epithelial Cell-Modulated Collagen Density in Engineered Human Lung Tissue

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ABSTRACT

Tissue remodeling is a complex process that can occur in response to a wound or injury. In lung tissue, abnormal remodeling can lead to permanent structural changes that are characteristic of important lung diseases such as interstitial pulmonary fibrosis and bronchial asthma. Fibroblast-mediated contraction of three-dimensional collagen gels is considered an *in vitro* model of tissue contraction and remodeling, and the epithelium is one factor thought to modulate this process. We studied the effects of epithelium on collagen density and contraction using two-photon laser scanning microscopy (TPLSM). TPLSM was used to image autofluorescence of collagen fibers in an engineered tissue model of the human respiratory mucosa—a three-dimensional co-culture of human lung fibroblasts (CCD-18 lu), denatured type I collagen, and a monolayer of human alveolar epithelial cell line (A549) or human bronchial epithelial cell line (16HBE14o⁻). Tissues were imaged at days 1, 8, and 15 at 10 depths within the tissue. Gel contraction was measured concurrently with TPLSM imaging. Image analysis shows that gels without an epithelium had the fastest rate of decay of fluorescent signal, corresponding to highest collagen density. Results of the gel contraction assay show that gels without an epithelium also had the highest degree of contraction (19.8% ± 4.0%). We conclude that epithelial cells modulate collagen density and contraction of engineered human lung tissue, and TPLSM is an effective tool to investigate this phenomenon.

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INTRODUCTION

BRONCHIAL AND ALVEOLAR EPITHELIA have many significant roles in pulmonary physiology, including the maintenance of normal lung tissue architecture. The epithelium serves not only as a physical barrier to the external environment, but also as an active participant in repair responses that include production and reorganization of the extracellular matrix (ECM), or tissue remodeling. Although the mechanisms of tissue remodeling are poorly understood, the phenomenon of fibroblast-mediated contraction of three-dimensional collagen gels is thought to be an *in vitro* model of connective tissue contraction and remodeling.^{1,2} In lung tissue, abnormal remodeling can result in an imbalance between collagen production and degradation, causing permanent structural changes.³ These alterations are recognized features of lung diseases such as interstitial pulmonary fibrosis and bronchial asthma,^{4–6} and are thought to play an important role in disease progression. Thus, it is critical to understand the mechanisms by which the epithelium modulates the progression of collagen production by subepithelial fibroblasts.

Collagen production and gel remodeling have previously been studied using electron microscopy⁷ and immunohistochemistry.¹ However, a limitation of these studies is tissue fixation at specific points in time; thus, these studies do not provide dynamic information about living tissue. Confocal microscopy allows imaging of live tissue, but limited imaging depth and concerns about photodamage and photobleaching from prolonged imaging are shortcomings of this approach.⁸ Two-photon laser scanning microscopy (TPLSM) is a relatively new, noninvasive biological imaging technique that can be used for high-resolution fluorescence imaging in live tissues. Compared with other techniques, TPLSM can be used to monitor key structural features of the ECM in live tissue at depths of up to 500 μm , with submicron resolution and minimal scattering.⁹ TPLSM uses pulses of near infrared (NIR) light to excite the endogenous fluorescence (*i.e.*, autofluorescence) of UV/visible-absorbing proteins in thick tissues. As a result, it is an effective technique for high-resolution imaging in turbid environments at depths of several hundred microns with minimal photobleaching and phototoxicity.^{10,11}

The growth, differentiation, and function of airway cells depend on the presence of the surrounding cells. For example, the bronchial epithelium modulates the migration¹² and proliferation of fibroblasts through chemical secretion as well as physical contact,^{13,14} and also secretes an unidentified chemical factor that modulates the responsiveness of the bronchial smooth muscle.¹⁵ The co-culture of pulmonary epithelial cells with a number of different cell types has been reported including fibroblasts,^{12–14} lymphocytes,¹⁶ and macrophages.¹⁷ However, the characterization of the collagen gel and its dynamic remodeling, including the interactions between the epithelium and the fibroblast, remain largely unexplored.

The goal of the current study is two-fold: (1) to test the hypothesis that epithelial cells modulate fibroblast-mediated collagen remodeling in anatomically arranged engineered human lung tissue co-culture, and (2) to investigate the use of TPLSM as a novel means of assessing collagen density in engineered tissue models. TPLSM was used to probe collagen density by imaging the autofluorescence of collagen fibers in a three-dimensional tissue model of the human respiratory mucosa. The model, which is applicable to pulmonary lung cells and potentially other tissue constructs with collagen, consists of a fibroblast-embedded collagen gel with an overlying epithelium, and is engineered using a co-culture of a human lung fibroblast cell line, and human lung epithelial cell lines (alveolar, A549; bronchial, 16HBE14o⁻). The excitation and emission maxima for collagen (350–390 nm and 400–500 nm, respectively) allow for efficient excitation of autofluorescence by the two-photon absorption.¹⁸ Although TPLSM has been reported as an effective method of imaging living cells in thick, multicellular environments such as brain slices and developing embryos,¹⁹ this is an initial report of the use of TPLSM to monitor collagen density *in vitro*.

