

Bronchial mucosa produced by tissue engineering: A new tool to study cellular interactions in asthma

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Background: The use of fiberoptic bronchial biopsies has improved our understanding of the immunopathology of asthma. However, this approach offers a limited ability to perform mechanistic studies observing cell-cell and cell-matrix interactions, which are a key issue in the study of airway remodeling. Tissue engineering is a technique that combines the use of biology and engineering expertise to generate a limitless amount of tissue from small samples. This technology allows for the study of cell interactions under conditions as close as possible to the natural environment.

Objective: The aim of this study was to evaluate the feasibility of an engineered human bronchial mucosa as a model to study cellular interactions in asthma.

Methods: Human bronchial fibroblasts from normal and asthmatic donors were incorporated into collagen gel. Bronchial epithelial cells were seeded over this gel and then cultured in an air-liquid interface in the presence or the absence of T lymphocytes. Biopsy specimens from these engineered mucosa were taken for structural and ultrastructural analysis, and T lymphocytes were harvested and used to localize IL-5.

Results: Histologic analysis showed that engineered mucosa with normal bronchial cells presented a pseudostratified ciliated epithelium with the presence of mucus secretory cells. The electron microscopy analysis confirmed these histologic results. These features were comparable with those observed in normal bronchial tissues. However, in engineered mucosa from asthmatic subjects, the tissue structure was disorganized, particularly the epithelial cell arrangement. The percentage of IL-5⁺ lymphocytes was significantly ($P = .03$) higher in engineered bronchial mucosa from asthmatic subjects ($87\% \pm 2\%$) compared with mucosa from normal volunteers ($2\% \pm 0.3\%$).

Conclusion: Using tissue engineering, we produced an in vitro model of bronchial mucosa from normal and asthmatic subjects.

These models could be a valuable tool to better understand key mechanisms involved in inflammation and airway repair. (*J Allergy Clin Immunol* 2001;107:36-40.)

Key words: Asthma, resident cells, engineered bronchial tissue, T cells, fibroblasts, epithelial cells, IL-5

Cellular culture in a monolayer system is a basic biologic procedure for investigation of the pathogenesis of diseases. However, isolated cells can only simulate a small part of the body's complexity and often lose their morphology and functional properties. It is well known that cells need an appropriate matrix as an optimal cell support to develop their typical differentiated phenotype in vitro. Tissue engineering is a new field that applies the principles of biology and engineering to the development of functional substitutes for damaged tissue. The 2 major goals of tissue engineering are the production of functional tissues that can be grafted into human subjects and the development of in vitro models that are superior to conventional cell cultures, mainly because monolayer cultures lack an adequate extracellular matrix. These in vitro models offer the possibility to study different mechanisms involved in many diseases and have a great potential in replacing, in some cases, experiments performed on animals. Tissue engineering efforts are currently being undertaken for every type of tissue. At the front of this development is the creation of an artificial skin for burned patients^{1,2} and generation of artificial cartilage and bone to treat articular joint disease³ and blood vessels for vascular grafts.⁴

Cellular interactions between epithelial and mesenchymal cells play a key role in regulating airway repair in asthma.⁵ These resident cells, in addition to their implication in tissue repair, produce a wide range of cytokines and chemokines that are important in the activation and recruitment of inflammatory cells in the bronchial mucosa of asthmatic subjects.⁶ This suggests that these cells may be involved in the maintenance and chronicity of the inflammatory response.⁷

The available information on resident cells in the bronchial mucosa were obtained from studying fixed bronchial biopsy specimens or from studies conducted on cells grown in monolayers.⁸ Such models have improved our understanding of the immunopathology of asthma but have some limits, including the absence of a matrix environment, which plays an important role in

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Supported by the Fonds de Recherche en Santé du Québec (FRSQ). Dr Chakir, Dr Hamid, and Dr Rouabhia are the recipients of Chercheur-Boursier awards from the Fonds de la Recherche en Santé du Québec.

Received for publication August 18, 2000; revised September 26, 2000; accepted for publication September 26, 2000.

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0091-6749/2001 \$35.00 + 0 1/83/111929

doi:10.1067/mai.2001.111929

Abbreviations used

cAMP: Cyclic adenosine monophosphate
EHBM: Engineered human bronchial mucosa

intercellular communications.⁹ We therefore designed this study to evaluate the feasibility of an *in vitro* model of bronchial mucosa produced by tissue engineering. This model will take into account the matrix environment and cell-cell contact and will enable us to study cell interactions in conditions that are close to the *in vivo* situation in the human airway.

