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Control of Embryonic Lung Branching Morphogenesis by the Rho Activator, Cytotoxic Necrotizing Factor 1

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Background. **Lung development is sensitive to physiological stresses, and its development may be impaired by physical distortion, as in patients with congenital diaphragmatic hernia. Yet, little is known about how mechanical forces can influence lung morphogenesis. Studies with cultured cells suggest that cytoskeletal tension may play a key role in growth control. Since the small GTPase Rho plays an important role in the control of cell tension generation, we carried out studies to test the hypothesis that changes in Rho-mediated cell tension may influence branching morphogenesis.**

Methods. **Embryonic lung buds from timed pregnant Swiss Webster mice were microdissected on Embryonic Day 12 (E12), and whole organs were cultured in serum-free medium in the presence of the Rho activator cytotoxic necrotizing factor 1 (CNF-1) for 48 h. Serial measurements of the degree of epithelial branch formation and tissue maturation were performed using light microscopy and computerized image analysis.**

Results. **At 48 h, embryonic lungs treated with 2 ng/ml CNF-1 increased their terminal bud count by 236 ± 18% ($P = 0.01$) compared with 132 ± 2% for untreated controls. However, dose–response experiments revealed biphasic behavior: at a higher dose of CNF-1 (200 ng/ml), bud number was actually decreased relative to controls (43 ± 1%, $P < 0.001$). Histological analysis revealed that individual glands appeared to be more highly developed at low-dose CNF-1, whereas the high dose produced gland contraction.**

Conclusions. **These data support a potential role for Rho and cytoskeletal tension in control of epithelial pattern formation during lung development.**

Key Words: **morphogenesis; Rho; cytotoxic necrotizing factor 1; pulmonary hypoplasia; forces; tension; tissue patterning; cytoskeleton.**

INTRODUCTION

It has been known for more than a century that mechanical forces dictate pattern, growth, and function of multiple tissues and organs [1]. In the case of the fetal lung, it is well documented that mechanical variables, including lung volume, fetal breathing movements, size of the intrathoracic space, and amniotic fluid volume, regulate organ growth and maturation [2]. A specific example is the increased lung growth and differentiation that result from fluid distension caused by tracheal occlusion during the fetal period [3]. Imbalances in physical cues also may interfere with normal lung expansion and result in pulmonary hypoplasia, a major cause of death in the neonate with congenital diaphragmatic hernia [4]. However, the mechanism responsible for mechanical stress-induced changes in lung development is not known.

Morphogenesis of developing organs requires that cell proliferation be tightly controlled locally within different regions of the tissue to generate normal tissue microarchitecture, such as formation of branching epithelium with characteristic lobulated form. While the mechanisms that establish this crucial spatial heterogeneity of growth remain poorly understood, local changes in cell tension and in the balance of forces that are transmitted between epithelium and mesenchyme across their intervening extracellular matrix, the basement membrane (BM), have been suggested to be central to this process [5, 6]. This concept is based on the

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observation that living tissues exist in a state of isometric tension. Tensional forces that are actively generated within the cytoskeleton (CSK) of both epithelial and mesenchymal cells are transmitted via transmembrane integrin receptors and BM, thereby establishing a mechanical force balance [5, 6]. Regional changes in BM integrity (mechanical stiffness/compliance) may control cell proliferation locally by modulating this force balance, altering CSK tension, and/or changing cell shape. This hypothesis is supported by the observation that inhibitors of CSK tension interfere with branching morphogenesis in other organs [7]. Additionally, the growth of cultured cells can be inhibited by either preventing cell spreading, disrupting CSK integrity, or specifically inhibiting tension generation within the CSK [8]. Thus, CSK tension represents a potential control point for local growth control during tissue morphogenesis.

Studies on control of CSK tension generation in contractile cells, such as smooth muscle cells, have traditionally focused on the role of intracellular calcium and myosin light chain kinase (MLCK) [9, 10]. More recently, a new pathway has been described that involves the small GTPase Rho, which promotes MLCK phosphorylation and stimulates CSK contraction through activation of Rho-kinase (ROCK) [11, 12]. Rho has been shown to be important during vertebrate morphogenesis with respect to head formation in *Xenopus* embryos [13]. Active Rho also appears to be required for normal development during early stages of chick and mouse embryonic morphogenesis [14]. However, its role in branching morphogenesis remains unknown.

