TGFβ1 inhibits branching morphogenesis and N-myc expression in lung bud organ cultures

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SUMMARY

Lung buds isolated from 11.5 days post coitum mouse embryos survive and undergo branching morphogenesis in culture. This organ culture system was used to examine the role of TGFβ1 and N-myc expression in lung branching morphogenesis. By 24 hours, TGFβ1 reversibly inhibited branching morphogenesis in a concentration-dependent manner. N-myc is known to be expressed during embryonic development in epithelial cells involved in branching morphogenesis and homozygous null N-myc mice have defects in lung development. In the present study, TGFβ1 was shown to inhibit the steady-state level of N-myc RNA 3- to 4-fold at 14 and 48 hours of treatment as measured by northern blot and RNase protection analysis. Suppression of N-myc expression in epithelium was confirmed by in situ hybridization. Since inhibition of N-myc occurred prior to the observed changes in morphology and previous genetic studies have demonstrated an important role for N-myc in lung development, a model is proposed in which TGFβ1 inhibits tracheobronchial development by inhibiting expression of N-myc.

Key words: lung development, TGFβ, N-myc, c-myc, mouse

INTRODUCTION

Lung development begins in mouse at 9.5 days post coitum (p.c.) as two endodermally derived epithelial buds from the primitive foregut grow into surrounding mesoderm (reviewed in Ten Have-Opbroek, 1991). The epithelium develops into the conducting and respiratory airways of the adult lung, whereas the mesoderm becomes the lung stroma. The pseudoglandular stage of lung development in mouse is between 9.5 and 15.5 days p.c. and is characterized by rapid growth and branching of primitive lung epithelium to form the initial pattern of the lung. Branching morphogenesis is a combination of the growth of epithelial buds and the formation of epithelial clefts. Expression of transforming growth factor beta (TGFβ) isoforms has been detected in the lung at critical times during development (Heine et al., 1990; Pelton et al., 1991). Specifically, during the pseudoglandular stage, TGFβ protein accumulates in the stroma along the proximal airways and underlying cleft points of epithelial branching and co-localizes with fibronectin and type I and III collagen (Heine et al., 1990). The expression pattern and known biological activities of TGFβ suggest an important role for this factor in tracheobronchial development.

TGFβ represents a family of polypeptides involved in growth control, extracellular matrix production and embryonic development (reviewed in Moses, 1990). TGFβ1, TGFβ2 and TGFβ3 have marked stimulatory effects on connective tissue formation, are indirect mitogens for certain mesenchymal cells and stimulators of extracellular matrix deposition (reviewed in Moses et al., 1990 and Massague et al., 1990). The TGFβs are also potent inhibitors of epithelial cell proliferation. It has been suggested that growth suppression by TGFβ1 is mediated by regulation of c-myc expression (Moses et al., 1990). TGFβ1 has been shown to down-regulate the expression of c-myc in specific epithelial cell types (Coffey et al., 1988; Mulder et al., 1988; Fernandez-Pol et al., 1987) and inhibition of c-myc expression by antisense oligonucleotides was shown to be sufficient to inhibit DNA synthesis in mouse keratinocyte cell lines (Pietenpol et al., 1990). A homozygous c-myc null mutation is lethal in embryos between 9.5 and 10.5 days p.c. and development of these embryos is retarded relative to non-transgenic littermates (Davis et al., 1993).