METHODS

Bronchial epithelial cell and fibroblast cultures

Epithelial cells and fibroblasts were isolated from bronchial biopsy specimens of asthmatic ($n = 7$) and normal ($n = 5$) subjects. The asthmatic subjects, as diagnosed according to American Thoracic Society¹⁰ criteria (methacholine PC₂₀ <8 mg/mL), used only an inhaled β_2 -agonist on demand. None used inhaled or systemic corticosteroids. All asthmatic subjects were atopic nonsmokers. Normal subjects (methacholine PC₂₀ >16 mg/mL) were nonatopic nonsmokers with no history of asthma or systemic diseases. Bronchial biopsy specimens were obtained by means of bronchoscopy from asthmatic and normal subjects. Fibroblasts and epithelial cells were isolated from bronchial biopsy specimens¹¹ and characterized by immunofluorescence and flow cytometry by using anti-cytokeratin, anti-vimentin, and a mouse anti-human fibroblast antigen Ab-1 antibody from Calbiochem (San Diego, Calif) that shows no cross-reactions with epithelial cells, endothelial cells, smooth muscle cells, and many other cell types. This identification confirmed the purity of our bronchial cell culture, as described previously.¹² During the culture period, the media of both cell types were changed 3 times a week. When the cultures reached 70% to 80% confluence for epithelial cells and 100% for fibroblasts, the cells were detached, expanded, and used to produce engineered human bronchial mucosa (EHBM) at earlier passages (passage 2 for epithelial cells and passages 3-5 for fibroblasts).

Preparation of EHBM

Bovine type I collagen (2.7 mg/mL; type III Sigma) was mixed with a normal or asthmatic human fibroblast suspension (1.5×10^6 cells) and poured into a Petri dish (35-mm diameter) containing an anchorage. The cells were cultured in 10% FCS-supplemented culture medium for 4 days. Epithelial cells ($9 \times 10^4/\text{cm}^2$) of the same subject were seeded onto the gel to obtain EHBM, which were grown submerged in Dulbecco's modified Eagle's-hydrocortisone-free culture medium. When epithelial cells reached 100% confluence, an interface air-liquid interface was performed for cell differentiation.

T lymphocyte-EHBM coculture

Lymphocytes were isolated from the peripheral blood of asthmatic patients ($n = 8$) by using Ficoll-Paque gradients. Adhering cells were removed by incubation in a culture flask for 1 hour at 37°C. Nonadhering cell suspensions were then filtered through a

Nylon column to enrich them in T lymphocytes. The purity of T cells was about 90%. T cells (10^6) were added into the lower chamber underneath the engineered tissue. Twenty-four hours later, T-cell pellets were harvested, centrifuged, and fixed in 2% paraformaldehyde. Cytospin preparations were then prepared and immunostained with anti-IL-5 (Genzyme, Cambridge, Mass) by using the ABC-peroxidase technique.

Histologic assessment of EHBM

For the histologic studies, biopsy specimens were taken from normal and asthmatic EHBM, fixed in 4% paraformaldehyde, and embedded in paraffin. Thin microtome sections (5 μm) were prepared from each biopsy specimen, and Masson trichrome staining was used to evaluate the structure of the different tissues. For immunohistochemical assessments, biopsy specimens were embedded in OCT (Miles, Elkhart, Ind) and frozen in liquid nitrogen, and cryostat sections (8 μm) were prepared for immunostaining. Sections were first overlaid with specific goat anti-laminin or anti-type IV collagen (Chemicon, Temecula, Calif) mAbs for 45 minutes in a 95% humidified chamber at room temperature. After the first incubation, the sections were rinsed extensively with PBS-1% BSA. Laminin was detected by using immunofluorescence and type IV collagen by using immunocytochemistry with the ABC-peroxidase system. Sections were mounted, observed, and photographed.

Electron microscopy

Biopsy specimens were taken from the EHBM for ultrastructural studies. The samples were fixed in 2% glutaraldehyde (0.1 mol/L cacodylate buffer, pH 7.5), postfixed in 1% osmium tetroxide, and dehydrated by using graded ethanol. They were then embedded in Epon 812, stained with toluidine blue, and observed under a light microscope. Ultrathin sections were obtained from representative areas, stained with uranyl acetate and lead citrate, and examined under a JEOL electron microscope.