MATERIALS AND METHODS

Cell culture

The immortalized human lung fibroblast cell line CCD-18lu (ATCC, Rockville, MD) was used as a model of the lung interstitial fibroblast. Transformed human bronchial cell line 16HBE14o⁻ was a generous gift of Dr. D.C. Gruenert (UCSF) and served as a model of the bronchial epithelium. Human alveolar cell line

A549 (ATCC, Rockville, MD) was used as a model of the pulmonary alveolar epithelium. All cells were passaged in 75-cm² tissue culture flasks (Falcon Labware, Oxnard, CA) using growth medium composed of minimum essential medium (MEM; supplemented with 100 U/mL of media penicillin/streptomycin, 100 μ g/mL of amphotericin-B, 2.5 μ g/mL of L-glutamine, and 10% fetal bovine serum (FBS; HyClone, Logan, UT). All cell cultures were incubated at 37°C in 5% CO₂, and harvested using a 0.1% trypsin/0.05% EDTA solution (Sigma, St. Louis, MO).

Collagen gels

Type I collagen is the most abundant ECM protein in pulmonary connective tissue; thus, type I collagen gels were prepared using rat tail tendon collagen (RTTC; Collaborative, Bedford, MA). Fibroblasts were harvested upon reaching 75–80% confluency, and added (seeding density of 3×10^5 fibroblasts/mL of final volume) to an iced mixture of collagen (4.0 mg/mL), $5 \times$ concentrated DMEM, and $10 \times$ reconstitution buffer comprised of NaHCO₃, HEPES buffer (Gibco, Grand Island, NY), and NaOH. Aliquots (0.9 mL) of the mixture were pipetted into wells of a 24-well tissue culture plate (Costar, Cambridge, MA) with a growth area of 2.0 cm²/well. Collagen gels were allowed to “gel” (cross-link) at 37°C in 5% CO₂ for 24 h before the addition of culture medium. Harvested epithelial cells were then seeded (1.5×10^5 cells/cm²) directly on top of the polymerized collagen gels and allowed to reach confluency (24–48 h). Six sets of gels were prepared for comparison: (1) control (collagen, no fibroblasts) (2) control + alveolar epithelial cells, (3) control + bronchial epithelial cells, (4) collagen and fibroblasts, (5) collagen + fibroblast + alveolar epithelial cells, and (6) collagen + fibroblast + bronchial epithelial cells. After the epithelial monolayer reached confluency, the gels were released from the surface of the tissue culture plates using a sterile spatula.

Contraction assay

Gel contraction was analyzed at 24-h intervals following release from the surface of the well by measuring the major and minor axes of the collagen gels using a stereomicroscope with an eyepiece micrometer. The area of a gel was calculated as an ellipse, and expressed as a percentage of the initial area of each gel immediately after release from the well. The contraction assay was conducted until the contraction reached a plateau (12–15 days).

TPLSM

The density of collagen fibers was studied in the engineered tissue using TPLSM (Laser Microbeam and Medical Program facility at the Beckman Laser Institute). TPLSM provides high-resolution, three-dimensional images of tissue at depths greater than those obtained by confocal techniques employing equivalent ultraviolet (UV) excitation sources.¹⁹ TPLSM uses pulses of NIR light to excite the autofluorescence of proteins in live tissue. Because the NIR source is scattered minimally,²⁰ TPLSM is an effective technique for imaging thick tissues (100–500 μ m). The two-photon microscope system utilizes a Titanium:Sapphire ultrafast laser (Coherent, Mira 900F), which serves as the two-photon excitation source, and an inverted microscope (Zeiss, Axiovert 100). The Titanium:Sapphire laser is pumped by a 532-nm solid-state laser (Coherent, Verdi). Fluorescence is detected by a single-photon counting detection system consisting of two orthogonal photomultiplier tubes (PMTs) (Hamamatsu; green, R7400P; red, R7400P-01). Because the excitation autofluorescence maxima for collagen is 350–390 nm,²¹ the excitation is achieved by tuning the 100 femtosecond Ti:Sapphire laser to a wavelength of 780 nm, corresponding to a UV one-photon wavelength of 390 nm.¹⁸ The emitted light (400–500 nm) is transmitted through a green wide-pass barrier filter that allows passage of light in the 400 to 550-nm range, and detected using the photomultiplier tube. The system also includes a 675-nm short-pass dichroic mirror at the excitation before the sample and a 580-nm long-pass dichroic mirror at the emission before the PMTs (see diagram of instrumentation in Fig. 1). Tissues are imaged over a 35- μ m \times 35- μ m scan region, integrated over 10 scans, on days 1, 8, and 15, at 10 depths within the tissue (20- μ m intervals, using a 63 \times , 1.2 N.A., c-apochromat, water-immersion objective with a working distance of 250 nm). Resolution is approximately 0.4 μ m in *x,y* and 1 μ m in *z* image planes, with an area of 0.019 μ m² per pixel.