The myc proto-oncogene family is involved in regulation of growth and differentiation. Myc proteins bind DNA, contain helix-loop-helix and leucine zipper motifs, and may regulate the expression of specific genes (Marcu et al., 1992; Luscher and Eisenman, 1990). N-myc is primarily expressed in the developing embryo (Zimmerman et al., 1986; Jakobovitz et al., 1985; Stanton and Parada, 1992) and in organs with epithelial and mesenchymal compartments, N-myc is expressed in the epithelium while c-myc is expressed in the stroma (Mugrauer et al., 1988; Hirning et al., 1991; Stanton et al., 1992). N-myc expression is low or undetectable in most differentiated tissues, but is highly expressed in tumors derived from embryonic or undifferentiated progenitor cells (reviewed in Stanton and Parada, 1992). This expression pattern suggests that N-myc plays a role in maintaining cells in an undifferentiated and/or proliferative state. The role of N-myc in development has been
analyzed using N-myc null mice (Sawai et al., 1993; Stanton et al., 1992; Charron et al., 1992). Embryos homozygous for the N-myc disruption die at 10-12 days p.c. and show multiple defects, including decreased growth and branching in the lungs. These experiments demonstrate that N-myc has functions that cannot be compensated for by other Myc proteins. Mice expressing lower than normal levels of N-myc protein have also been generated by insertion of a neomycin resistance cassette into the first intron of the N-myc gene. The cassette, intended to disrupt the gene, is occasionally spaced from the mRNA, resulting in low levels of a normal N-myc transcript (Moens et al., 1992, 1993). Mice homozygous for this mutation survive to birth but then die because of lung defects; the lungs are smaller and have less branching than non-transgenic controls. Lungs of homozygous mutant mice express 25% the level of N-myc relative to wild-type embryos whereas expression of N-myc in the kidneys of the same mice, which were not affected by the mutation, was 32% the level in wild-type embryos. These data suggest that the lungs may be particularly sensitive to reduced levels of N-myc.

Lung buds in organ culture undergo branching morphogenesis and can be easily manipulated to study the effect of specific agents on lung development (Jaskoll et al., 1988; Ganser et al., 1991). Organ cultures provide a useful experimental model for studying growth factor action since the response of cells attached to native matrix rather than plastic can be studied. In addition, most cultured cells that are known to express N-myc, such as neuroblastoma and teratocarcinoma cells, do not respond to TGFβ (Massague et al., 1990 and L. Dagnino and H. L. M., unpublished observations). It has been suggested that local autocrine and paracrine factors are signals for lung branching morphogenesis and TGFβ has been localized to the lung during this process (Heine et al., 1990). The objective of this study was to use lung buds in organ culture to test the hypotheses that TGFβ1 can regulate lung branching morphogenesis and N-myc expression. The data presented suggest a model in which TGFβ1 acts to regulate branching morphogenesis by altering N-myc expression.

MATERIALS AND METHODS

Lung bud organ culture

ICR strain (Harlan) female mice were mated to (C57BL/6 x DBA) F1 (Harlan) males. Noon on the day of vaginal plug is 0.5 days p.c. Lung buds were isolated from 11.5 days p.c. embryos and cultured on 13 mm diameter, 8 μm pore size nucleopore membranes (Thomas Scientific) in a chemically defined medium (Ganser et al., 1991). One or two lung buds were cultured on each membrane floating on 1 ml of media in each well of a 24-well plate. The medium consisted of a 1:1 mixture of Ham’s F12 and DMEM with 1 μg BSA/ml, 10 μg transferrin/ml and 50 μg gentamicin/ml. Lung buds were grown at 37°C in a humidified, 5% CO2 incubator. Cultures were allowed to incubate at 37°C for 2 to 4 hours before TGFβ1 in 4 mM HCl or 4 mM HCl alone was added. The medium was not changed for the duration of each experiment unless otherwise noted. TGFβ1 was obtained from R and D Systems (Minneapolis, MN).

Lung buds in culture on the nucleopore membrane were photographed using an Olympus SZH10 dissecting microscope with bright-field illumination. For histological analysis, lung buds were washed in PBS, fixed 2 to 16 hours in 4% paraformaldehyde in PBS, dehydrated and embedded in paraffin wax. Sections (5 μm) were stained with hematoxylin and eosin. The relative levels of stroma and epithelium were determined from photographs of stained lung sections. The number of intercepts from a square grid in each compartment were counted. Per cent stroma and epithelium were calculated relative to the total number of intercepts counted.