Statistical analysis

Results are presented as means $\pm$ SEM. Comparisons between groups were performed by using the Student *t* test. Normality and variance assumptions were fulfilled. The results were considered significant if *P* values were less than .05. Data were analyzed by using the statistical package program SAS (SAS Institute Inc, Cary, NC).

RESULTS

EHBM was obtained from asthmatic ($n = 7$) and normal ($n = 5$) subjects. Fig 1, A, shows that in EHBM with normal cells, the epithelium was pseudostratified, a structure typical of the bronchial epithelium. Electron microscopy analysis confirmed this structure (Fig 1, C). The basal layer contained small cells with a low cytoplasm/nucleus ratio, whereas the upper layer contained differentiated cells with a high cytoplasm/nucleus ratio. Histologic examinations revealed the presence of mucus in some cells, suggesting the *in vitro* conservation of the secretory profile. Fibroblasts were oriented horizontally to the collagen fibers and had dense nuclei and small cytoplasm. Fig 1, B, shows that in EHBM with asthmatic cells, the epithelium was less organized than in EHBM of normal cells, and the number of cell layers was reduced. Large epithelial cells with faint nuclei, large cytoplasm, and the presence of vacuoles were observed in the asthmatic model.

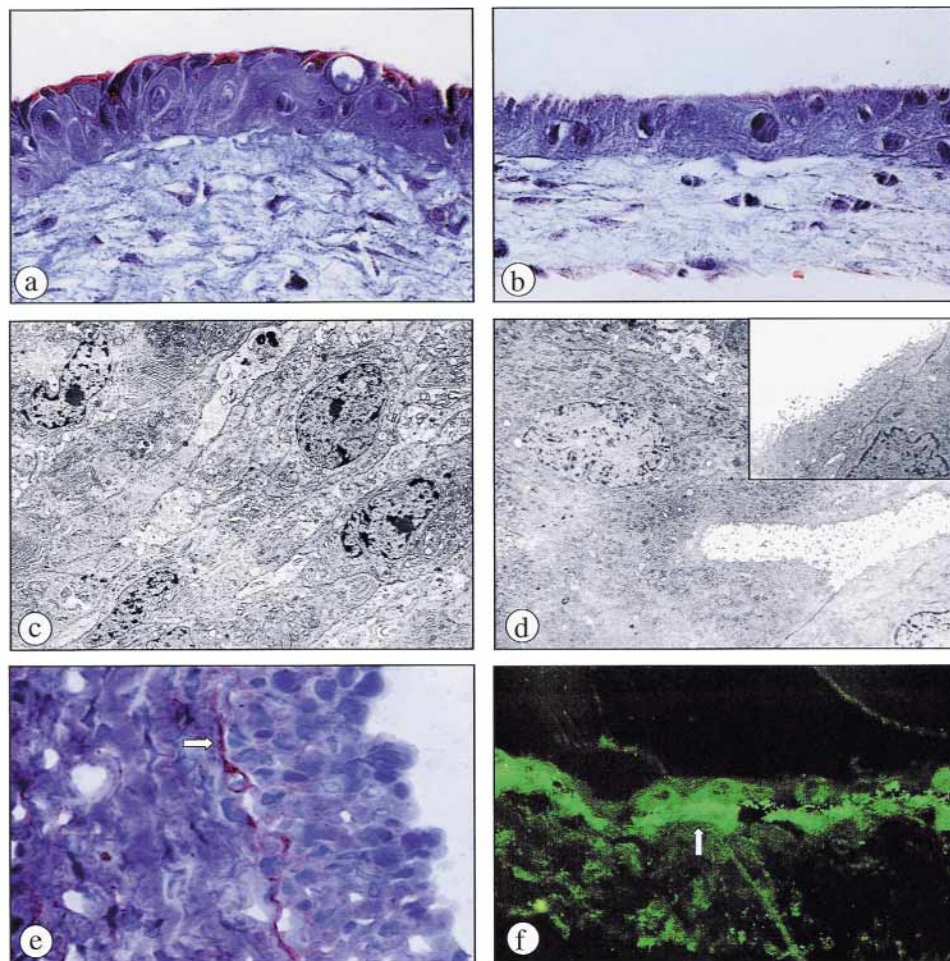


FIG 1. Micrographs of a typical example of EHBMs with cells from normal (**A**) and asthmatic (**B**) donors (original magnification, 400 $\times$). **C** and **D**, Electron microscopy micrographs showing the ultrastructure and the formation of rudimentary cilia (original magnification, 2700 $\times$). **E**, Positive staining for type IV collagen in the EHBMs (original magnification, 400 $\times$). **F**, Immunofluorescence-positive staining for laminin in the EHBMs (original magnification, 400 $\times$).