Given the role of cell tension in growth control, which in turn is critical for tissue patterning, we reasoned that Rho-dependent tension generation within embryonic cells also may be important during branching morphogenesis in the lung. To test this hypothesis, we studied the effects of a specific activator of Rho, cytotoxic necrotizing factor 1 (CNF-1), on embryological lung development in an organ culture model. We demonstrate here that low levels of Rho activation can stimulate branching morphogenesis in embryonic lungs whereas high levels of Rho activity are inhibitory, thus demonstrating a role for the Rho pathway and CSK tension generation in lung development.

METHODS

Experimental system. Embryos from Day 12 (E12) timed-pregnant Swiss Webster mice (Taconic Farms, MA) were removed aseptically and placed into bacteriological dishes containing Waymouth's MB medium (Gibco-BRL). Lung rudiments were microdissected *en bloc* (with all lobes still attached to the trachea), washed in serum-free medium, and transferred to a semipermeable membrane (Falcon cell inserts, 0.4-μm pore size) that was placed over 2.5 ml of serum-free BGJb medium (Fitton–Jackson modification, Gibco-BRL) supplemented with penicillin, streptomycin, and ascorbic acid (0.2 mg/ml) in a six-well plate. Three lungs were placed in each well and subjected to the same dose of CNF-1 (0, 2, or 200 ng/ml). This was repeated in three separate experiments, and thus, the results presented were from a total of nine whole lungs per condition. The Rho activator, CNF-1 (gift from Dr. K. Aktories), was added to individual wells at 0 h and again at 24 h with fresh BGJb medium. *In vitro* development was monitored within whole organs by serial measurements of branch points (number of buds) at 12-h intervals from 0 to 48 h using light microscopy; the observer was blinded to the treatment protocol. Results were expressed as percentage increase in number of terminal lung buds formed at each branch point relative to Time 0 baseline controls (n = 9 lungs/condition). Data were analyzed using an analysis of variance (ANOVA) single-factor test and the two-sample independent t test. In parallel studies, lungs were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned (3 μm), and stained with hematoxylin and eosin (H&E) for light microscopic analysis.

RESULTS

E12–E14 murine lung bud cultures. Lung buds were dissected at E12 when the trachea, primary bronchi, and five lobes had formed; at this point, >90% of the buds had 9–13 peripheral branchpoints (Fig. 1). While the branching pattern of the primary bronchial buds was monopodial, the secondary bronchi underwent dichotomous branching. Serial light microscopic images illustrated that with time, each original bud enlarged and formed clefts, resulting in two or three smaller buds within 48 h (Fig. 1). After 48 h in serum-free culture, the number of peripheral branches increased by 132 ± 2%, i.e., a 2.32-fold increase relative to Time 0 (n = 9 organs analyzed) (Fig. 2).

E12 lung buds were treated for 48 h with various doses of CNF-1. Compared with untreated controls, lungs treated with 2 ng/ml CNF-1 exhibited a significant acceleration of terminal bud formation, as indicated by a 236 ± 18% increase in their terminal bud count (Fig. 2) and a more mature morphology, including smaller buds and deeper clefts (Fig. 1). However, dose–response experiments revealed biphasic behavior: at higher doses of CNF-1 (200 ng/ml), bud number increased by only 43 ± 1% in the same period (Fig. 2). These results indicate nearly complete inhibition of new bud formation during the 48 h of organ culture.

Morphologically, by 48 h embryonic lung explants treated with low-dose CNF-1 formed buds that were tighter and smaller with progressively smaller lumens than those in untreated control lungs (Figs. 1, 3A, 3B), indicating that they proceeded to the next generation of branching. However at the high dose of 200 ng/ml CNF-1, the bud number was decreased compared with low-dose or control lungs at 48 h (Fig. 2) and the lumens appeared to be either poorly formed or excessively compressed when analyzed in histological sections (Fig. 3C). This inhibition of lung development did not appear to be due to general drug toxicity, since extensive survey of hematoxylin–eosin-stained lung sections did not reveal any sign of necrosis.