In situ hybridization

In situ hybridization of lung bud sections was performed as described (Pelton et al., 1991). Briefly, 5 μm paraffin sections of lung buds that had been fixed in 4% paraformaldehyde were hybridized to [35S]UTP-labeled riboprobes. The 500 bp antisense RNA probe, unique for N-myc, was transcribed from the T7 promoter of BS16 (the kind gift of Dr. L. Parada) (Stanton et al., 1992) after linearization with StyI. The c-myc-specific probe was constructed by insertion of the 400 bp BamHI/SacI fragment derived from the c-myc first exon into a Blue-script vector (Stratagene). Antisense RNA was synthesized from the T7 promoter of the BamHI linearized plasmid. An 800 bp nonsense riboprobe was used as a negative control. Hybridization was detected after 14 days exposure to autoradiographic emulsion. Phase-contrast and dark-field photomicrographs were taken of each section with a Zeiss axiophot microscope. They were scanned into a Macintosh computer using a Mirror Scan PS scanner and Photoshop software. Equal color levels were applied to each picture so that the phase-contrast pictures would appear blue and the dark-field pictures appear red. The two pictures were then superimposed so that autoradiographic grains, in red, could be easily localized. For all in situ hybridizations, the TGFβ1-treated sample and the untreated control were processed together.

Northern blot hybridization

RNA was isolated from cultured lung buds by lysis in guanidine thiocyanate followed by phenol extraction and isopropanol precipitation as previously described (Chomczynski and Sacchi, 1987). Total RNA concentration was determined spectrophotometrically. For northern blot analyses, equal amounts of formamide-denatured RNA were separated by electrophoresis on a 1.4% agarose gel containing formaldehyde. The rRNA concentration in each lane was estimated visually in the ethidium bromide-stained gels to confirm that each lane contained equal amounts of total RNA. RNA was transferred to Nitroplus 2000 membranes (Micron Separations, Inc., Westboro, MA) and the membranes were baked at 80°C for at least 2 hours. Blots were prehybridized for 24 hours at 42°C in 50% formamide, 5x SSC, 5x Denhardt’s, 50 mM Na2HPO4 pH 6.5, 0.2% SDS and 0.5 mg/ml salmon sperm DNA. Probes corresponding to the same regions used for in situ analysis were used on northern blots. The PstI fragment of BS16 was used as a specific N-myc probe (Stanton et al., 1992). A 400 bp SacI/BamHI fragment from the c-myc first exon was used as the c-myc probe. DNA fragments were labeled using random primer extension (kit purchased from Boehringer Mannheim Biochemical) to a specific activity of 1-1010 cts/minute/μg DNA and hybridized to membranes in prehybridization buffer containing 1 mM EDTA. Hybridization was performed at 42°C for 24 hours. Filters were washed twice in 2x SSC at room temperature and twice in 0.1x SSC with 0.1% SDS for 30 minutes at 55°C. The filters were then rinsed in 2x SSC, partially dried and exposed to Kodak XAR-5 film at −70°C. Labeled DNA was stripped off the blots by washing in distilled water at 90°C for 10 minutes. Each blot was hybridized to labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Sabath et al., 1990) to confirm that each lane contained equal amounts of mRNA. Relative levels of mRNA were quantified using a Molecular Dynamic Phosphorimager.

RNase protection

RNase protection assays were performed as described (Melton et al., 1984). The N-myc DNA construct used for in situ hybridization was also used to make 32P-labeled riboprobe for RNase protection analysis. Relative levels of mRNA were quantified with a Molecular
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Dynamic PhosphorImager. Expression levels were normalized to the level of GAPDH expression.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Zhou et al., 1993). Briefly, digoxigenin-labeled riboprobes were hybridized to lung buds that had been fixed in 4% paraformaldehyde, dehydrated in methanol, bleached with hydrogen peroxide and permeabilized with protease K. The same N-myc and c-myc DNA constructs used for in situ hybridization of sectioned tissue were used to make digoxigenin-labeled riboprobes. Hybridization was detected with alkaline phosphatase-conjugated anti-digoxigenin antibodies and NBT and BCIP substrates. Photographs were taken under dark-field illumination. An 800 bp nonsense riboprobe was used as a negative control. For each experiment, the TGFβ1-treated sample, the TGFβ1-released sample and the untreated control were processed together.

