Role of laminin polymerization at the epithelial mesenchymal interface in bronchial myogenesis

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SUMMARY

Undifferentiated mesenchymal cells were isolated from mouse embryonic lungs and plated at subconfluent and confluent densities. During the first 5 hours in culture, all the cells were negative for smooth muscle markers. After 24 hours in culture, the mesenchymal cells that spread synthesized smooth muscle α-actin, muscle myosin, desmin and SM22 in levels comparable to those of mature smooth muscle. The cells that did not spread remained negative for smooth muscle markers. SM differentiation was independent of cell-cell contact or proliferation. In additional studies, undifferentiated lung mesenchymal cells were cocultured with lung embryonic epithelial cells at high density. The epithelial cells aggregated into cysts surrounded by mesenchymal cells and a basement membrane was formed between the two cell types. In these cocultures, the mesenchymal cells in contact with the basement membrane spread and differentiated into smooth muscle. The rest of the mesenchymal cells remained round and negative for smooth muscle markers. Inhibition of laminin polymerization by an antibody to the globular regions of laminin β1/γ1 chains blocked basement membrane assembly, mesenchymal cell spreading and smooth muscle differentiation. These studies indicated that lung embryonic mesenchymal cells have the potential to differentiate into smooth muscle and the process is triggered by their spreading along the airway basement membrane.

Key words: Smooth muscle, Myogenesis, Lung, Cell spreading, Laminin, Basement membrane, Mouse

INTRODUCTION

Smooth muscle (SM) constitutes a significant portion of developing organs including those of the gastrointestinal, urogenital and respiratory tracts, and the vascular system. Most SM originates by differentiation of local mesenchymal cells, which in mid-gestation begin to express and accumulate SM proteins (Ruzicka and Schwartz, 1988; Sawtell and Lessard, 1989; Kedinger et al., 1990; Roman and McDonald, 1992; McHugh, 1995; Li et al., 1996) in a process that progresses in a cranial-to-caudal gradient. With the exception of the aorta, visceral SM a-actin, desmin and myosin are first detected in the trachea and proximal segments of the main bronchi, along with transcripts for muscle myosin and SM22 (Miano et al., 1994; Li et al., 1996). The last two also are detected in the proximal segments of pulmonary arteries (Miano et al., 1994; Li et al., 1996). The non-bronchovascular lung mesenchyme does not express SM proteins at any time during embryogenesis (Woodcock-Mitchell et al., 1993; Miano et al., 1994; McHugh, 1995; Li et al., 1996). However, in the postnatal lung, some of the mesenchymal cells become contractile interstitial cells or alveolar myofibroblasts (Kapanchi et al., 1974) and express SM α-actin (Leslie et al., 1990).

Although our understanding of SM biology has grown considerably over the years, the factors that induce and regulate SM differentiation remain largely unknown. Epithelial-mesenchymal interaction (Kedinger et al., 1990; Duluc et al., 1994; Baskin et al., 1996), growth factors (Boström et al., 1996; Zhou et al., 1996) and hormones such as retinoic acid (Blank et al., 1995) have all been implicated in the differentiation process. The best studied among these is the inductive epithelial effect on mesenchymal SM differentiation. In tissue recombinant experiments, Duluc et al. (1994) demonstrated that epithelium derived from the intestine can induce SM differentiation in associated mesenchyme. Interestingly, the photomicrographs presented in their study seemed to show a change in mesenchymal cell shape from round to elongated concomitantly with the expression of SM α-actin. Similarly, during embryogenesis most of the
mesenchymal cells are round/cuboidal and adopt an elongated/spread configuration only around hollowed epithelial structures. This change in cell shape from cuboidal to elongated preceeds the appearance of SM at the same sites (Theiler, 1989). Although the shift in cell shape may be secondary to the underlying differentiation process, we recently found that embryonic lung explants cultured in the presence of antibodies to laminin α1 chain contained round, instead of elongated, peribronchial mesenchymal cells. These explants synthesized lower levels of SM-specific proteins, suggesting that changes in cell shape may play an active role in stimulating SM development (Schuger et al., 1997).