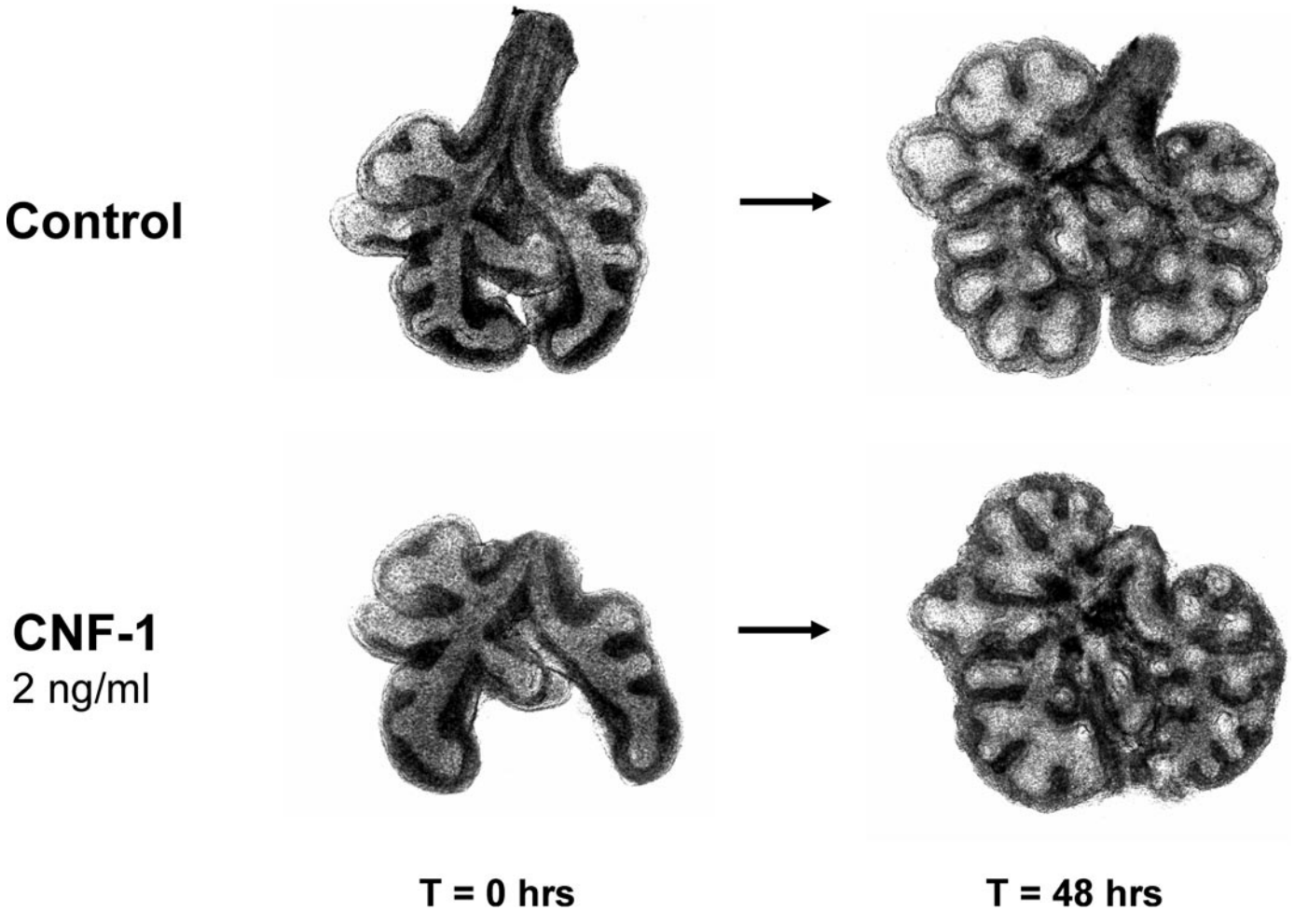

FIG. 1. Light microscopic images of embryonic lungs maintained in organ culture for 0 or 48 h in the absence or presence of CNF-1 (2 ng/ml).