RESULTS

Effects of TGFβ1 on lung branching morphogenesis

To test whether TGFβ1 regulates branching morphogenesis during lung development, organ cultures were treated with varying concentrations of TGFβ1 for 72 hours, at which time the morphology of lung buds was observed. TGFβ1 inhibited lung bud development, including branching morphogenesis, in organ culture in a dose-dependent manner. The effects of TGFβ1 were visible with 10, 30 and 100 ng/ml of TGFβ1 (Fig. 1). The higher doses required for an effect in the organ cultures as compared to cell culture is likely due to the TGFβ1 accessing the responsive cells by diffusing through the nucleopore membrane and throughout the tissue. The morphological effects of TGFβ1, including decreased branching and overall lung size relative to untreated controls, were visible by 18 to 24 hours of treatment and persisted up to 96 hours of treatment (Fig. 2A). The effect of TGFβ1 was shown to be reversible after up to 48 hours of treatment (Fig. 2B). Lung buds treated for 24 or 48 hours with TGFβ1 and then removed from the medium and placed in fresh medium without TGFβ1 resumed branching morphogenesis by 24 hours (Fig. 2B). This result indicates that treatment with TGFβ1 causes reversible inhibition of developmental processes and does not cause extensive cell death.

Hematoxylin- and eosin-stained sections of untreated lung buds and lung buds treated with TGFβ1 for 14, 48 and 96 hours were examined to characterize further the effects of TGFβ1 on lung bud morphology. At 14 hours there was little detectable difference in the histology of TGFβ1-treated lung buds relative to the untreated controls (Fig. 3A,B). Columnar epithelium lined the airways, branching was apparent and the stroma appeared similar in sections of untreated and TGFβ1-treated lungs. By 48 hours, however, differences in the histology of the two samples were obvious (Fig. 3C,D). Branching of the columnar epithelium was evident in sections from untreated lungs while there were short unbranched airways in the sections from TGFβ1-treated samples. Although there were striking alterations in the architecture of TGFβ1-treated lungs, morphometric analysis revealed little difference in the relative area of stroma and epithelium in untreated or TGFβ1-treated lung cultures at 48 hours (untreated stroma 63.1±2.8%; treated stroma 73.1±6.2%; untreated epithelium 37.4±8.8%; treated epithelium 26.9±6.2%; epithelia/stroma ratio: untreated 0.59±0.11%, treated 0.38±0.12%). By 96 hours, many branch points were detected in the cuboidal epithelium at the periphery of the untreated lung; columnar epithelium lined the more proximal airways (Fig. 3E). In the TGFβ1-treated cultures, most of the stroma consisted of cartilaginous material and there were some areas

![Fig. 1. Dosage effect of TGFβ1 on lung bud morphology. Lung bud cultures were treated with TGFβ1 (1-100 ng/ml) for 72 hours. Concentration-dependent inhibition of branching morphogenesis is visible in comparison with the untreated control cultures. Triplicate cultures are shown for each condition.](image-url)
of apparent necrosis in the non-cartilaginous stroma; columnar epithelium lined most of the airways and no branching was detected (Fig. 3F).

In situ hybridization of lung buds in organ culture

Reduced expression of N-myc inhibits lung development in vivo (Moens et al., 1992, 1993; Stanton et al., 1992). To examine possible mechanisms of TGFβ1 inhibition of branching morphogenesis in lung bud organ cultures, we used in situ hybridization to test the hypothesis that TGFβ1 regulates N-myc expression. Lung buds were cultured in the presence and absence of TGFβ1 for 14 or 48 hours, and then fixed, sectioned and hybridized to 35S-labeled N-myc or c-myc riboprobes. As previously reported for embryonic lungs in vivo (Mugrauer et al., 1988; Hirning et al., 1991; Stanton et al., 1992), c-myc RNA was localized to the stroma (Fig. 4A-D) and N-myc expression was localized to cells lining the airways of lung buds (Fig. 4E-H). TGFβ1 treatment caused little or no obvious change in the pattern or level of expression of c-myc. The level of N-myc hybridization detected at 14 and 48 hours of treatment (Fig. 4F,H) was diminished relative to untreated controls (Fig. 4E,G).