In the present study, we show that undifferentiated mesenchymal cells isolated from embryonic lungs have the potential to differentiate into SM upon spreading. Conversely, SM differentiation does not occur if the cells retain their original round shape. Additional studies using epithelial-mesenchymal cocultures demonstrated that cell spreading and SM differentiation are restricted to mesenchymal cells close or apposed to the newly formed BM. Furthermore, the prevention of laminin polymerization by a monoclonal antibody against the globular regions of laminin β1/γ1 chains blocked BM formation and abolished mesenchymal cell spreading and differentiation into SM. This study therefore identified cell spreading/elongation as a trigger for SM myogenesis and suggests a critical role for the airway BM in the development of bronchial SM.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibody to desmin and rabbit polyclonal antibody to low and high molecular weight cytokeratins were purchased from Dako (Carpinteria, CA). Mouse monoclonal antibody to SM α-actin was obtained from Boehringer Mannheim Biochemica (Indianapolis, IN). Rabbit polyclonal antibody to SM22 (Sartaren et al., 1987; Almendral et al., 1989) was a gift from Dr Rodrigo Bravo (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). Delipidized rabbit whole antisem to myosin and rabbit polyclonal antibody to laminin-1 were purchased from Sigma (St Louis, MO). A rabbit polyclonal antibody to mouse laminin-1 was purchased from Sigma. A monoclonal antibody to laminin β1/γ1 chains was generated against murine laminin-1 by immunization of male LOU/MNCr rats. The preparation, purification and characterization of this antibody have been previously described (Skubitz et al., 1987, 1988). This monoclonal antibody reacted with laminin by solid phase radioimmunoassay and radioimmunoprecipitation and did not cross react with type IV collagen, thrombospondin or fibronectin in an enzyme-linked immunosorbent assay (ELISA). Rotary shadowing and electron microscopy demonstrated that the antibody binds to the globular segments of the lateral short arms (β1 and γ1). Normal rat immunoglobulin was purchased from Cappel (Malvern, PA). Matrigel, a B M extract obtained from the EHS mouse tumor, was purchased from Collaborative Biomedical (Boston, MA).

Mesenchymal cell cultures

CD-1 strain (Charles River, Portage, MI) mice were mated and the day of finding a vaginal plug was designated as day zero of embryonic development. Lungs were removed at days 11 and 12 of gestation. Peripheral mesenchyma away from SM, was microdissected from day 12 lungs after immersing the lungs in PBS containing 0.3% trypsin and 0.1% EDTA for 2 minutes. Mesenchymal cells were then dissociated by trypsinization and plated at subconfluent densities in 6-well plates (Fisher, Itasca, IL) (approximately 1×105 cells/well) or in 8-chamber Lab-Tek slides (Nunc, Inc., Naperville, IL) (approximately 2×105 cells/well) in minimal essential medium (MEM) (Gibco, Grand Island, NY) containing non-essential amino acids, 0.29 mg/ml L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 mg/ml amphotericin B, and either 1% or 0.5% fetal bovine serum (Irvin Scientific, Santa Ana, CA). The lower serum concentration was used to minimize cell proliferation. Mesenchymal cells from day 11 lungs (prior to SM appearance in the lung), were obtained by differential plating as previously described (Schuger et al., 1993). Briefly, the mixed lung cell populations obtained by trypsinizing the lungs were plated in 6-well plates or 8-chamber slides and incubated for 2 hours to allow the mesenchymal cells to attach. The non-attached cells, essentially epithelial cells, were then removed.

Primary confluent cultures were generated by plating the cells in numbers high enough to cover the entire dish surface by the time that their attachment was completed, approximately 1 hour later. Cells were plated at a density of 2.5×106 cells/chamber to generate a culture of tightly packed round cells, with average diameters of 9±2 µm; or at a density of 6×105 cells/chamber to generate a confluent culture of moderately spread cells with average diameters of 10±5 by 35±15 µm. The normal cell size of mesenchymal cells in culture is 60±20 by 100±30 µm. In additional studies, the mesenchymal cells were plated at subconfluent densities (approximately 1×105 cells/well in 6-well plate, approximately 2×105 cells/chamber in 8-chamber Lab-Tek slides) in poly-L-lysine-coated dishes. In such cases, the dishes were pretreated for 2 hours with 0.1% poly-L-lysine in water (Sigma), washed and used right away.