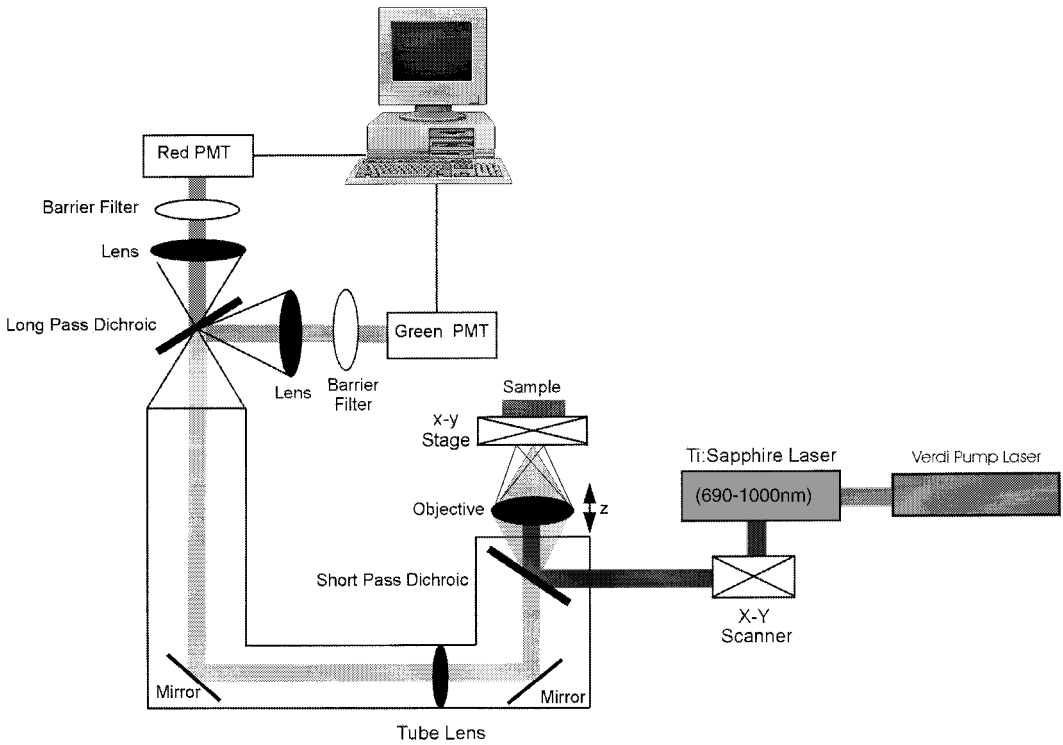


FIG. 1. Two-photon laser scanning microscope instrumentation. The TPLSM system includes a solid-state laser that pumps a Titanium:Sapphire laser that serves as the two-photon excitation source, and an inverted microscope. The emitted light passes through a filter and is detected by a photomultiplier tube.

Data analysis

The raw autofluorescence signal $S(x,y,t)$ for each image were saved as 16-bit TIFF images where t is time in days. Each image represented 255×255 pixels in the x - y plane. The mean autofluorescence (\bar{S}) of each image was determined by taking the average pixel value over the $35\text{-}\mu\text{m} \times 35\text{-}\mu\text{m}$ image ($255 \times 255 = 65,025$ pixels/image) according to the following relationship:

$$\bar{S}(t) = \frac{\sum_{x=1}^{255} \sum_{y=1}^{255} S(x, y, t)}{\sum_{x=1}^{255} \sum_{y=1}^{255} 1} = \frac{\sum_{x=1}^{255} \sum_{y=1}^{255} S(x, y, t)}{65,025} \quad (1)$$

This analysis was facilitated by Scion Image (National Institutes of Health, Bethesda, MD). $S(t)$, considered an index of collagen concentration, was modeled according to the following simple exponential relationship⁹:

$$\bar{S}(t) = \bar{S}_0(t) e^{-z/\zeta(t)} \quad (2)$$

where $\bar{S}_0(t)$ is the mean autofluorescence at time zero, z is the focal depth (μm) into the tissue, and $\zeta(t)$ is the exponential decay constant. The mean signal at infinite depth is very close to zero (<0.1) and was thus set equal to zero. The autofluorescent signal decays with increasing focal depth due to light scattering of both the excitation light as well as the emission light. $\zeta(t)$ is a parameter that captures both of these effects as well as the intrinsic collection efficiency of the system.⁹ The natural logarithm of the dimensionless mean

density $\bar{S}(t) = \bar{S}(t)/\bar{S}_0(t)$ was plotted as a function of depth. The exponential decay constant was determined using a linear least squares fit (inverse slope of the exponential decay).⁹

Statistical Analysis

Results are expressed as mean \pm standard error of samples in triplicate. Data were compared using a paired Student *t*-test. A $p < 0.05$ level of probability was used as the criterion of significance, except when described otherwise.