Electron microscopy analysis showed the presence of microvilli and the formation of rudimentary cilia, suggesting that epithelial cells can differentiate in both normal and asthmatic models (Fig 1, *D*). Immunohistochemical and immunofluorescence staining showed deposition of type IV collagen and laminin at the junction between the epithelium and fibroblasts, suggesting formation of a basement membrane (Fig 1, *E* and *F*).

Using our bronchial mucosa engineered model, we also evaluated interaction between immune cells (T cells) and bronchial resident cells. Fig 2 shows that 87% of T lymphocytes produced IL-5 when cultured with asthmatic EHBMs, whereas only 2% produced IL-5 in the normal engineered tissue ($P = .03$). There was no significant difference in IL-5 production when T cells were cultured alone compared with coculture with the normal EHBMs ($2\% \pm 0.8\%$ vs $2\% \pm 0.3\%$).

DISCUSSION

We have developed a bronchial mucosa model by tissue engineering with primary fibroblast and epithelial cells isolated from normal and asthmatic human bronchial biopsy specimens. This model exhibits morphologic, histologic, and functional features of bronchial mucosa. Our study showed that in engineered tissue from normal volunteers, fibroblasts were well organized in the extracellular matrix, and the epithelial cells proliferated and differentiated to produce a pseudostratified structure. Epithelial cells and fibroblasts in this model were able to produce basement membrane proteins. This confirmed that there was functional *in vitro* cooperation between cells through laminin and type IV collagen synthesis. Recently, Zhang et al¹³ showed that epithelial cells controlled the proliferation

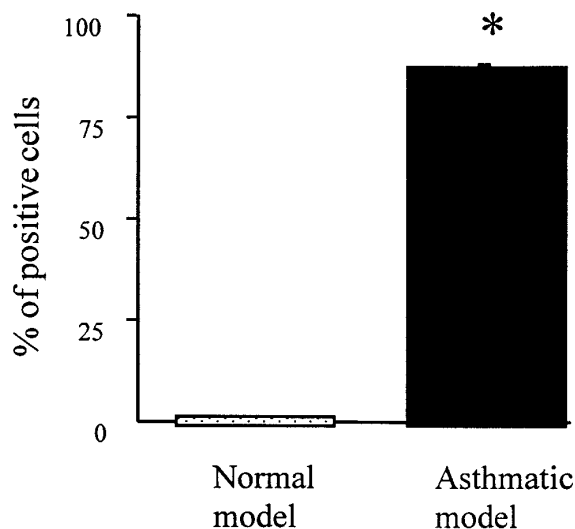


FIG 2. Percentage of IL-5⁺ cells after lymphocyte coculture with EBM.

of bronchial fibroblasts grown in a 3-dimensional model. This supports the hypothesis that the tissue environment and cell contact are key factors in reproducing in vitro the cascade of events that occur in vivo.

We also showed that in an engineered model from asthmatic subjects, epithelial cells lose the capacity to have an organized structure compared with normal cells. Our finding is in concordance with different observations previously reported in asthma studies and suggests that the asthmatic epithelium acquired an intrinsic abnormal function and an altered ability to repair.^{14,15}

The involvement of resident bronchial cells in modulating and maintaining the inflammatory response is likely important. We showed that the asthmatic bronchial cell environment promoted IL-5 production by T cells. Several factors may be involved in orienting the immune response to a T_H2 response, notably certain chemokines, such as monocyte chemoattractant protein 1, as well as certain mediators that increase cyclic adenosine monophosphate (cAMP) levels, such as prostaglandins.¹⁶ Although the mechanisms by which monocyte chemoattractant protein 1 stimulates a T_H2 response (production of IL-4) at the expense of a T_H1 response are unknown, cAMP is known to act specifically on a transcription factor (GATA-3) that is essential for the expression of the *IL5* gene.¹⁷ This transcription factor seems to be expressed only by T_H2 cells.¹⁸ We previously noticed that asthmatic bronchial fibroblasts in monolayer cultures produce significantly more PGE₂ than normal cells (data not shown). It was suggested that in our system, PGE₂ may induce an increase in intracellular cAMP, which stimulates the specific expression of GATA-3. This transcription factor is translocated to the nucleus, where it binds to a specific site on the IL-5 promoter, inducing the gene transcription. The role of PGE₂ in inducing IL-5 production by T cells in our model is currently being addressed.