DISCUSSION

Our results show that Rho plays a key role in tissue patterning during lung organogenesis. Activation of Rho by addition of low-dose (2 ng/ml) CNF-1 resulted in acceleration of bud formation and maturation without producing overall enlargement of the lung. Interestingly, the response to CNF-1 was also found to be biphasic since a high dose (200 ng/ml) inhibited branching morphogenesis.

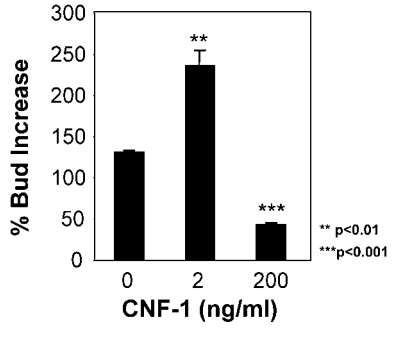

FIG. 2. Biphasic effect of CNF-1 treatment on lung branching at 48 h. Bars represent the average percentage increase in bud number compared with 0 h in lungs treated with 0, 2, or 200 ng/ml CNF-1 (n = 9; error bars indicate SD; P values are calculated relative to Time 0).

These studies were carried out as a first attempt to test a model of morphogenetic control in which changes in mechanical forces within the CSK due to Rho-dependent activation of ROCK [11, 12] can influence cell growth and pattern formation during tissue development [5, 6]. According to this model, lung tissue experiences isometric tension or "prestress" due to contractile forces generated within the cytoskeleton of epithelial and mesenchymal cells; these forces are resisted by the intervening extracellular matrix (i.e., the BM) and by opposing cells. Past studies have demonstrated accelerated BM turnover and local thinning of the BM at the sites of prospective buds [15, 16] that may increase BM flexibility. Increased compliance of the BM would result in changes in the level of tension

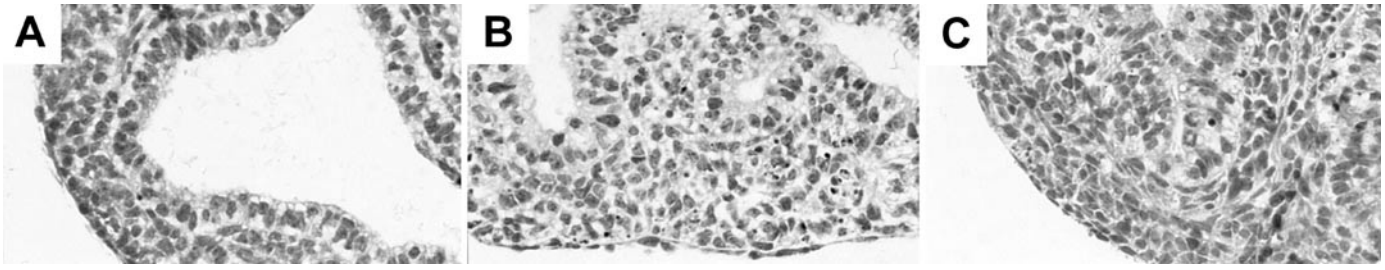

FIG. 3. Histological staining of embryonic lungs treated with 0 (A), 2 (B), or 200 ng/ml (C) of CNF-1 for 48 h. Stained with H&E; 63×.

experienced by adherent epithelial cells and thereby produce local cell distortion (Fig. 4). As previously demonstrated [8], cells become responsive to soluble growth factors when they physically extend whereas they remain quiescent or undergo apoptosis when spreading is prevented [17–19]. Thus, cells adherent to neighboring regions of the BM that exhibit low turnover rates, remain thicker, and maintain their stiffness would neither extend nor respond to growth stimuli in the same tissue microenvironment. In this manner, the requirement for cell spreading or tension for cell cycle progression may translate into localized cell proliferation and budlike tissue expansion (Fig. 4). In line with this model, previous studies on mouse salivary glands suggest that epithelial buds outgrow from highly proliferating areas where extracellular matrix turnover is increased and the BM is discontinuous [15, 16]. Moreover, Cardoso found that although local cell proliferation is a requirement for bud formation, spatial differences of cell proliferation rates were not responsible for the initiation of buds [20], suggesting that local mechanical changes in the mesenchyme could be the primary triggering events.

