ORIGINAL ARTICLE

Preliminary Study of the Cellular Characteristics of Primary Bronchial Fibroblasts in Patients With Asthma: Expression of α–Smooth Muscle Actin, Fibronectin Containing Extra Type III Domain A, and Smoothelin

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■ **Abstract**

Background: The relationship between fibroblasts, myofibroblasts, and smooth muscle cells within the airway wall remains poorly understood.
Objective: The cellular characteristics of primary bronchial fibroblasts from patients with asthma were investigated by evaluating the expression of 3 proteins: α–smooth muscle actin (SMA), fibronectin containing extra type III domain A (EDAcFN), and smoothelin.
Methods: Expression of SMA, EDAcFN, and smoothelin was evaluated in primary fibroblasts from 3 patients with asthma of varying symptom severity, embryonic fibroblasts, and a healthy control. In addition, primary bronchial fibroblasts from patients with asthma were assessed for SMA at various incubation times (4 hours to 76 hours) and with different extracellular matrices (ECMs). Immunofluorescence was assessed by manually counting cells that stained positively as fine filamentous structures under a fluorescence microscope.
Results: Expression of filamentous SMA tended to increase with the length of incubation. The positive to total cell ratio for filamentous cells did not differ significantly between the various kinds of ECMs onto which cells were plated ($P > .05$). Primary bronchial fibroblasts from asthma patients produced more prominent expression of EDAcFN than control fibroblasts. Smoothelin was not expressed in any fibroblasts.
Conclusions: More than 50% of primary bronchial fibroblasts were defined as myofibroblasts. Primary bronchial fibroblasts in patients with asthma had more potential for tissue fibrosis than control fibroblasts. No mature smooth muscle cells were observed in primary bronchial fibroblasts in patients with asthma.

Key words: Asthma. Fibroblast. Smooth muscle actin. Fibronectins.

■ **Resumen**

Antecedentes: Todavía se sabe poco de la relación entre los fibroblastos, los miofibroblastos y las células del músculo liso (ML) en la pared de las vías respiratorias.
Objetivo: Se investigaron las características celulares de los fibroblastos bronquiales primarios de pacientes con asma mediante la evaluación de la expresión de 3 proteínas: la α-actina de músculo liso (α-AML), la fibronectina con extradominio A de tipo III (FN EDA) y la *smoothelin*.
Métodos: Se evaluó la expresión de α-AML, FN EDA y smoothelin en fibroblastos primarios de 3 pacientes asmáticos con síntomas de distinta gravedad, fibroblastos embrionarios y controles sanos. Asimismo, se evaluaron los fibroblastos bronquiales primarios de pacientes asmáticos en cuanto a la expresión de α-AML en distintos tiempos de incubación (de 4 horas a 76 horas) y con diferentes matrices extracelulares (MEC). La inmunofluorescencia se evaluó mediante el recuento manual de las células que se tiñeron positivamente como estructuras filamentosas finas en el microscopio de fluorescencia.
Resultados: La expresión de α-AML filamentosa tendió a aumentar a medida que aumentaba el tiempo de incubación. La relación entre

células positivas y células totales de las células filamentosas no difirió significativamente entre los distintos tipos de MEC en las que se cultivaron células (*p*>0,05). Los fibroblastos bronquiales primarios de pacientes asmáticos produjeron una expresión más destacada de FN EDA que los fibroblastos de los controles. Ningún fibroblasto expresó smoothelin.

Conclusiones: Más del 50% de los fibroblastos bronquiales primarios se definieron como miofibroblastos. Los fibroblastos bronquiales primarios de pacientes asmáticos presentaban una mayor probabilidad de fibrosis tisular que los fibroblastos de los controles. No se observaron células del ML maduras en los fibroblastos bronquiales primarios de pacientes asmáticos.

Palabras clave: Asma. Fibroblasto. Actina de músculo liso. Fibronectinas.

