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A method for identification of vocal fold lamina propria fibroblasts in culture

Susan L. Thibeault, PhD, Wenhua Li, MS, and Stephanie Bartley, BS

Department of Surgery, Division of Otolaryngology–Head and Neck Surgery, University of Wisconsin Madison; and Department of Surgery, Division of Otolaryngology–Head and Neck Surgery, The University of Utah

Abstract

OBJECTIVE—Vocal fold biology research is emerging as a vital area of study in laryngology. One impediment is the lack of both commercially available vocal fold lamina propria fibroblasts and a constitutively expressed specific marker for fibroblasts. We present an in vitro technique that allows for identification of fibroblasts by ruling out the possibility of the cells belonging to other lineages that are found in vocal fold tissue.

STUDY DESIGN—An in vitro study.

METHODS—Two primary vocal fold fibroblast cell lines and one immortalized vocal fold fibroblast cell line were cultured. Immunohistologic staining for α -actinin, cytokeratin 19, and von Willebrand factor was completed for the three fibroblast lines in addition to skeletal, endothelial, and epithelial cell lines. Cell type was differentiated by positive staining for α -actinin, cytokeratin 19, and von Willebrand factor.

RESULTS—Fibroblast cultures did not express α -actinin, cytokeratin 19, and von Willebrand factor, whereas skeletal muscle, endothelial, and epithelial cultured cells expressed each respectively.

CONCLUSIONS—This simple rule-out methodology for fibroblast confirmation is an important step when establishing cell culture, and it establishes sound internal validity particularly in the early stages of this emerging area of study.

Homeostasis of the vocal fold (VF) lamina propria (LP) extracellular matrix (ECM) is maintained by the fibroblast.¹ Under normal physiologic conditions, regulation of the ECM is tightly regulated by fibroblasts that sustain a balance between synthesis and degradation of proteins. Fibroblasts are the most abundant cell in the ECM and are regarded as the most important cell for the production and degradation of the ECM and are assumed to play a pivotal role in normal tissue architecture, tissue function, and mechanical support for tissues.¹ Alterations in homeostasis of the ECM are thought to produce pathogenesis of the LP such as benign lesions, scarring, and sulcus vocalis. Unfortunately, many physiologic and pathophysiologic aspects of vocal fold fibroblast function are at present only poorly

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Corresponding author: Susan L. Thibeault, PhD, Department of Surgery, Division of Otolaryngology–Head and Neck Surgery, University of Wisconsin Madison, Madison, WI. thibeault@surgery.wisc.edu.

AUTHOR CONTRIBUTIONS

Susan L. Thibeault, study design, analysis, writer; **Wenhua Li**, study design, data collection, analysis; **Stephanie Bartley**, data collection, analysis.

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understood. The use of cultured VF LP ECM fibroblasts under defined in vitro conditions represents a powerful tool for elucidating many hypotheses.

Since its introduction early in the 20th century, tissue culture has developed into an invaluable technique allowing the study of cells from a variety of tissues in a more highly defined system. However, one of the major problems yet to be satisfactorily overcome is that of overgrowth of the more specialized cells when cultured from tissues that are composed of a variety of cell types. Different techniques for the isolation and culture of fibroblasts have been reported, including the cultivation from outgrowths of minced tissue^{2,3} and the selective removal of contaminating cells by various methods.^{2,4} Several aspects have to be considered when culturing fibroblasts for the VF LP ECM. Primary cell culture from laryngeal samples is more often than not heterogeneous and can include striated muscle, endothelial, and epithelial cell types. In the laryngology literature, it has been commonplace for the morphologic characteristics of the cells in culture to be used to identify cell type. However, fibroblast identification in culture exclusively by morphologic criteria is hazardous especially in mixed cultures with multiple cell types. Similar morphologic or phenotypic features can exist between cell types. Epithelial cells in culture have been reported to have fibroblast morphology depending on culture conditions.⁵ Furthermore, different morphologic phenotypes can exist for fibroblasts depending on the state of differentiation as reported in kidney and skin fibroblasts.^{6,7} Because of the potential for erroneous conclusions by relying on morphologic criteria, Wheeler and Burkitt⁸ have strongly advocated that fibroblasts should not be defined morphologically but rather be defined as cells that are not of other lineages.