RESULTS

Contraction of engineered human lung tissue

Figure 2 shows a plot of collagen gel contraction over 14 days. The gels without an epithelial layer (control) had the highest degree of contraction reaching a minimum area of $19.8\% \pm 4.0\%$ of the original gel area. The gels with an alveolar epithelial layer (A549) had the next highest degree of contraction ($34.3\% \pm 8.9\%$), followed by the gels with a bronchial epithelium (16HBE14o⁻, $37.7\% \pm 7.0\%$). The rate of contraction appears exponential, and the data were plotted as the natural log of dimensionless area, \hat{A} , versus time (not shown) according to the following relationship:

$$\ln\left(\frac{A - A_\infty}{A_0 - A_\infty}\right) = \ln(\hat{A}) = -\frac{t}{\tau} \quad (3)$$

where *A* is the area of the gel (absolute or percent of initial), *A*₀ is the initial area, and *A*_∞ is the area at infinite time (12–15 days in our experiment). The time constant τ was determined as the inverse slope of the line determined by linear least squares ($R^2 > 0.93$ in all cases). τ for the fibroblast-only gel was 2.5 days which was slightly longer ($p < 0.05$) than the τ for the cases where the epithelial cells were present (2.1

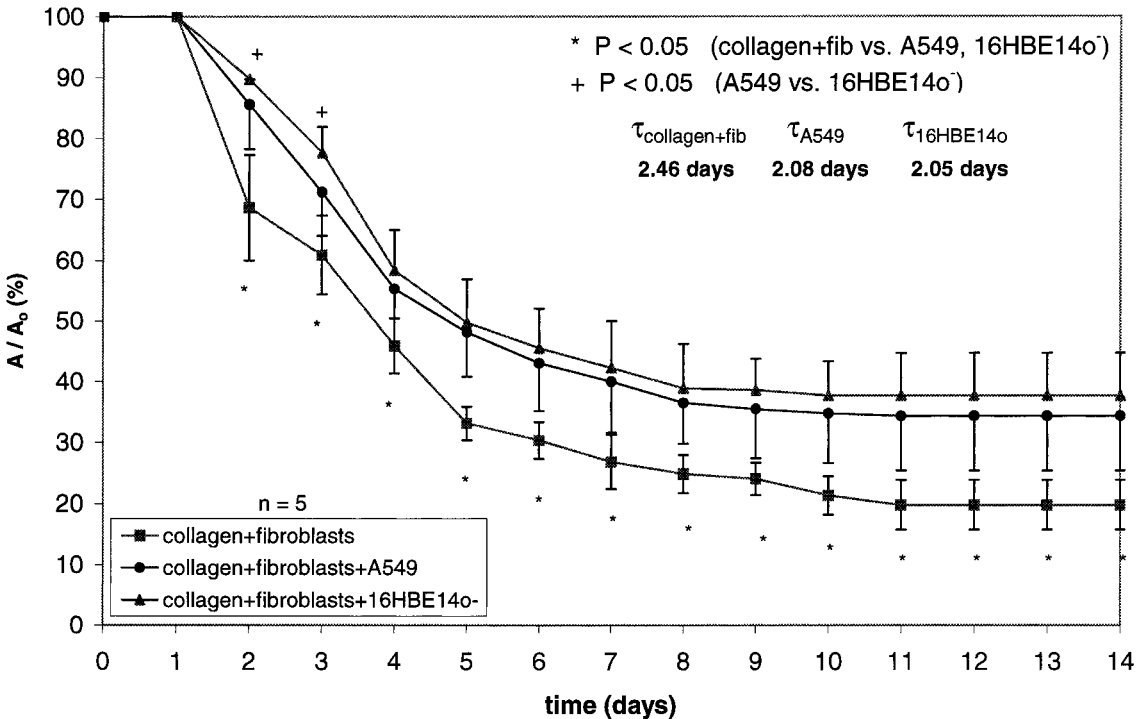


FIG. 2. Collagen gel contraction (control, with alveolar epithelium, with bronchial epithelium) as a function of time. $p < 0.05$.