Introduction

Chronic inflammation and remodeling of the bronchial wall are basic hallmarks of asthma. It has become generally accepted that modulation of fibroblasts towards the myofibroblastic phenotype, with acquisition of specialized contractile features, is essential for connective tissue remodeling during wound healing in the airway wall of asthmatics [1]. However, the relationship between myofibroblasts, fibroblasts, and smooth muscle cells within the airway wall remains poorly understood [2].

Mesenchymal cells in the lamina reticularis underlying the basement membrane of the thickened airway wall of asthmatics predominantly display myofibroblast phenotypes and express α–smooth muscle actin (SMA) [3].

In vitro, primary human bronchial fibroblasts transform into myofibroblasts under the influence of transforming growth factor ß (TGF-ß), mechanical stress, and fibronectin containing extra type III domain A (EDAcFN) [1]. Cellular fibronectin, a multimeric form, is synthesized by mesenchymal, epithelial, and inflammatory cells. It is deposited in the fibrils of the extracellular matrix and contains variable proportions of the extra type III domain A and B (EDA and EDB) sequences [4]. These domains are thought to be involved in the contribution of EDAcFN to fibroblast activation [5] and wound healing [6].

Differentiated myofibroblasts do not express smoothelin [7,8], a recently described late differentiation marker of both vascular and parenchymal smooth muscle cells [9]. The expression of smoothelin is currently the most reliable criterion by which to distinguish between the differentiated myofibroblast and the mature smooth muscle cell [8].

Although ours is a preliminary study performed in a small group of asthmatics and a healthy control, we evaluated expression of SMA in primary bronchial fibroblasts at varying incubation times and in various extracellular matrices (ECMs) to determine the effect of mechanical stress and ECMs on differentiation of fibroblasts into myofibroblasts. We also evaluated expression of EDAcFN and smoothelin in order to characterize various kinds of cells in primary bronchial fibroblast culture.

Materials and Methods

Reagents

ECMs (laminin, collagen I and IV, fibronectin), mouse monoclonal anti–smooth muscle antibody (anti-SMA), 7-aminoactinomycin D, mouse immunoglobulin (Ig) G1 isotype control, mouse anti-SMA fluorescein isothiocyanate (FITC) conjugate, mouse IgG_{2a} FITC conjugate isotype control, fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Poole, UK). Polyclonal rabbit anti-mouse Ig/FITC rabbit F(ab')2 was purchased from Dako Ltd. (High Wycombe, UK). Mouse monoclonal antibody to smoothelin and mouse monoclonal antibody (IST-9) to fibronectin (EDAcFN) were obtained from Abcam (Cambridge, UK). Hoechst 33342 stain and the Zenon labeling kit (Alexa fluor 647) were obtained from Invitrogen (Paisley, UK), Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, and trypsin were purchased from Gibco Life Technologies Ltd. (Paisley, UK). L-glutamine was obtained from Biowhittaker (Wokingham, UK).

Primary Bronchial Fibroblast Isolation and Culture

Fibroblasts were grown as previously described [10] from 2 sources: explants of bronchial biopsy tissue from a healthy nonatopic nonsmoking male adult volunteer with a mean forced expiratory volume in 1 second (FEV_1) of 93.8% of predicted and a provocation concentration of inhaled methacholine required to reduce FEV_1 by 20% (methacholine PC_{20}) >16 mg/mL;

Table. Clinical Characteristics of Study Participants

Number	Sex	Age, y	PC_{20}	FEV_1 (%)	Atopy	Medication
1	Male	25	7.45	3.51 (82.0)	Yes	ICS only
2	Male	47	1.1	4.00 (76.7)	Yes	ICS+LABA
3	Male	53	0.66	2.14 (57.8)	No	ICS+LABA+LTRA

Abbreviations: FEV_1, forced expiratory volume in 1 second (% predicted); ICS, inhaled corticosteroids; LABA, long-acting ß$_2$ agonist; LTRA, leukotriene receptor antagonist; PC_{20}, provocation concentration of methacholine required to reduce FEV_1 by 20%.