Mesenchymal-epithelial cocultures

Peripheral lung parenchyma, which includes epithelial and mesenchymal cells but does not contain SM, was microdissected from day 12 lungs. The cells were then dissociated by trypsinization and plated at a density of 2.5×106 cells/chamber directly or over a thin film of Matrigel (less than 0.5 mm thick). Coating the dish with Matrigel increased by 30 fold the number of epithelial cells that attached. In additional studies, mesenchymal cells isolated from day 11 lungs were mixed in a 1 to 5 ratio with lung epithelial cells obtained from primary monolcultures as described (Schuger et al., 1997).

Treatment of mesenchymal-epithelial cocultures with monoclonal antibodies against laminin β1/γ1 chains

Antibody or control immunoglobulin (100 µg/ml) was added to cocultures at the time of plating as previously described (Schuger et al., 1995). The cocultures were incubated for 24 to 72 hours and then immunostained with anti-cytokeratin antibody to determine the number of polarized and unpolarized epithelial cell clusters (Schuger et al., 1995) per 4 mm2 surface. Previous studies indicated that polarized epithelial cells form spheroids and cysts, whereas unpolarized epithelial cells grow as a regular monolayer (Schuger et al., 1993, 1995). In additional experiments, at the end of the culture period, the cells were fixed for the immunohistochemical studies described below.

Treatment of mesenchymal cell cultures with Matrigel

To determine the effects of the BM in the absence of cell spreading, high cell density primary confluent cultures (2.5×106 cells/chamber) were plated on a thin film of Matrigel, or the latter was added to the confluent cultures in concentrations ranging from 20 to 850 µg/ml (25 µl/ml). In additional studies similar concentrations of Matrigel were added to primary subconfluent round cell cultures established on poly-L-lysine-coated wells. After 24 hours, the cultures were evaluated for changes in cell shape and SM-specific protein expression.
Western blotting
Lungs isolated at days 11, 12, 13, 14 and 15 of gestation, as well as day 11 and day 12 cell cultures, were lysed by boiling for 10 minutes in reducing sodium-dodecyl-sulfate (SDS) sample buffer. 30 μg of sample were resolved in a 12% acrylamide gel. The samples were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and blocked with 5% non-fat dry milk in TBS-T (20 mM Tris base, 137 mM sodium chloride, 0.05% Tween-20, pH 7.6, all from Bio-Rad). The membranes were then blotted for 1 hour with antibodies to either SM α-actin (0.25 μg/ml), desmin (1.125 μg/ml), SM22 (0.2 μg/ml) or myosin (1:150 dilution). This was followed by 1 hour of incubation with a 1:3000 dilution of the appropriate secondary antibody. The bands were detected by chemiluminescence using a commercial kit (Amersham Life Science, Arlington Heights, IL) according the manufacturer’s instructions.

Immunohistochemistry
5 μm frozen sections from embryonic lungs obtained at days 11, 12, 13, 14 and 15 of gestation, mesenchymal cell cultures and mesenchymal-epithelial cocultures established in 8-chamber Lab-Tek slides were fixed for 5 minutes in absolute alcohol. The slides were then treated with either 1 μg/ml of monoclonal antibody to SM α-actin or 5 μg/ml of monoclonal antibody to desmin for 45 minutes at room temperature. The sections were washed in PBS and exposed to a 1:50 dilution of the appropriate secondary antibody (biotin-conjugated goat anti-mouse or anti-rabbit IgG; Sigma) for 30 minutes at room temperature and staining was completed using a commercial peroxidase-anti-peroxidase kit (Dako), following the manufacturer’s instructions. To identify epithelial cells, the cultures were immunostained with anti-cytokeratin antibodies using a commercial peroxidase-anti-peroxidase kit (Dako), following the manufacturer’s instructions. Immunostaining with anti-laminin antibodies was performed as previously described (Schuger et al., 1995).