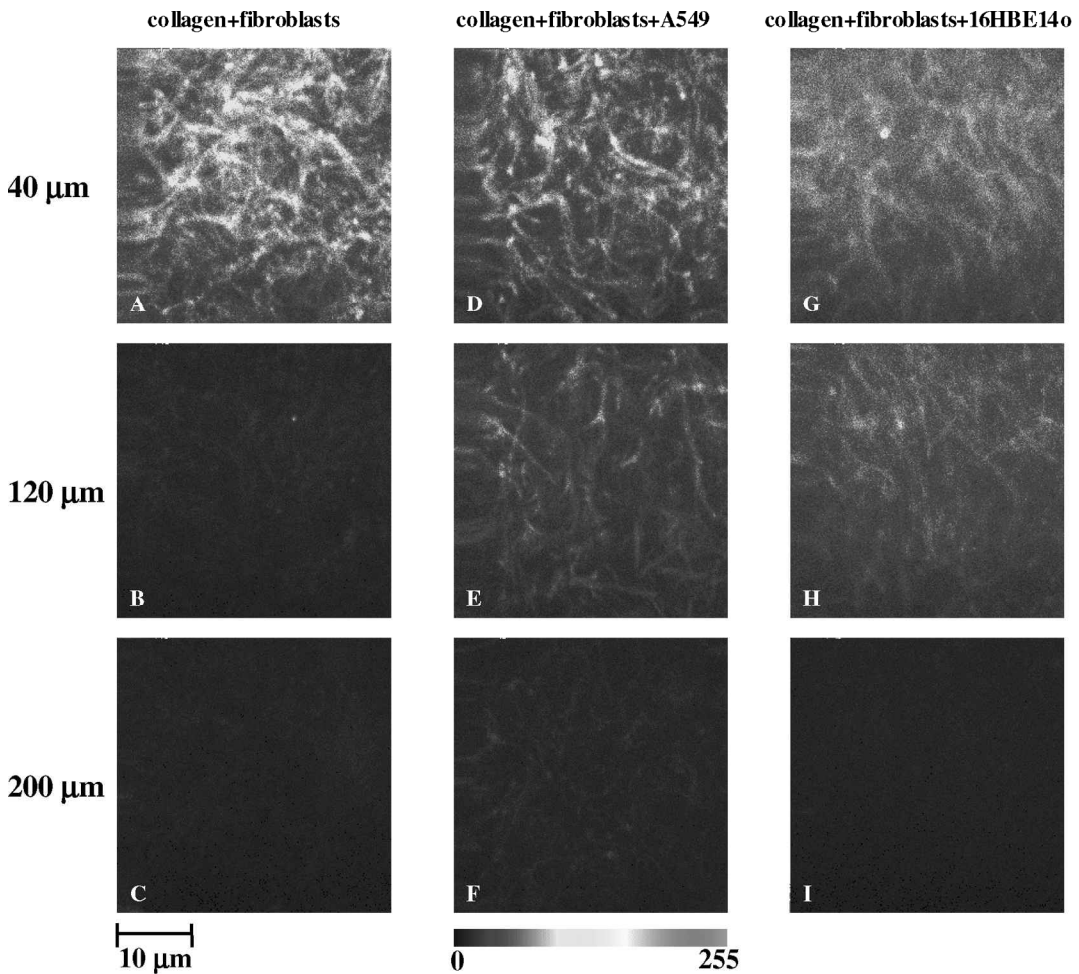


FIG. 3. Two-photon fluorescence micrographs of collagen + fibroblast gels (A–C), with alveolar epithelium (D–F), and with bronchial epithelium (G–I). Taken on day 15 at depths of 40 μm (A, D, G), 120 μm (B, E, H), and 200 μm (C, F, I). (Magnification: $\times 2200$).

days for both). Thus, although the *degree* of contraction was larger in the absence of epithelial cells, the *rate* of contraction was smaller. Fibroblast-free gels (both with and without epithelial cells) showed negligible contraction (data not shown).

Two-photon micrographs

Figure 3 shows a sampling of the two-photon micrographs used in the measurement of collagen density. These images (day 15) demonstrate the presence of collagen fibrils, and show that the autofluorescence decays with depth. These images also show that the signal drops off faster in the collagen + fibroblast gels (Fig. 3A–C) than in the gels with collagen + alveolar epithelium (Fig. 3D–F) or gels with collagen + bronchial epithelium (Fig. 3G–I). These results are consistent with the trends in gel contraction, described above. Similar results were found at day 8 (not shown).

Tissue histology

TPLSM and histology were used to determine the viability of the engineered human lung tissue. Two-photon micrographs (Fig. 4) show active fibroblasts embedded in the collagen gels. The intense fluores-

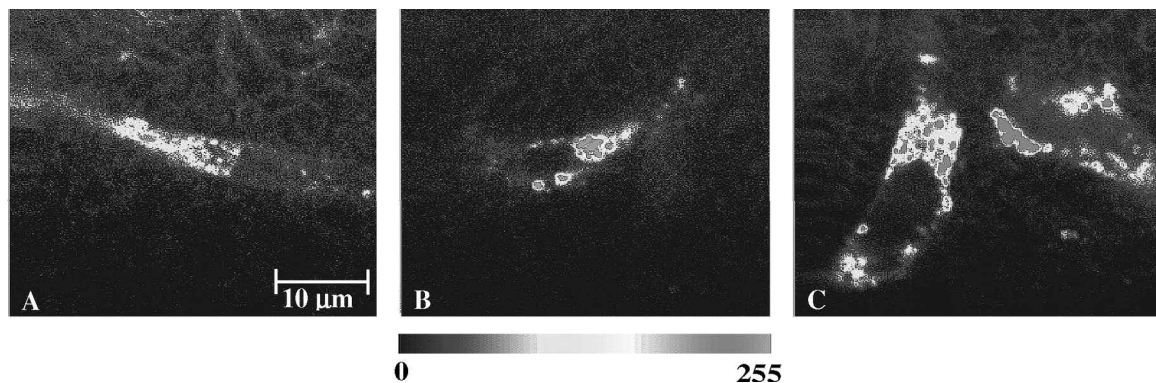


FIG. 4. Two-photon fluorescence micrographs of fibroblasts embedded in the collagen gels. Collagen + fibroblast (A), with alveolar epithelium (B), with bronchial epithelium (C). Taken on day 15 at depths of 82 μm (A), 84 μm (B), and 76 μm (C). (Magnification: $\times 2200$).

cence in the cytoplasm represents NADH within mitochondria, and an intact nuclear membrane is present. These features are characteristic of viable cells. Figure 5 shows histological staining (H&E) of the three sets of gels. The gels were formalin-fixed on day 15, and subsequently embedded and stained. The histological images show confluent alveolar and bronchial epithelia (when present) and spindle-shaped fibroblasts within the subepithelial collagen matrix.