and from 3 male donors with mild, moderate, and severe persistent asthma according to the Global Initiative for Asthma (GINA) guidelines [11] (Table). Human embryonic tissue was collected, staged, and processed as previously described [12]. Participants had been free of respiratory tract infections for at least 4 weeks prior to the study. Written informed consent was obtained from all participants in accordance with the Southampton and South West Hampshire Joint Research Ethics Committee. Two submucosal biopsies from each participant were obtained as previously described [10]. Biopsies were cut into pieces with sterile scalpel blades in a petri dish containing DMEM with 10% heat-inactivated FBS, 50 IU/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM nonessential amino acids. The tissue was cultured in a humidified incubator (37°C, 5% CO_2) for approximately 1 week, during which time fibroblasts migrated from the tissue and proliferated on the base of the culture dish. Separate cultures were maintained from each donor; cultures were fed every 2 days and passaged weekly. All studies were performed using cultured fibroblasts between the third and sixth passage.

Immunocytochemistry

SMA staining: Fibroblasts were cultured on 96-well culture plates (Sigma-Aldrich). Primary fibroblasts from patients with asthma and embryonic fibroblasts were plated in each well at a concentration of 3000 cells/well. After 52 hours of incubation, cells were washed with DMEM, fixed in cold methanol for 10 minutes, and washed again. After washing 3 times in phosphate-buffered saline (PBS) for 5 minutes, nonspecific binding was reduced by incubation with DMEM containing 10% FBS and 1% BSA for 30 minutes. Mouse monoclonal anti-SMA primary antibodies were added to wells and incubated for 1 hour at 1:150 dilutions. After washing, rabbit F(ab') polyclonal secondary antibody to mouse IgG/FITC was added to the wells and left to incubate for 1 hour at 1:50 dilutions. Immunostaining was visualized using the nuclei counterstained by 7-aminoactinomycin D. Immunofluorescence of SMA was assessed based on its fine filamentous morphology under a fluorescence microscope.

SMA staining at different incubation times and ECMs: Prior to incubation of fibroblasts from patients with mild persistent asthma, 4 kinds of ECM were coated onto each well. The dilutions for each ECM (collagen I, collagen IV, fibronectin, and laminin) were 1:500, 1:120, 1:100, and 1:40, and incubation times were 30 minutes 24 hours, 30 minutes, 1.5 hours, and 2 hours, respectively. After plating the same number of cells for each condition, incubation was maintained for 4, 28, 52, and 76 hours. The immunofluorescence of SMA was assessed based on its fine filamentous morphology under the microscope.