Importantly, our findings are consistent with this mechanical model of morphogenetic control. Changes in mechanical tension within the CSK produced by low-dose CNF-1 treatment that activates Rho and thereby promotes activation of ROCK [11, 12] promoted cell growth and accelerated bud formation in the developing lung. This suggests that tensional forces generated within the CSK of individual cells and resisted by extracellular matrix that locally differs in its mechanical compliance may provide the driving force that guides pattern formation during tissue morphogenesis. But, if the forces generated are too great, as observed with high-dose CNF-1, then cell contraction may be promoted and development will cease. It is important to note that this interpretation may be complicated by the finding that there are many Rho effectors in addition to ROCK, including some signaling molecules that are involved in control of cell proliferation [6]. However, if this effect were dominant, it would result in uniform growth stimulation, and could not explain the regional patterns of growth that shape the tissue during budding morphogenesis, as observed in the lung.

CNF-1 was administered to the organ uniformly in serum-free medium. It is thus intriguing that a global increase in tissue tension resulted in enhanced budding, i.e., in organized growth with preservation of tissue patterns (Fig. 1). Past work has shown that the mesenchyme controls tissue-specific pattern formation [7, 21], and thus, one possibility is that CNF-1 may preferentially influence mesenchymal cell contractility. Alternatively, the drug may produce similar relative increases in contraction in both mesenchyme and epithelium; however, the mesenchymal cells will still dominate because they commonly generate higher levels of force than epithelium. An increase in mechanical tension in the mesenchyme relative to the epithelium might then accelerate tissue growth while retaining normal patterns by further distorting the preexisting regions of the gland that normally exhibit the highest rate of BM turnover and, thus, the highest mechanical compliance. These normal spatial differentials in BM turnover are also established by the mesenchyme [15, 16]. Thus, the actual force that drives localized BM distortion and promotes cell growth may be the change in the cellular force balance due to Rho-dependent alterations in CSK tension. However, further studies involving positive and negative modulation of Rho and ROCK while simultaneously quantitating CSK tension generation within developing lung are needed to confirm this hypothesis. Experiments in which mechanical tension is targeted to specific regions of the growing epithelium (e.g., using micromanipulation) also may be used to demonstrate a positive role for mechanical distortion in bud initiation. Conversely, ablation of Rho's tension-generating activity by drugs, such as Rho kinase inhibitors, or by antisense oligonucleotides should slow normal development or lead to a disorganized tissue morphology.

A higher dose of CNF-1 (200 ng/ml) that produced excessive tension generation within lung cells and glandular compaction had an inhibitory effect on bud morphogenesis, perhaps by abrogating regional stretching. This inhibitory effect did not appear to result from generalized toxicity; however, additional studies are required to determine whether regional