RESULTS

SM development in the mouse lung
SM α-actin, myosin and SM22 are first detected in the lung by immunoblotting on day 12 of gestation, while desmin appears on day 13 (Fig. 1A). From then on, SM-specific proteins increase over time (Fig. 1A). Immunohistochemical studies show that the first SM α-actin-positive mesenchymal cells appear around the tracheal epithelium on day 11 of gestation (not shown). This is followed by the appearance of desmin approximately 24 hours later. By day 12 of gestation, SM cells are identified not only in the trachea, but also in the proximal segments of main bronchi (Fig. 1B,C). The number of positive cells and immunohistochemical intensity increase over time (Fig. 1D,E). As previously reported (Mitchell et al., 1990; Woodcock-Mitchell et al., 1993; Miano et al., 1994; McHugh, 1995), vascular SM development lags in comparison to that of the airways. SM α-actin is first detected in the main blood vessels on day 14 of gestation (Fig. 1E) and desmin is not detected at all in the vasculature during the developmental period encompassed by this study.

Differentiation of embryonic lung mesenchymal cells towards a SM lineage upon spreading
Western blots indicated that mesenchymal cells isolated from the peripheral mesenchyme of day 12 lungs did not express SM markers at the time of isolation (Fig. 2A). However, after 24 hours the mesenchymal cells in subconfluent cultures became positive for desmin, SM α-actin, SM22 and muscle myosin (Fig. 2A). Immunohistochemical studies confirmed the absence of SM-specific proteins in the mesenchymal cells at the time of plating and during the first 4 hours (Fig. 2B,C). Over the next 20 hours, all the cells progressively began to immunoreact with antibodies to SM α-actin, increasing in intensity during successive hours and reaching stable levels after 24 hours in culture (Fig. 2D,F). Similarly, all the cells began to immunoreact with antibodies to desmin, reaching full intensity after 48 hours in culture (Fig. 2E,G).

Mesenchymal cells isolated from day 11 lungs (before SM cells can be detected in the lung) differentiated into SM in a similar fashion as day 12 mesenchymal cells (Fig. 3). In contrast to myofibroblasts (Masur et al., 1996), SM differentiation did not revert when the cells reached confluence (Fig. 3, lanes 3, 6). SM differentiation was neither affected by cell proliferation. This was demonstrated by studies in which cell growth was arrested by reducing fetal bovine serum concentration in the culture medium. A reduction in serum concentration from 10 to 0.5% reduced cell proliferation by over 90%. This was established by determining the number of cells/well at time zero (2 hours after plating) and after 48 hours in culture (8.9±2×104 at time zero; 9.3±3×103 after 48 hours in 0.5% FBS; 8.2±2×105 after 48 hours in 10% FBS). Under these conditions, mesenchymal cells differentiated into SM in a manner identical to that observed for proliferating cells (Fig. 4).

Fig. 1. SM differentiation in the developing mouse lung. (A) Immunoblots demonstrating the presence of muscle myosin heavy chain, SM22 and SM α-actin in the lung starting on day 12 of gestation and the presence of desmin, starting on day 13. An increment in synthesis occurs along with development. *Mature intestine, used as control. (B-E) Immunohistochemical studies demonstrating the distribution of SM in the developing lung using antibodies to SM α-actin and desmin. On day 12 of gestation, SM α-actin (B) and desmin (C) are detected in one or two layers of mesenchymal cells surrounding the main bronchi (arrows). On day 14 of gestation the intensity of the immunostaining increase, along with its extension to the proximal portions of the second order bronchi (arrow in C). SM α-actin, but not desmin, is present in the walls of the main blood vessels (arrow in D). Scale bar, 200 μm.
SM differentiation does not occur in the absence of cell spreading

In these experiments, the cells were plated at a density sufficient to cover the entire dish surface by the time their attachment was completed (approximately 1 hour). Plating a total of 2.5×10^6 cells/chamber (50 to 60% plating efficiency) resulted in a confluent culture of tightly packed round cells, with average cell diameters of 9±2 μm. Plating a total of 6×10^5 cells/chamber (60 to 70% efficiency) resulted in a confluent culture of spread cells with average diameters of 35±15 μm. After 24 hours in culture, the monolayers composed of round cells were negative for SM markers (Fig. 5A). However, the confluent monolayers composed of elongated cells synthesized SM-specific proteins in a fashion similar to their subconfluent counterparts (Fig. 5B). These results were confirmed by immunoblots (Fig. 5C). After 24 hours, the concentration of fetal bovine serum was reduced to 0.5% to reduce cell proliferation and prevent cell detachment. Under these conditions, the cultures composed of round cells remained negative for SM markers for several days until eventually detached and died. Similarly, the cells plated on poly-L-lysine remained round in shape and did not differentiate into SM cells (Fig. 6). However, when the cells cultured on poly-L-lysine-coated plates were detached and replated onto plastic dishes they spread and differentiate into smooth muscle. This observation demonstrated that the cells cultured onto poly-L-lysine-coated dishes have the potential to differentiate and will do so if placed under conditions that allow cell spreading.