EDAcFN staining: alone and combined with SMA: Staining was as for SMA, except for the dilution of the primary antibody (1:400). In double staining of SMA and EDAcFN, the antibodies used included monoclonal anti-SMA FITC conjugate, mouse IgG_{2a} FITC conjugate isotype control, mouse monoclonal antibody (IST-9) to fibronectin, and mouse IgG1 isotype control with the Zenon labeling kit (Alexa fluor 647). Immunostaining was visualized using the nuclei counterstained by Hoechst 33342.
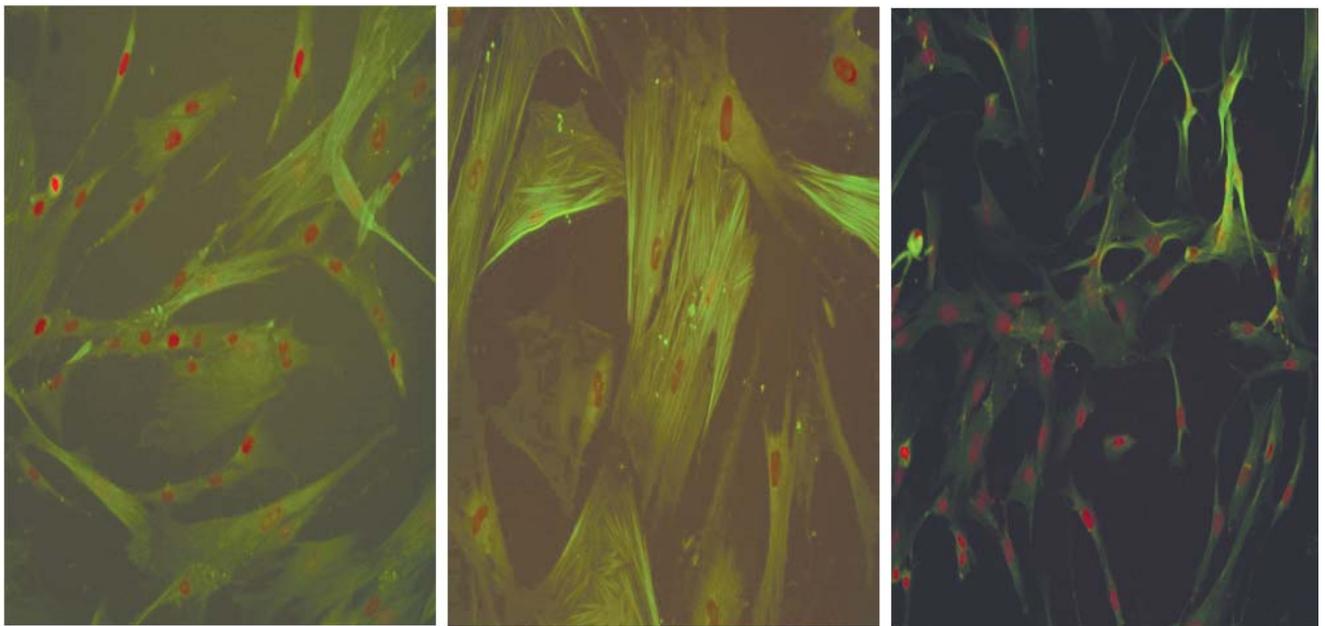
Figure 1. Expression of SMA stains in primary fibroblasts from patients with asthma and embryonic fibroblasts. Cells were stained with SMA (1:150). Filamentous SMA staining can be seen in most fibroblasts from patients with asthma. From left to right; mild persistent asthma, moderate persistent asthma, and embryonic fibroblasts. SMA indicates α–smooth muscle actin.

Smoothelin staining: Procedures were as for SMA immunocytochemistry in patients with severe asthma.

Statistical analyses were performed using SPSS version 15 (SPSS Inc, Chicago, Illinois, USA). The ratios of SMA-positive cells to total cells in 5 groups with different ECMs were compared using the Kruskal-Wallis test followed by a post hoc analysis. Statistical significance was set at $P<.05$.

Results

Expression of SMA in Embryonic Fibroblasts and in Fibroblasts From Patients With Asthma