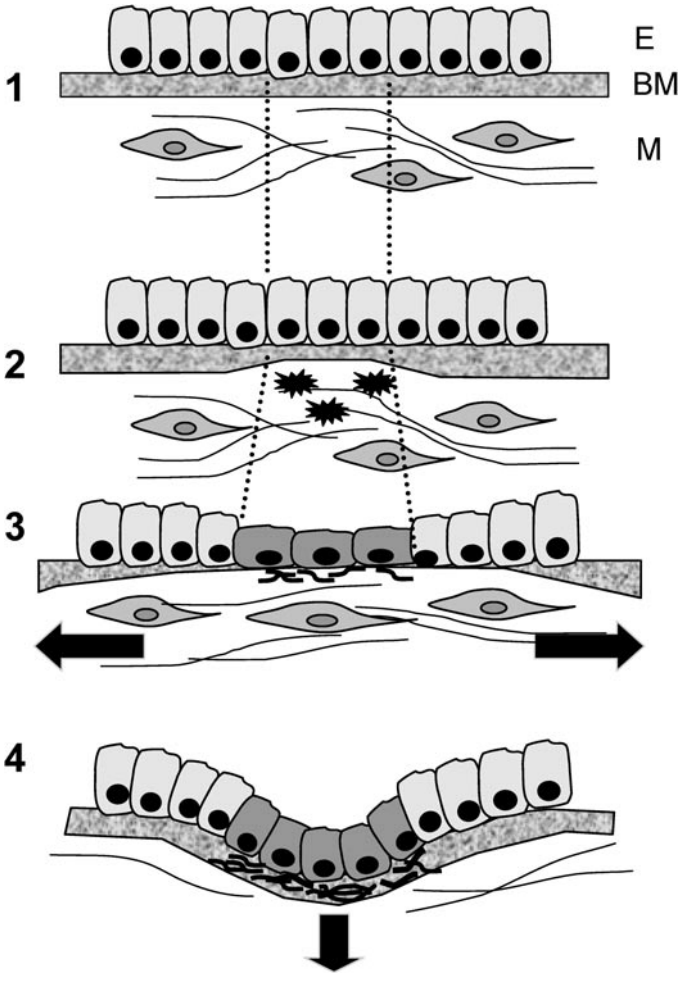

FIG. 4. Model for role of BM turnover, tissue tension, and local cell proliferation in epithelial morphogenesis. (1) In the quiescent state, epithelial cells (E) on the basement membrane (BM) do not proliferate even when stimulated with soluble mitogens because the mechanical forces generated by epithelium and mesenchyme (M) are balanced by each other and by the ability of the BM to resist distortion, and thus, the cells cannot extend. (2) Proteases (black stars) released locally by the mesenchyme promote regional ECM degradation, leading to local thinning of the BM. (3) The thinned BM extends laterally because of the residual tension or prestress in the tissue. The net force is exerted downward and outward because the mesenchymal cells exert greater tension than the epithelium. Physical distortion of this small region of the BM results in stretching of the adherent epithelium and a concomitant increase in new BM deposition (black curved lines). (4) Local cell distortion and/or changes in CSK tension trigger cell cycle progression in the presence of soluble growth factors, resulting in cell proliferation (dark-colored cells) and localized bud outgrowth. Neighboring cells (light gray) on the thicker and less compliant BM remain quiescent despite the presence of soluble mitogenic factors, because the local forces they experience remain in balance and they cannot extend.

apoptosis and cell cycle arrest contribute to this response. For example, the glandular contraction also may have led to cell retraction and rounding. As shown previously, cell retraction inhibits growth and induces G_1 arrest [8] whereas cell rounding or detachment from matrix induces apoptosis [17]. Cells that are made hypercontractile by transfection with a constitutively active form of MLCK are also both smaller and growth-inhibited [22].

How do the present results translate to the clinical situation? Increasing mechanical distension by tracheal occlusion or continuous infusion of intrapulmonary perfluorocarbon have been shown to accelerate lung growth in animal models [23–27] and in human infants [28, 29]. Moreover, abnormal loading conditions can result in developmental defects [30–33]. For instance, pulmonary hypoplasia due to compression by intraabdominal organs released into the thorax through a congenital diaphragmatic hernia might correspond to our experiments with high-dose CNF-1 where internal tissue contraction appeared to prevent epithelial development and organ expansion. This findings raise the intriguing possibility that CSK tension and the Rho–ROCK pathway may represent potential therapeutic targets for developmental diseases that are characterized by hypoplastic or poorly developed tissues, such as in infants with bronchopulmonary dysplasia.

CONCLUSION

Our observations support a model in which CSK tension controlled by Rho plays an important role in lung branching morphogenesis. Pathological conditions that compromise or in some way alter the normal balance of forces within embryonic organs may interfere with this process and inhibit normal tissue development. Results from future investigations into this phenomenon may pave the way to new approaches to prevent, minimize, or correct congenital anomalies, such as pulmonary hypoplasia, as well as provide alternative methods to accelerate lung maturation in premature infants.

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