Irreversibility of the SM phenotype

Once the lung mesenchymal cells expressed SM markers, they remained positive for the entire culture period (a maximum of 4 passages and over 20 days). Upon reaching confluence, SM-differentiated cells showed an increase in SM-specific proteins (Fig. 3). In myofibroblasts, however, the SM-like phenotype is transient, disappearing when cells become confluent (Masur et al., 1996). Lastly, the SM phenotype persisted after inducing the lung cells to re-adopt a spherical shape by plating them at high confluency density (2.5×10^6 cells/chamber) (Fig. 7), indicating that changes in cell shape induce, but do not revert, the SM phenotype.
Differentiation of mesenchymal cells from adult mouse lung

Mesenchymal cells isolated from adult mouse lung showed a mixed cell population at the time of plating, including cells positive for SM markers. The latter, which constituted approximately 10% of the total cell number, likely represented the mature SM cells normally found in the lung. The number of cells expressing SM α-actin increased overnight (approximately 40% of the total number) while the proportion of cells expressing desmin remained constant (approximately 10% of the total number). This was consistent with the expansion of a myofibroblast subpopulation, since most myofibroblasts synthesize only SM α-actin (Gabbiani, 1996).

Restricted SM differentiation in mesenchymal-epithelial cocultures

As previously reported (Schuger et al., 1995, 1996), in organotypic cultures, the epithelial cells formed small three-dimensional spheroids and cysts completely encased by mesenchymal cells that spread over the epithelial surface (Fig. 8A-C). The rest of the mesenchymal cells remained round in shape and attach to the dish surface as a monolayer covering it entirely (Fig. 8A). In these cocultures, a BM-like structure was formed between the two cell types (Schuger et al., 1995), (Fig. 8E). A 1.0 µm thick section through one of the cysts (Fig. 8B) shows a central lumen (lu), polarized epithelium (e) and elongated/spread mesenchymal cells (m) encasing the cysts. Immunohistochemical studies showed that the mesenchymal cells spread over the newly formed BM expressed SM proteins whereas the rest of the mesenchymal cells remained round in shape and negative for SM markers over the whole culture period (Fig. 9).

Mesenchymal cell primary cultures in which the cells were forced to maintain a round configuration by plating them at very high cell density (2.5×10^6 cells/chamber) showed not SM differentiation when treated with Matrigel (Fig. 10). Likewise, Matrigel in concentrations of up to 180-200 µg/ml did not induce cell spreading or synthesis of SM markers in cells plated on poly-L-lysine-coated dishes (not shown). High concentrations of Matrigel (over 200 µg/ml) induced cell spreading and SM differentiation in poly-L-lysine-plated cells when added after 2 hours of cell plating. However, when added after 6 hours, Matrigel did not stimulate cell spreading or SM differentiation at any concentration studied (not shown).
Blocking BM assembly in mesenchymal-epithelial cocultures prevents mesenchymal cell differentiation

Treatment of the cocultures with 100 μg/ml of a monoclonal antibody against laminin β1/γ1 chains reduced the number of epithelial spheroids/cysts by 68±8% compared to cocultures exposed to the same concentration of control immunoglobulin (Fig. 11A). Previous studies indicated that the decrease in cyst formation is due to a lack of organotypic rearrangement, due to blockage of laminin polymerization at the epithelial-mesenchymal interface, rather than a decrease in the number of epithelial cells (Schuger et al., 1995). In cocultures exposed to the monoclonal antibody against laminin β1/γ1 chains the epithelial cells cluster together but, instead of forming three-dimensional structures, they attach to the dish surface forming keratin-positive islets within the mesenchymal monolayer.
Fig. 11. Organotypic cocultures were established in the presence of a monoclonal antibody against laminin α1/β1 chains or control IgM. After 24 hours the cultures were immunostained with anti-keratin antibodies and the number of epithelial cysts and unpolarized epithelial islets per well was determined. (A) Anti-laminin β1/γ1 chains antibody inhibited cyst formation. (B) The number of unpolarized epithelial islets was directly proportional to the concentration of anti-laminin α1/β1 chains antibody. The bars represent standard deviations (SD). The means and SD are based on four samples in a single experiment. The experiment was repeated four times with similar results.