Expression of SMA in embryonic fibroblasts was represented by diffuse irregular cytoplasmic staining with a
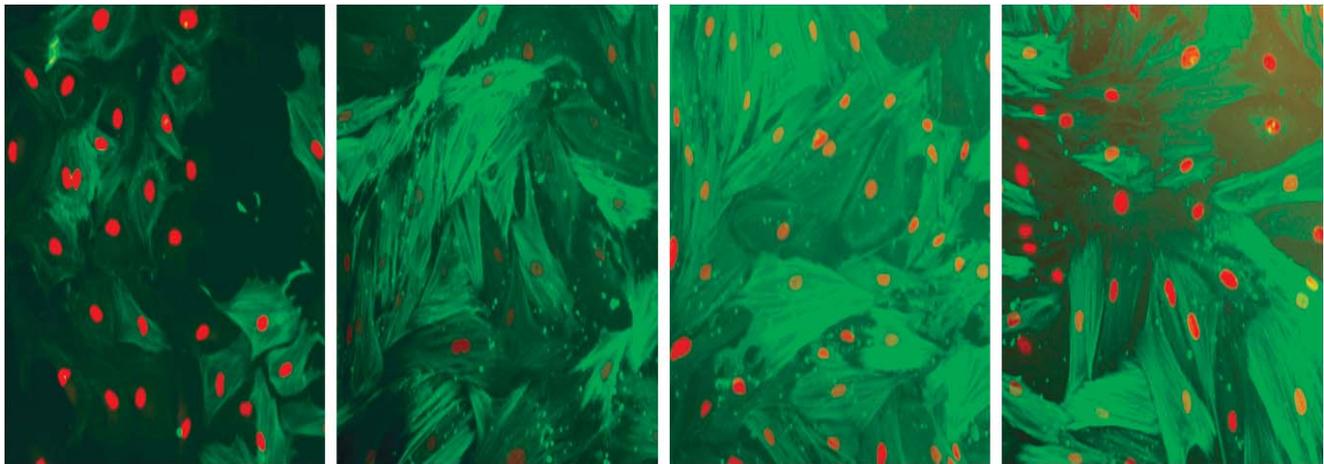
Figure 2. Expression of SMA in primary fibroblasts from patients with mild persistent asthma plated on ECM (collagen I). As incubation time increased, the circular cellular morphology became spindle-shaped while filamentous SMA expression became more prominent. Incubation time was 4, 28, 52, 76 hours from left to right. SMA indicates α–smooth muscle actin.
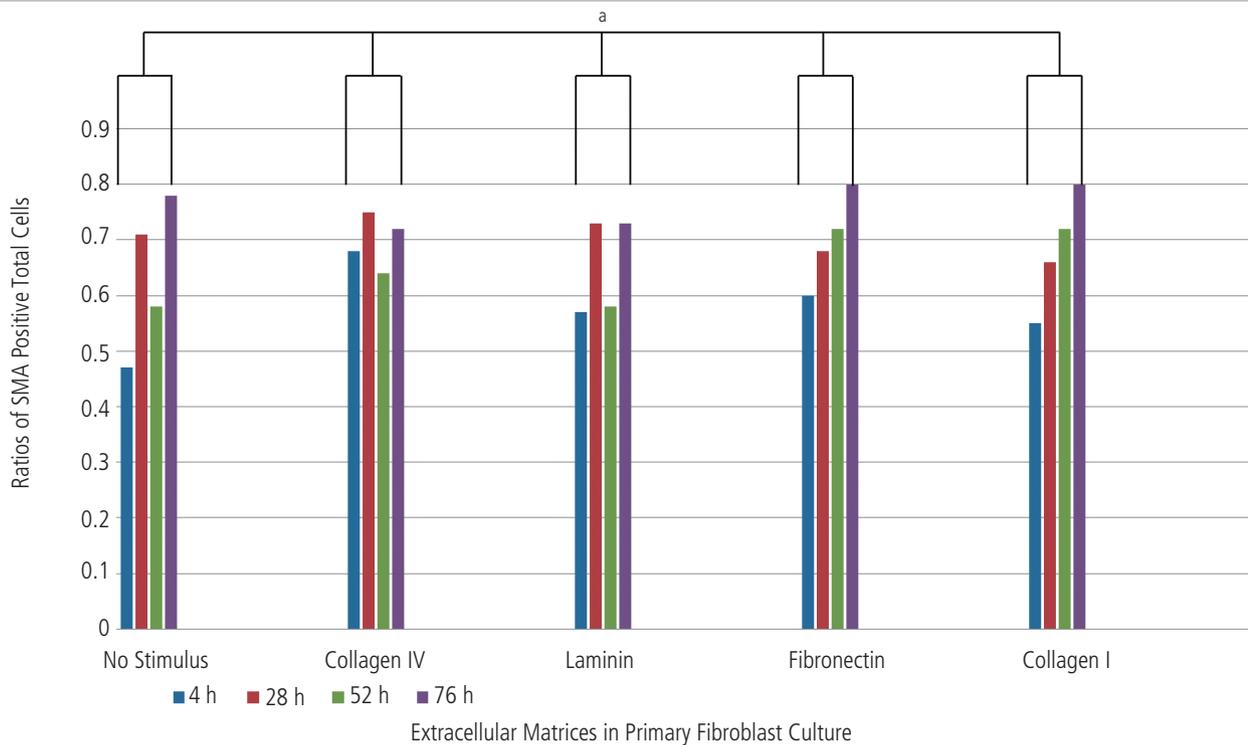
$^aP>.05$

Figure 3. Ratio of filamentous SMA-positive cells to total cells with respect to cell incubation time and ECMs in primary fibroblast cultures from patients with mild persistent asthma. There were no significant differences in the ratios of SMA-positive cells to total cells among the various kinds of ECMs onto which cells were plated ($P>.05$). ECM indicates extracellular matrix; SMA, α–smooth muscle actin.

minimally filamentous pattern. However, in asthmatic patients, the expression pattern of SMA was primarily high-intensity filamentous and more prominent than in embryonic fibroblasts (Figure 1).