Analysis of N-myc and c-myc expression

To obtain better quantitation of the change in the level of expression of N-myc and c-myc in response to TGFβ1, RNA isolated from untreated lung buds and lung buds treated with TGFβ1 for 14 and 48 hours was analyzed using RNase protection or northern blot hybridization (Fig. 5A). N-myc expression was inhibited by 3-fold at 14 and 48 hours of treatment (Fig. 5C). We have also observed a 50% reduction in N-myc RNA levels at 10 hours of TGFβ1 treatment. Inhibition of N-myc expression occurred prior to the observed changes in morphology. TGFβ1 inhibited c-myc RNA expression by only 27% and 16% at 14 hours and 48 hours of treatment (Fig. 5B,C).

To strengthen the case for a direct relationship between TGFβ1, N-myc and lung branching, whole-mount in situ hybridization was used to determine if N-myc expression resumed, as did branching, after removal of explants from...
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Explants that were either grown in the absence of TGFβ1 for 48 hours, treated with TGFβ1 for 48 hours, or treated with TGFβ1 for 24 hours and then grown in the absence of TGFβ1 for 24 hours were fixed, permeabilized and hybridized to a digoxigenin-labeled N-myc riboprobe. N-myc expression and branching were barely detectable in TGFβ1-treated explants but N-myc expression and branching were clearly detectable in explants that had been removed from TGFβ1 for 24 hours (Fig. 6).

DISCUSSION

We present the first evidence that TGFβ1 can negatively regulate lung development and suppress expression of N-myc. The observed effects of TGFβ1 on tracheobronchial development could be a direct effect of the protein on epithelial growth and/or differentiation, or an indirect effect mediated by changes in the stroma, including increased accumulation of extracellular matrix (ECM). We have shown that lung buds in organ culture expressed N-myc in the epithelium and c-myc in the stroma and that TGFβ1 inhibited N-myc expression before detectable changes in lung bud histology. These data lead to the hypothesis that TGFβ1 inhibits branching morphogenesis by directly affecting the expression of N-myc in the epithelium.

DNA synthesis is highest in morphogenetically active regions of lung epithelia (buds) and lower in proximal airway epithelium and in branching clefts (Goldin and Opperman, 1980). Moreover, branching morphogenesis, specifically bud formation, cannot proceed without proliferation (Goldin et al., 1984). TGFβ1 is known to inhibit the growth of epithelial cells (Moses et al., 1990) and we have observed a reduction in the per cent of [3H]thymidine-labeled nuclei in TGFβ1-treated lung epithelium relative to untreated controls (Serra and Moses).
Fig. 4. In situ hybridization of lung bud sections. Untreated (A,C,E,G) or TGFβ1-treated (100 ng/ml; B,D,F,H) lung buds were hybridized to 35S-labeled c-myc (A-D) or N-myc (E-H) probes. Lung buds were cultured in the presence or absence of TGFβ1 for 14 hours (A,B,E,F) or 48
Inhibition of lung development observed in this report may therefore be the result of TGFβ1 inhibiting DNA synthesis in the epithelium and thus prohibiting bud growth.

TGFβ1 may regulate growth by modulating the expression of Myc proteins (Moses et al., 1990). TGFβ1 inhibits c-myc expression in many epithelial cell types (Coffey et al., 1988; Mulder et al., 1988; Fernandez-Pol et al., 1987), but has not previously been shown to regulate N-myc expression, which occurs primarily in the developing embryo. A role for N-myc in lung branching morphogenesis was suggested in experiments with mice having targeted disruption of the N-myc gene. Mice deficient in N-myc expression had less developed lungs than the non-transgenic controls (Moens et al., 1992, 1993; Stanton et al., 1992), similar to what we observed for lung organ cultures treated with TGFβ1. In the present study, TGFβ1 treatment resulted in inhibition of N-myc expression in lung epithelial cells before inhibition of lung development was observed and N-myc expression resumed when branching resumed after removal of explants from TGFβ1, suggesting that the effects of TGFβ1 could be the result of N-myc inhibition. The mechanism by which N-myc regulates branching morphogenesis is not known. N-myc has been shown to regulate transcription, cell proliferation and differentiation (Luscher and Eisenman, 1990; Marcu et al., 1992; Stanton and Parada, 1992). Reduced levels of N-myc, as a result of gene disruption or TGFβ1 treatment, may directly inhibit DNA synthesis in lung buds, inhibiting continued branching by prohibiting bud formation.