(Schuger et al., 1995). Our previous observations were confirmed in this study, which showed an increase in the number of epithelial islets in the cocultures exposed to the antibody against laminin β1/γ1 chains proportional to the decrease in the number of cysts (Fig. 11B). When cocultures treated with the monoclonal antibody against laminin β1/γ1 chains were immunostained with anti-desmin and anti-keratin antibodies (and counterstained with hematoxylin), they showed no mesenchymal cell spreading/elongation or SM differentiation around the epithelial clusters (Fig. 12A,B). Additional studies in which cocultures treated with monoclonal antibody against laminin β1/γ1 chains or control immunoglobulin were immunostained with anti-laminin and anti-keratin antibodies confirmed the absence of BMs (Fig. 12C) in the cocultures exposed to the antibody and their presence in the controls (Fig. 12D,E).

DISCUSSION

During lung development, only a small number of cells undergo SM differentiation. This study however suggests that most lung mesenchymal cells have the potential to become SM cells and will follow this differentiation pathway upon spreading. The process of SM differentiation is rapid, irreversible and independent of cell proliferation or cell-cell contact. Moreover, if spreading is prevented, the cells remain undifferentiated suggesting that cell spreading/elongation plays a key role in initiating SM development.

The potential triggering effect of cell spreading is already suggested by the normal cytoarchitecture of the developing lung. During lung organogenesis, the mesenchymal cells apposed to the airway begin to spread and elongate around the trachea and then the main bronchi (Theiler, 1989; L. S. and Y. Y., unpublished observations). Cell elongation is rapidly followed by the synthesis of SM proteins and eventually those cells become the bronchial SM. The rest of the mesenchymal cells remain round in shape and negative for SM markers. The key role of cell spreading in SM myogenesis was revealed by our studies using organotypic cocultures. In these cocultures, likewise in the normal lung, the only mesenchymal cells that spread, elongated and differentiated into SM were those encasing epithelial structures. The rest of the mesenchymal cells remained round in shape and did not express SM markers through the whole period studied.

We recently observed that exposure of embryonic lung explants to a monoclonal antibody against laminin α1 chain resulted in a decreased synthesis of SM proteins by the whole explants (Schuger et al., 1997). These explants also showed deficient peribronchial mesenchymal cell elongation. Based on our findings and on the close proximity between the cells differentiating into SM and the BM, we reasoned that the latter may serve as a substratum for peribronchial mesenchymal cell attachment and spreading. In such a case, blocking BM assembly should have an impact on mesenchymal cell differentiation around the epithelial clusters (E). Scale bars, 10 μm in A, 15 μm in B and 20 μm in C,D.
spreading around epithelial cysts as well as on their differentiation to SM.

Similar to what occurs in vivo, in organotypic cocultures, a BM-like structure is formed between epithelial and mesenchymal cells (McAteer et al., 1988; Schuger et al., 1995, 1997). To determine the possible role of the BM in SM differentiation, we exposed the organotypic cocultures to a monoclonal antibody against the globular domains of laminin \( \beta_1/\gamma_1 \) chains. These are the sites whereby laminin molecules assemble into a polymer (Yurchenco and Cheng, 1993). Our previous studies showed that this anti-laminin \( \beta_1/\gamma_1 \) chains antibody blocks laminin assembly at the epithelial-mesenchymal interface of organotypic cocultures (Schuger et al., 1995), thus preventing epithelial cell polarization into cysts. The unpolarized epithelial cells rearrange into clusters that attach to the culture surface and grow as a simple monolayer (Schuger et al., 1995). The anti-laminin \( \beta_1/\gamma_1 \) chains antibody blocked in vitro BM formation and prevented mesenchymal cell spreading/elongation and SM differentiation around the epithelial cysts. Furthermore, Matrigel added to the cultures did not induce SM differentiation in the absence of cell spreading. These experiments, therefore, suggested that the airway BM plays a critical role in bronchial SM development by facilitating cell spreading/elongation. It should be stressed that the antibody against laminin \( \alpha_1 \) chain used in the previous studies (Schuger et al., 1997) prevented organotypic cell rearrangement (Y. Y. and L. S., unpublished observation) and therefore was not suited for these studies.