One of the best methods to determine cell lineage is to label cells with specific antibodies.^{9,10} Unfortunately, a constitutively expressed, specific marker for fibroblasts does not exist, despite a substantial number of markers having been suggested. An antibody against proline-4-hydroxylase has been used to detect fibroblasts; however, proline-4-hydroxylase is a marker of collagen-producing cells and is therefore not restricted to fibroblasts.^{11,12} Furthermore, this antibody fails to stain quiescent fibroblasts with little or no collagen production. Other antibodies or strains (ie, vimentin, ASO2, and FSP1) used in fibroblast identification show cross-reactivity with monocytes, macrophages, muscle cells, and matrix components or they react only with a part of the whole fibroblast population.^{13–15} Histologic stains and immunohistochemical antibodies that have been suggested for fibroblast identification are not specific and show cross-reactivity with smooth and striated muscle, endothelial cells, and monocytes.^{16,17}

Given the lack of a specific marker for fibroblasts, it is essential for future progress in the investigation of the role of VF LP ECM fibroblasts in growth, development, and pathological processes that a methodology for identifying fibroblasts in culture be ascertained particularly when first establishing primary or immortalized cultures for research purposes. This is regardless if the fibroblasts are being cultured from normal, benign, or malignant tissue. Based on Wheeler and Burkitt's⁸ recommendations, we have developed a subtractive immunohistochemical methodology to identify our cultured VF LP ECM fibroblasts using specific antibodies to cells of potentially contaminating ECM cell lineages (skeletal muscle, endothelial, and epithelial cells). We present our methodology that allows for identification of fibroblasts by ruling out the possibility of the cells belonging to other lineage that are found in vocal fold tissue (skeletal muscle, endothelial, or epithelial cells).

METHODS

VF LP Fibroblast Isolation and Culture

Two primary cell lines and one immortalized cell line were assessed in this investigation. Human vocal fold tissue was obtained from one patient undergoing surgery for supraglottic cancer (male, 63 years of age) and one patient (male, 21 years of age) 4 hours after death in accordance and with approval from the Institutional Review Board at The University of Utah. The larynx was removed, and the vocal folds were judged to be normal without any evidence of disease by an otolaryngologist. The left vocal fold was resected to the thyroarytenoid muscle and removed. This tissue sample contained epithelium, LP, and trace muscle tissue. This was processed within 30 minutes of sample harvesting. As previously published,^{18–20} our culture methodology involved cutting tissue samples into small pieces (1×1 mm) with a sterile scalpel blade and placing these into cell culture dishes precoated with minimum essential medium Eagle with Earle's balanced salt solutions supplemented with $1 \times$ nonessential amino acids and 10% fetal bovine serum. Using a pipet, a drop of medium was carefully placed above each of the tissue pieces to prevent the tissue pieces from floating and to ensure adherence to the Petri dish. The dishes were kept at 37°C in a cell culture incubator with 5% CO₂-95% air. After 3 days, 2 mL of culture medium (described earlier) was carefully added to the explants. The medium was changed every 2 to 3 days until cells had migrated from the explant. The remaining tissue pieces were removed, and cells were allowed to reach subconfluence (ie, 90% confluent). Fibroblast cells were trypsinized and passed to T75 flasks. For this experiment, fibroblast cells from passage three were used. The last cell line used in this study was from of an immortalized fibroblast cell line developed from the 21-year-old donor.

Epithelial, Skeletal, and Endothelial Cell Culture

Human small airway epithelial cells (SAEC) (Cambrex-Bio Science Walkersville, Inc, Walkersville, MD), skeletal muscle cells (SkMC) (Cambrex-Bio Science Walkersville, Inc), and human umbilical vein endothelial cells (HUVEC) (gift from the laboratory of Guy Zimmerman, PhD, The University of Utah) were plated and grown to passage three with the following mediums: small airway (Cambrex-Bio Science Walkersville, Inc), skeletal muscle (Cambrex-Bio Science Walkersville, Inc), M199 with 2% human sera, and 2 mm glutamine.

Immunofluorescent Cell Staining

Cells' lineages were determined by immunofluorescent staining with cytokeratin 19, von Willebrand factor (vWF), and α -actinin antibodies for identification of epithelial, endothelial, and skeletal muscle cells, respectively. Fibroblasts were differentiated by the absence of staining for cytokeratin 19, vWF, and α -actinin. Staining was repeated in triplicate. SAEC, SkMC, HUVEC, and fibroblasts were seeded into separate sterile Chamber glass slides at the density of 10×10^4 cells per mL or 2.5×10^3 cells per mL (fibroblasts) and grown in a 37°C incubator with 5% CO₂ until they reached 50% to 90% confluence. SkMC and correspondent fibroblasts were treated with fusing medium (DMEM plus 2% horse serum) for 5 to 7 days. Cells were rinsed three times with phosphate-buffered saline (PBS). SAEC, HUVEC, and corresponding laryngeal fibroblasts were fixed with ice-cold methanol for 15 minutes, air dried, and stored at 4°C. SkMC were rinsed 2 times with HEPES-buffered saline solution (HBSS). They were then fixed with 2 percent formaldehyde solution (prepared in HBSS) for 30 minutes. Cells were rinsed two times in HBSS; each rinse was left on the cells 5 to 10 minutes before aspirating off. After second wash, cells were covered in a solution of 1% NP-40 (prepared in HBSS) and left on the cells for 10 minutes. Cells were again washed two times in HBSS, as described previously, but the last wash was left on the cells until the staining procedure was performed.