In conclusion, using tissue engineering technology, we produced well-organized normal bronchial mucosa tissues. This model is a very powerful tool that will open the way to cutting edge technologies in asthma research. It is a stable and reproducible technique for comparative studies. It has the advantage of being more versatile and allowing studies in a step-by-step design for each type of inflammatory cell on resident cells and vice versa. In addition, it is composed of human cells from normal or asthmatic subjects and may lead to a better understanding of some mechanisms involved in inflammation and airway repair in asthma.

We thank Dr M. Boutet for the electron microscopy and G. Ross for her technical assistance.

REFERENCES

1. Aubock J, Irschick E, Romani N, Kompatscher P, Hopfl R, Herold M, et al. Rejection, after a slightly prolonged survival time, of Langerhans cell-free allogeneic cultured epidermis used for wound coverage in humans. *Transplantation* 1988;45:730-7.
2. Larochelle F, Ross G, Rouabhia M. Permanent skin replacement using engineered epidermis containing fewer than 5% syngeneic keratinocytes. *Lab Invest* 1998;78:1089-99.
3. Wakitani S, Goto T, Young RG, Mansour JM, Goldberg VM, Caplan AI. Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel. *Tissue Eng* 1998;4:429-44.
4. Ogle BM, Mooradian DL. The role of vascular smooth muscle cell integrins in the compaction and mechanical strengthening of a tissue-engineered blood vessel. *Tissue Eng* 1999;5:387-402.
5. Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* 2000;105:193-204.
6. Gaudie J, Jordana M, Cox G, Ohtoshi T, Dolovich J, Denburg J. Fibroblasts and other structural cells in airway inflammation. *Am Rev Respir Dis* 1992;145:14-7.
7. Raeburn D, Webber SE. Proinflammatory potential of the airway epithelium in bronchial asthma. *Eur Respir J* 1994;7:2226-33.
8. Marini M, Vittor E, Hollemborg J, Mattoli S. Expression of the potent inflammatory cytokines, granulocyte-macrophage colony-stimulating

- factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 1992;89:1001-9.
9. Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. *Development* 1993;117:1183-98.
 10. American Thoracic Society Statement. Standardization of spirometry—1987 update. *Am Rev Respir Dis* 1987;136:1285-98.
 11. Dube J, Chakir J, Laviolette M, Saint-Martin S, Boutet M, Desrochers C, et al. In vitro procollagen synthesis and proliferative phenotype of bronchial fibroblasts from normal and asthmatic subjects. *Lab Invest* 1998;78:297-307.
 12. Goulet F, Boulet LP, Chakir J, Tremblay N, Dubé J, Laviolette M, et al. Morphological and functional properties of bronchial cells isolated from normal and asthmatic subjects. *Am J Respir Cell Mol Biol* 1996;15:312-8.
 13. Zhang S, Smartt H, Holgate ST, Roche WR. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma. *Lab Invest* 1999;513:395-405.
 14. Ricciardolo FLM, Ruchimann E, Rabe KF, Litvinov S, Sterk PJ, Van Krieken JHJM. Epithelial damage and expression of proliferation marker Ki67 and epithelial adhesion molecules Ecadherin and Ep-CAM in bronchial biopsies of atopic mild asthmatics. *Eur Respir J* 1999;14:66-7s.
 15. Puddicombe SM, Holgate ST, Davies DE. The role of the epidermal growth factor receptor (EGFR) in bronchial epithelial repair. *Eur Respir J* 1998;12:29S.
 16. Lukacs NW, Chensue SW, Karpus WJ, Lincoln P, Keefer C, Strieter RM, et al. C-C chemokines differentially alter interleukin-4 production from lymphocytes. *Am J Pathol* 1997;150:1861-8.
 17. Snijdwint FG, Kalinski P, Wierenga EA, Bos JD, Kapsenberg ML. Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J Immunol* 1993;150:5321-9.
 18. Siegel MD, Zhang DH, Ray P, Ray A. Activation of the interleukin-5 promoter by cAMP in murine EL-4 cells requires the GATA-3 and CLEO elements. *J Biol Chem* 1995;270:245-8.