SMA Expression of Primary Bronchial Fibroblasts Based on Incubation Time and ECMs

As incubation time increased, cell morphology changed from round to spindle-shaped. Immunofluorescence of SMA revealed a fine filamentous morphology, and the number of positive cells increased with incubation time (Figure 2). The

ratio of filamentous SMA expressed to total cells also increased with incubation time. However, there were no significant differences between the various kinds of ECMs onto which cells were plated (P>.05, respectively, Figure 3).

Expression of EDAcFN in Embryonic Fibroblasts and Primary Fibroblasts From Patients With Asthma and From a Healthy Control

Expression of EDAcFN in fibroblast cultures obtained from patients with asthma tended to show a more intense intracellular staining pattern than in the cells from the healthy

A
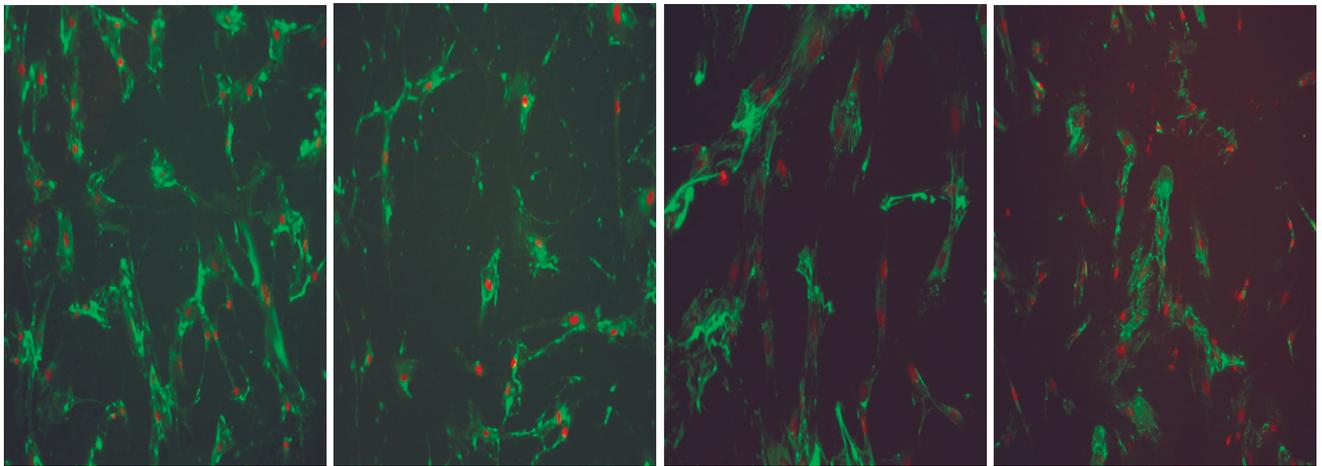
Figure 4A. Expression of EDAcFN alone and combined with SMA. Expression of EDAcFN in primary fibroblasts from asthmatics and the healthy control. In asthmatics, the expression of EDAcFN had a more scattered intracellular staining pattern than in the healthy control. From left to right; severe asthma, moderate asthma, mild persistent asthma, and healthy control.