All cells were washed with PBS and then incubated in a 37°C humidified incubator for 2 hours with the primary antibody of interest in 1 percent normal goat serum (DAKO Corporation, Carpinteria, CA). Primary antibodies were mouse antihuman cytokeratin 19 (1:100; DAKO Corporation) for SAEC, rabbit antihuman vWF (1:300, DAKO Corporation) against HUVEC, and mouse antihuman α -actinin (Sacromeric) (1:200; Sigma-Aldrich, St Louis, MO) against SkMC. Fibroblasts were incubated with all three primary antibodies. Additionally, a set of slides was washed and mounted with VECTASHIELD Mounting Medium with propidium iodine (Becton Laboratories, Inc, Burlingame, CA) to counterstain DNA fluorescent red to confirm the presence of live cells. Controls were performed in parallel with normal rabbit serum (DAKO Corporation) or normal mouse serum (DAKO Corporation) at the same concentration as the primary antibody. After washing three times with warm PBS, to visualize the cells, all were incubated with fluorescein isothiocyanate–conjugated secondary goat anti-mouse or goat antirabbit antibody (1:200; BioSource International, Inc, Camarillo, CA) in 37°C for 1 hour, which results in green fluorescence. All slides were then washed and mounted. Microscopy was performed with a Nikon Eclipse E600 fluorescent microscope (Nikon, Melville, NY). Pictures were taken with a Pixera color camera (Pixera, Los Gatos, CA). Slides were judged for the presence or absence of staining by three judges who were blinded to the conditions. All slides were remeasured for intrarater reliability ($P = 0.78$, paired t test).

RESULTS

To verify the homogeneity of primary cultures of fibroblasts from the VF LP ECM, outgrowths from minced biopsy specimens were studied. Primary VF LP ECM fibroblasts grew out from the biopsied tissue during the course of several days. In the initial phases of culture, the VF LP ECM fibroblasts presented as irregularly branched cytoplasm with a large ovoid nucleus. As cells approached confluence (approximately 7 days), they developed a more stellar shape. Immortalized vocal fold fibroblasts were also assessed for homogeneity. These cells were grown to confluence; morphology was similar to primary fibroblast lines.

Immunofluorescent Cell Staining

Uniform fluorescent staining was present for α -actinin (Fig 1B) in skeletal muscle cell cultures, cytokeratin 19 (Fig 1D) in epithelial cell cultures, and vWF (Fig 1F) for endothelial cells, showing the use of suitable cell markers. This is further substantiated by the absence of staining when the use of controls (no primary antibody) did not yield fluorescent staining for α -actinin (Fig 1A), cytokeratin 19 (Fig 1C), and vWF (Fig 1E); only a small amount of fluorescein isothiocyanate background can be detected.

VF LP ECM fibroblast cell cultures treated with antibodies for α -actinin, cytokeratin 19, and vWF showed only a small amount of background fluorescent staining as seen in Figures 2, 3, and 4A–C, respectively. Controls shown in 2A–C, 3A–C, and 4A–C show only a small amount of background staining. Confirmation of the presence of cells in culture (cells treated with propidium iodine) is shown in Figures 2, 3, and 4A–C. These results indicate that skeletal muscle, epithelial, or endothelial cells were not present in the fibroblast culture, rendering these markers reliable for distinction between cultured VF LP ECM fibroblasts and skeletal muscle, epithelial, and endothelial cells.

DISCUSSION

An initial step in establishing in vitro fibroblast models for the study of VF LP ECM homeostatic processes such as growth and development is to accurately culture fibroblasts. We describe our technique for fibroblast confirmation, which is based on a lack of

immunohistochemical staining for other possible cell lineages. Furthermore, we were able to show a lack of contamination from other LP cell types. Cell types for LP that could potentially compete with fibroblasts in culture, skeletal muscle, endothelial, and epithelial were cultured and shown to express markers that characterize their etiology, α -actinin, vWF, and cytokeratin 19, respectively.