Decay profile

The exponential decay constant of the fluorescence signal in the collagen gel (ζ) is inversely proportional to collagen concentration. These profiles demonstrate slight changes in the value of the decay constant (*e.g.*, $\zeta \sim 300\text{--}500 \mu\text{m}$ for fibroblast free gels without epithelium) over the 15-day imaging period (not shown). This finding is consistent with the fact that negligible contraction was measured in these gels. The decay profiles for the gels with fibroblast cells (Fig. 6) exhibit trends similar to the contraction rate of the gels: the collagen + fibroblast gels had the fastest rate of decay of fluorescent signal for all three days of imaging, followed by fibroblast + A549, and then by fibroblast + 16HBE14o⁻. The decay constants on day 15 (remodeling complete) range from 80 (fibroblast-only) to 140 μm (fibroblast+16HBE14o⁻) (see Fig. 7). For all 3 days, the decay constants of fibroblast-free gels + epithelial cells were statistically different from the decay constant of the collagen-only gels ($p < 0.05$). For the case of the gels with fibroblasts, decay constants of gels with 16HBE14o⁻ were statistically different from the fibroblast-only gels on all 3 days. However, decay constants of gels with A549 were statistically different from the fibroblast-only gels on days 8 and 15 ($p < 0.05$), but not on day 1.

DISCUSSION

Fibroblast-mediated contraction of collagen gels is an *in vitro* model of connective tissue contraction and remodeling, processes that are crucial to normal wound repair.² Disruption of this phenomenon can result in changes in tissue architecture that are characteristic of various fibrotic pulmonary diseases. The collagen gels investigated in this study were three-dimensional engineered models of human lung tissue, consisting of collagen, a fibroblast cell line, and two epithelial cell lines (alveolar or bronchial). The results of the contraction study presented here show that the degree of contraction in gels without an epithelial layer was markedly higher than that of the gels with an alveolar or bronchial epithelium. Previous studies of epithelial cell-fibroblast interaction⁷ and epithelial cell modulation of collagen gel contraction^{2,22} have attributed this effect to the release of stimulatory and inhibitory factors such as transforming growth factor- β (TGF- β) or prostaglandin E₂ (PGE₂). The novelty of this study was in the use of two-photon microscopy to mon-

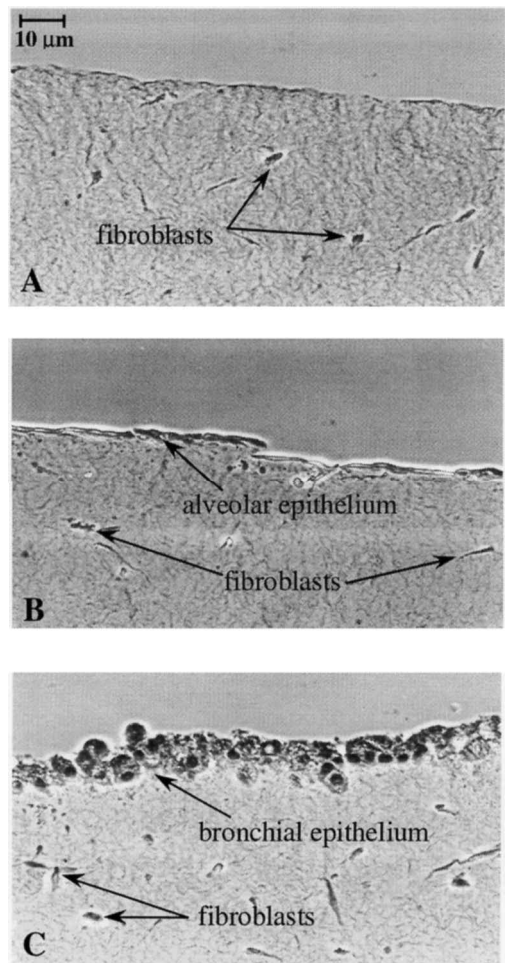


FIG. 5. Histology of 15-day engineered human lung tissue with fibroblasts. (A) Collagen + fibroblast. (B) Gels with alveolar epithelium. (C) Gels with bronchial epithelium. (A–C) Images of H&E-stained paraffin sections. (Magnification: $\times 400$).

itor both the collagen concentration in the gels and the three-dimensional arrangement of the pulmonary epithelial cells and fibroblasts to mimic the normal mucosa.

Two-photon micrographs, in conjunction with histology, confirmed the viability of the engineered tissue. Fibroblasts were seen to have a spindle-shaped form resembling myofibroblasts, which is characteristic of fibroblast cells cultured in a three-dimensional collagen gel.^{2,23,24} Two-photon microscopy also showed distinct differences in collagen density among the three sets of gels. These results were consistent with the contraction study, in that the control gels with fibroblasts had the highest collagen density for all 3 days of imaging. These results were also characteristic of the fibroblast-free gels, which showed negligible contraction and negligible differences in collagen density over the 15-day period of observation.