B
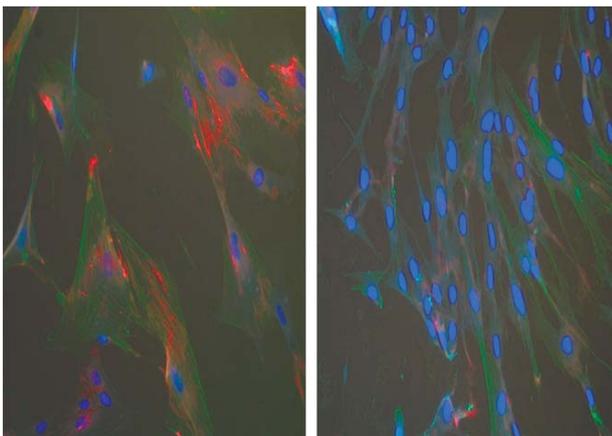
Figure 4B. Double immunofluorescence of SMA (green) and EDAcFN (red) in primary fibroblasts from a patient with asthma and embryonic fibroblasts. Expression of EDAcFN (red) in patients with asthma had an intracellular scattered pattern and was more prominent than in embryonic fibroblasts. From left to right; mild persistent asthma and embryonic fibroblast.
EDAcFN indicates fibronectin containing extra type III domain A; SMA, α–smooth muscle actin.

control (Figure 4A). In double staining of SMA and EDAcFN, EDAcFN (red) in fibroblasts cultured from patients with asthma was expressed as a scattered intracellular pattern of SMA (green) and was more prominent than in embryonic fibroblasts (Figure 4B).

Smoothelin Expression

Smoothelin was not expressed in the patient with severe persistent asthma at any concentration of primary antibody ranging from 1:400 to 1:100.

Discussion

This study showed that SMA expression increased proportionally with cell incubation time, regardless of the type of ECM presented for cells to adhere to. It also showed that expression of EDAcFN in the asthmatic patients was more prominent than in the healthy control and embryonic fibroblasts. Thus, we could infer that primary bronchial fibroblasts were susceptible to mechanical stimuli during differentiation into myofibroblasts. Furthermore, fibroblasts from asthmatics showed greater potential for tissue fibrosis than those from the healthy control and embryonic fibroblasts.

Expression of smoothelin, a late maturation marker of smooth muscle cells, was not noted in this study, suggesting that there were no mature smooth muscle cells present or that smooth muscle cells had already dedifferentiated to myofibroblasts in primary bronchial fibroblast culture. However, as ours was a preliminary study performed in a small group of asthmatic patients, larger-scale studies are required before these findings can be generalized to all asthmatic patients.

Myofibroblasts are contractile cells with morphologic and biochemical features that lie somewhere between those of fibroblasts and smooth muscle cells [13-17]. However, no specific markers have been identified for myofibroblasts, in part accounting for the difficulty in accurately defining the cell populations present, especially after cell culture of airway wall biopsy specimens. The most important finding in differentiation of myofibroblasts from fibroblasts was the expression of SMA, specifically a myofibrillar staining pattern [2,18,19]. In fibroblasts, SMA revealed diffuse irregular cytoplasmic staining with no filamentous pattern. In this study, more than 50% of cells expressed filamentous SMA after 4 hours of incubation. This expression increased with incubation time.

Previous studies revealed that TGF-ß, mechanical stress, and EDAcFN are the most important stimuli in the differentiation of fibroblasts into myofibroblasts [1]. TGF-ß1 is the central regulator of myofibroblast differentiation through its capacity to promote accumulation of intracellular contractile proteins, high collagen density, and EDAcFN [1]. In addition, autocrine production of TGF-ß1 by fibroblasts plays a key role in preserving fibrogenic activity once the inflammatory stimulus has ceased [20,21].