To date, numerous reports in the literature have used VF LP ECM fibroblasts.^{21–24} However, there has been no methodology presented that verifies cell phenotype. Even though light microscopy, which has been used in many of the previously mentioned studies, is a simple and direct technique to identify cells, it has critical shortcomings. In the absence of tissue architecture, there is plasticity of cellular morphology. The same cell type can take on differing morphology, and different cells may look the same. For example, epithelial cells when growing in the center of a confluent sheet are usually regular, polygonal, and have a clearly defined edge, whereas the same cells growing at the edge of a patch may be more irregular, distended, and become fibroblast-like in shape.⁵ Fibroblasts from the lung or skin assume multipolar or bipolar shapes and are well spread out on the culture surface, but at confluence they are bipolar and less well spread out. They can also form parallel arrays and whorls.⁵ Nerve cells grow like multipolar fibroblasts at low cell density and become epithelial-like at confluence.⁵ Each of these examples provides opportunity for error in assumption of fibroblast presence when morphology alone is used for identification.

To minimize errors in cell identification, one may try to establish in vitro models using primary fibroblasts from other tissue types that are commercially available and whose identification has been well documented. Unfortunately, only the study of primary or immortalized VF LP ECM fibroblasts may be appropriate for the study of vocal fold phenomena. This may be because of the heterogeneous nature of fibroblasts. Differing fibroblast phenotypes have been documented depending on the tissue's physiologic and pathophysiologic states.⁴ Fibroblasts from the kidney change shape and alter protein production when fibrotic compared with normal fibroblasts.⁴ Moreover, fibroblasts from various tissues may very well secrete different heterogeneous proteins and matrix components than the VF LP ECM. Further investigation detailing the VF LP ECM fibroblast phenotype in various conditions is crucial, and an adequate methodology for isolation is the first step in this process.

Another advantage of this methodology is that it can be used to differentiate fibroblasts from all tissue types and disease states such that for otolaryngology head and neck surgery, fibroblasts from normal, benign, or malignant tissue can be grown and distinguished using the described methodology. The tissue need not be normal or from the vocal folds only. Consequently, any in vitro research that uses fibroblast lines that have not been previously characterized can use this easy methodology. Once fibroblasts are confirmed, further study typically requires medical history that may include whether or not tissue was from a patient with advanced-stage laryngeal carcinoma, smoking history, medications, and other medical history that may be pertinent. It should be recognized that each of these conditions acting independently, let alone in combination with one another, could have had significant adverse pathologic effects on the constituent vocal fold fibroblast in vitro cultures.

CONCLUSIONS

Cultured VF LP ECM fibroblasts represent a useful tool to examine multiple aspects of vocal fold biology under defined conditions in vitro. However, given the complex morphologic and functional heterogeneity of the fibroblasts, strict consideration has to be given to its confirmation in culture. Using an established methodology that confirms the identity of the fibroblast might eliminate future discrepancies in study outcomes and is

important in establishing sound internal validity particularly in the early stages of this emerging area of study.

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Figure 1. Skeletal cell negative (A) and positive (B) expression for α -actinin (20 $\times$ magnification). Epithelial cell negative (C) and positive (D) expression for cytokeratin 19 (40 $\times$ magnification). Endothelial cell negative (E) and positive (F) expression for vWF.

Figure 2. Primary fibroblast culture from a 63-year old: positive live cell presence in culture (**A**, **B**, and **C**). Cell cultures stained for α -actinin, cytokeratin 19, and vWF show a lack of staining in A+, B+, and C+, respectively. Negative controls for α -actinin, cytokeratin 19, and vWF show a lack of staining in A-, B-, and C-, respectively. All magnified at 20 $\times$.

Figure 3. Primary fibroblast culture from a 21-year old: positive live cell presence in culture (**A**, **B**, and **C**). Cell cultures stained for α -actinin, cytokeratin 19, and vWF show a lack of staining in A+, B+, and C+, respectively. Negative controls for α -actinin, cytokeratin 19, and vWF show a lack of staining in A-, B-, and C-, respectively. All magnified at 10 $\times$.

Figure 4. Immortalized fibroblast culture from a 21-year old: positive live cell presence in culture (**A**, **B**, and **C**). Cell cultures stained for α -actinin, cytokeratin 19, and vWF show a lack of staining in A+, B+, and C+, respectively. Negative controls for α -actinin, cytokeratin 19, and vWF show a lack of staining in A-, B-, and C-, respectively. All magnified at 20 $\times$.