The fluorescent signal observed and quantified in the engineered tissue via TPLSM was assumed to be Type I collagen (original and newly synthesized), because collagen is the most abundant fiber in the connective tissue matrix.²⁵ This assumption is further supported by the difference between excitation spectra for collagen (350–400 nm) and other extracellular matrix proteins that could potentially be excited, such as elastin (310–340 nm),^{21,26} which is present in small amounts in lung connective tissue. Thus, excitation of elastin fibers is unlikely to contribute to signals obtained using the 780-nm source.

Contraction parameters A and τ were confirmed by determining the decay profiles for the different gels. The two-photon excited signal decays exponentially with increasing focal depth.⁹ When the depth-dependent fluorescence intensity is fit to an exponential form (Eq. 2), the slope of the exponential decay is proportional to collagen concentration. For all two-photon images acquired over the 15-day period, gels with-

TPLSM OF COLLAGEN DENSITY

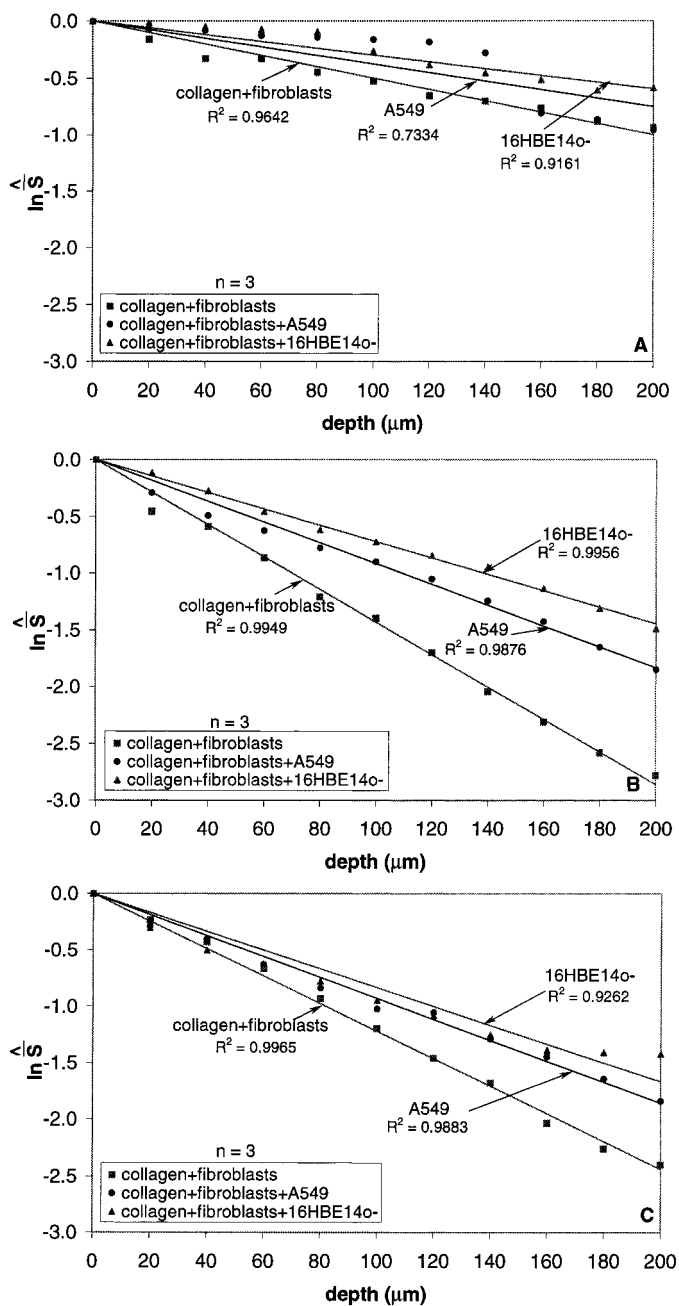


FIG. 6. Exponential decay profiles of fluorescent signal of collagen as a function of depth for gels with fibroblasts. (A) Day 1; (B) Day 8; (C) Day 15. Error bars indicate means \pm SE for $n = 3$.

out epithelia had the smallest ζ value, indicating a higher collagen density for the control gels compared to the gels with epithelia. All fibroblast-free gels showed insignificant differences in ζ values, which agree with the negligible differences found in the contraction parameters A and τ .

The physical significance of this fluorescence decay can be considered to be a function of the optical properties of the engineered tissue. This is due to the fact that collagen serves as both the source of fluorescence and the principal light-scattering structure. As a result, collagen light scattering degrades the two-photon fluorescence efficiency.