The effect of mechanical stimuli on cellular proliferation has been addressed in the literature [22,23]. Hinz et al [22] reported that mechanical stimuli resulting from splinting in an animal model containing artificially made granulation tissue could express increased SMA and EDAcFN concentrations without changing the expression levels of TGF-ß1. Using cultured corneal fibroblasts, Masur et al [23] showed that culture density also modulates cellular phenotype. Corneal fibroblasts differentiated to a myofibroblastic phenotype when cultured at low density in serum-enriched media but maintained their phenotype in high-density seedings. These results suggest that, in addition to the regulatory effects of TGF-ß, loss of cell-cell contact is an important determinant of myofibroblast differentiation. Underlying mechanisms of mechanical stretch–induced cellular proliferation, with activation of protein tyrosine kinase secondary to calcium influx, have been suggested [24]. Gene expression and protein synthesis of platelet-derived growth factor (PDGF) and PDGF receptor (PDGFR) are increased by mechanical stress. These factors then stimulate cell proliferation through autocrine/paracrine mechanisms [24]. Fibroblasts cultured in vitro are mechanically activated by contact with the rigid substrate and possess a proto-myofibroblastic phenotype, which represents an intermediate step between fibroblasts and myofibroblasts in vivo [1]. Because these cells are exposed to tractional forces during the process of cell growth, they eventually differentiate into myofibroblasts. This study was performed using a plastic culture plate with no additional stimulation by TGF-ß.

Therefore, we assume that the most important stimulus was a mechanical force enacted during traction on the culture plate. However, the lack of positive controls stimulated with TGF-ß to provide a basis of comparison is a limitation of this study.

Interaction of cells with their surrounding matrix is mediated mainly through integrins, a group of heterodimeric transmembrane glycoproteins [25]. Airway myofibroblasts express a range of integrin receptors, such as α1, α3, α4, α5, αv, and ß1, of which the most important are α5 and ß1, as they exert a strong survival signal in airway myofibroblasts [26,27]. The range of integrin receptors expressed appears to be similar to the receptors from very-early-passage airway myofibroblast cultures [2]. We observed no significant variations in differentiation of myofibroblasts on the ECMs, suggesting that the corresponding proportions of integrins change according to the degree of differentiation of fibroblasts. Furthermore, we believe the most important stimuli in differentiation of myofibroblasts in this study were mechanical stimuli, while the effect of ECMs on differentiation of myofibroblasts might be less relevant.

In experimental models, EDAcFN is expressed early after lung injury and before collagen deposition [28]. The physiologic factors that induce EDAcFN production are poorly defined, although the molecular mechanisms regulating alternative EDA exon splicing have been well described [29,30]. Interestingly, TGF-ß1 promotes inclusion of EDA into fibronectin [31]. Although this study was a preliminary experiment using only a small group of patients with asthma, expression of EDAcFN in asthmatics was more prominent than in the healthy control and the embryonic fibroblasts, suggesting that fibroblasts from asthmatics have more potential for tissue fibrosis. These results were consistent with those of a previous study of patients with idiopathic pulmonary fibrosis [32] and with those of a recent report [33].

We did not observe expression of smoothelin in primary bronchial fibroblasts from patients with bronchial asthma. When airway smooth muscle cells are placed into primary cultures of animal and human lung biopsy specimens, a population of cells with typical myofibroblastic characteristics is obtained, suggesting that airway smooth muscle cells are capable of dedifferentiating into airway myofibroblasts, at least in vitro [34]. However, at present, it is unclear whether myofibroblasts and smooth muscle cells are distinctly different cell types or whether the myofibroblast represents a step within a continuous differentiation spectrum between the fibroblast and the smooth muscle cells [7,8,9,35].

Our study has several limitations. First, we did not apply a quantitative method to evaluate the degree of expression of each protein. Second, the number of participants was very small; therefore, our results may not be representative of all patients with asthma. In terms of perspective, we think that the precise phenotypic markers for different cell types in primary bronchial fibroblast culture should be identified. Larger samples of asthma patients and more extensive quantitative evaluation of proteins expressed during differentiation of fibroblasts into myofibroblasts are necessary. It would also be interesting to evaluate the in vivo expression of these proteins.

In conclusion, we found that more than 50% of primary bronchial fibroblast cultures were myofibroblasts and that

the remaining fibroblasts had yet to acquire myofibroblastic characteristics. Primary bronchial fibroblasts from patients with asthma had a greater potential for tissue fibrosis than control fibroblasts. There were no mature smooth muscle cells in primary bronchial fibroblasts from patients with asthma. Further studies are warranted to elucidate precise phenotypic markers for different cell types in primary bronchial fibroblast culture.

Acknowledgments

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