Based on proximity with epithelia, it has been proposed that epithelial-mesenchymal interactions are likely to play a role in SM differentiation. Recent studies using tissue recombinants, in which a fragment of epithelium is apposed to a fragment of mesenchyme, seems to support this hypothesis. SM differentiation has been shown in recombinants of embryonic mesenchyme localized outside the lung (Li et al., 1996). In such cases the differentiation of mesenchymal cells to SM has been attributed to epithelial influences. However, none of these tissue recombinants was evaluated for changes in mesenchymal cell shape. The latter could result from the production of an in vitro BM at the epithelial-mesenchymal interface, or from mechanical forces generated by the unequal growth of the two tissues. In this regard, it is interesting that the pictures presented by Duluc et al. (1994) seem to show a change in cell shape from round to elongated along with SM differentiation.

Diffusible factors/gradients have also been implicated in SM differentiation (Blank et al., 1995). These investigators reported that treatment with retinoic acid promotes stem cell differentiation towards a SM cell lineage. However, retinoic acid-induced SM differentiation required several days and involved the emergence and further growth of cell subpopulations within the culture. Similarly, cell subpopulations with a SM/myofibroblast phenotype have been amplified from primary cultures of mesenchymal cells (Mitchell et al., 1993; Babij et al., 1993; Galmiche et al., 1993; Iehara et al., 1996; Desmouliere et al., 1992; Arciniegas et al., 1992). In all these instances, the primary cultures were established from adult organs and required several days for significant SM \( \alpha \)-actin-positive cells to emerge, supporting their clonal origin from local SM cells. Alternatively, they may represent myofibroblasts. It has been shown that certain populations of adult fibroblasts and perhaps other mesenchymal cells are facultative myofibroblasts and can express SM \( \alpha \)-actin and occasionally other SM proteins in culture (Gabbiani, 1996). However, myofibroblasts differ from SM cells in that they express mainly SM \( \alpha \) actin and their phenotype is reversed by re-establishment of cell-cell contact (Gabbiani, 1996; Masur et al., 1996). Furthermore, the levels of SM markers synthesized by myofibroblasts are lower than those of SM cells (Gabbiani, 1996).

The role of cell spreading on the BM as a triggering factor for SM myogenesis is relevant for visceral SM development, but do not necessarily apply to vascular SM. In contrast with the local origin of visceral SM cells, the origin of vascular SM cells is not well characterized (Ruzicka and Schwartz, 1988, Sawtell and Lessard, 1989, Keding et al., 1990; Roman and McDonald, 1992; McHugh, 1995; Li et al., 1996). The current view is that these cells are likely to derive from mesodermal mesenchyme localized outside the lung (Li et al., 1996). Vascular SM cells are distinct from visceral SM cells in contractile properties and pharmacological responses (Akerlund, 1994; Zingg et al., 1995). Thus it will be of interest to determine whether cell spreading also promotes vascular SM differentiation and, in that case, what is the role of the vascular BM.

In summary, this study provided two new and important clues about bronchial SM myogenesis. First, it demonstrated that most lung embryonic mesenchymal cells are potential myogenic cells. Second, these results supported a model where bronchial SM differentiation is regulated by mesenchymal cell spreading on the airway BM. This model would explain two key questions about SM development which have remained elusive: (1) what triggers it, and (2) why it is localized around epithelia. While the molecular mechanisms that control differentiation of skeletal and cardiac muscles have begun to be elucidated (Weintraub et al., 1991; Olson and Klein, 1994; Bodmer, 1995; Lin et al., 1997) little is known of the genetic program that controls SM myogenesis and no transcription factors that regulate SM-specific gene expression have yet been identified. Just as the differentiation of 10T1/2 fibroblasts to striated muscle upon treatment with 5-aza-cytidine (Taylor and Jones, 1979) has provided an instrumental model for the study of striated muscle differentiation, the differentiation of embryonic mesenchymal cells to SM may be useful for studying SM myogenesis.

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