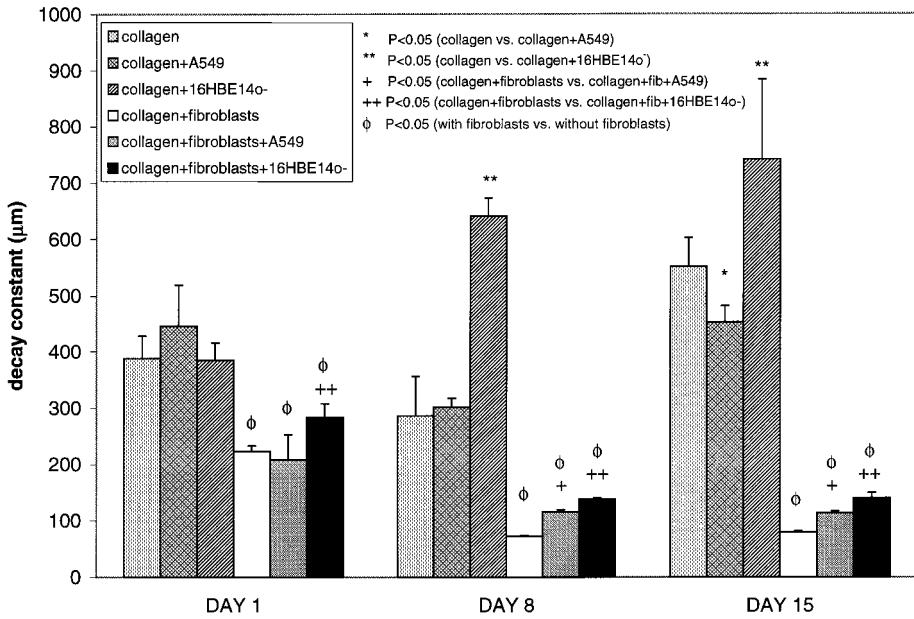


FIG. 7. Decay constants were calculated by analyzing the decay of the collagen autofluorescence signal with depth.

Previous studies of *in vitro* collagen gel contraction have addressed the effect of mechanical stress on the behavior of collagen gels. Anchored matrices differ markedly from floating gels in the distribution of tension, and this mechanical feature is responsible for notable differences in cell proliferation²⁷⁻²⁹ and the rate of gel contraction.³⁰ Because the results presented here are for floating collagen gels, mechanical tension was not considered a factor in the behavior of the gels. Therefore, the rates of contraction and determination of collagen density in this study are attributed to the chemical communication between epithelial cells and fibroblasts. Future studies must investigate the expression of potential chemical mediators such as TGF- β , fibronectin, and prostaglandins, which have been found to modulate fibroblast-mediated contraction and collagen production.^{2,22,31-35}

Evidence of this type of epithelial cell-fibroblast communication, with regard to ECM production, has previously been shown. In the presence of oral epithelial cells, cultured gingival fibroblasts exhibited increased collagen I fluorescence.⁷ Similarly, epithelial cell-conditioned medium caused an increase in collagen production and rate of gel contraction by lung fibroblasts.²² In addition, epithelial cells alone have been shown to contract collagen gels.^{1,2} Disparities between results found in the current study and those found in previous studies can be explained by differences in the components of the engineered tissue, such as collagen concentration, and the use of transformed cell lines versus primary cells.

For example, in the study by Mio *et al.*²² the epithelial cells and fibroblasts were not cultured in a normal anatomical arrangement, and when bronchial epithelial cells were shown to contract collagen gels,^{1,2} the collagen concentration was much lower (0.5–1.5 mg/mL compared to 4.0 mg/mL). Liu *et al.*^{1,2} further demonstrated that increasing collagen concentration markedly slows the rate of gel contraction by epithelial cells which can explain our results in Fig. 6 (collagen + epithelial cells). Finally, our studies have utilized transformed cell lines. The transformation process is useful in that it can maintain a consistent phenotype during passaging; however, it can also alter the phenotype. Thus, at this time we cannot rule out differences in constitutive production of cytokines and growth factors that may modulate the interactions between the epithelial cells and fibroblasts.

In addition to serving as a physical barrier to the external environment, the epithelium plays a significant role in repair response by signaling ECM production.²² In normal processes, this communication results in fibroblast activity in the form of production and reorganization of collagen fibrils. In abnormal pro-

cesses, however, the over-production of collagen (characteristic of lung diseases such as asthma and pulmonary fibrosis) can potentially be attributed to the epithelial response signal that stimulates collagen production to repair damaged epithelium. For example, asthma is characterized by elevated levels of the cytokines interleukin-1 (IL-1), IL-6, IL-8, and TGF- β , which may influence fibroblast activity.³⁶ Similarly, interstitial pulmonary fibrosis is characterized by elevated expression of IL-1, IL-4,³⁷ and IL-8.³⁸ Therefore, the epithelium-inhibited contraction seen in this study could be due to the absence of a repair response, because there was no insult to the epithelium on the collagen gels used in this experiment.

Our results indicate that in engineered human lung tissue, the presence of an unperturbed epithelium resulted in a lower degree of gel contraction and lower collagen concentration within the gel. These results confirm that the epithelium modulates fibroblast-mediated collagen gel contraction, and that TPLSM is a useful tool to probe these interactions. Our results provide a platform for future studies that will investigate extracellular matrix proteins and cytokines produced by the epithelium that are responsible for the modulation of fibroblast-embedded collagen gel contraction.

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