TGFβ1 may also affect the differentiation of epithelial cells. It has been shown that TGFβ1 will induce squamous differen-

Fig. 5. Effect of TGFβ1 on c-myc and N-myc RNA expression. (A) Total RNA (5 µg) extracted from untreated (−) and TGFβ1-treated (100 ng/ml; +) lung buds at 14 and 48 hours was analyzed by RNase protection with an N-myc riboprobe. Hybridization to a mouse GADPH probe was used to correct for RNA loading. (B) Total RNA (10 µg) extracted from untreated (−) and TGFβ1-treated (100 ng/ml; +) lung buds at 14 and 48 hours was analyzed by northern blot hybridization to a c-myc probe. Hybridization to a mouse GADPH probe was used to correct for RNA loading. (C) N-myc and c-myc RNA levels from northern blots and RNase protection assays were quantified using a Molecular Dynamics phosphorimager. Expression in untreated (shaded) and TGFβ1-treated (open) samples was normalized to expression of GADPH. Data for N-myc from three separate experiments are shown as the mean percent expression relative to the untreated control ± the standard deviation at each time point. Data for c-myc compiled from two separate experiments are shown as the mean percent expression relative to the untreated controls.

Fig. 6. N-myc expression after the removal of TGFβ1. Lung buds grown in the absence (A) or presence (B) of TGFβ1 for 48 hours, or in the presence of TGFβ1 for 24 hours then in the absence of TGFβ1 for 24 hours (C) were fixed, permeabilized and hybridized to digoxigenin-labeled N-myc riboprobe. Areas of hybridization appear purple. A representative of two separate experiments is shown. As a positive control, a lung bud treated with TGFβ1 for 48 hours and hybridized to c-myc riboprobe is shown (D). This demonstrates that probe and antibody can penetrate into the TGFβ1-treated lung bud. Untreated (E) and TGFβ1-treated (F) lung buds hybridized to nonsense digoxigenin-labeled riboprobes are shown as negative controls.
tiation of normal bronchial epithelium in culture (Masui et al., 1986; Jetten et al., 1986) and suppression of N-myc may promote differentiation in specific cell types (Hasegawa et al., 1991). Reduced N-myc expression in response to TGFβ1 could lead to premature differentiation of bronchial epithelium so that cells are no longer able to respond to proliferative or positioning signals required for branching morphogenesis. Alternatively, suppression of N-myc may be a consequence of TGFβ1-induced differentiation. These possibilities seem unlikely since epithelial cells in treated lung buds do not exhibit squamous differentiation morphologically and, unlike terminal differentiation, the effect of TGFβ1 is reversible.

Tissue- and differentiation-specific expression of N-myc has been studied and the regulatory elements controlling N-myc expression in specific cell types in vitro have been localized (Zimmerman et al., 1990; Hiller et al., 1991). Protein complex formation on specific DNA elements was shown to change as endogenous N-myc levels were altered in response to differentiating agents. Also, protein phosphorylation was shown to be necessary for complex formation (Hara et al., 1993; Reichel, 1992). TGFβ1 inhibits c-myc expression in many epithelial cell types (Coffey et al., 1988; Mulder et al., 1988; Fernandez-Pol et al., 1987), but not in fibroblastic cells (Chambard and Pouyssegur, 1988; Sorrentino and Bandyopadhyay, 1989). In the present study, N-myc and c-myc expression were regulated differently by TGFβ1. Suppression of N-myc expression was greater than that for c-myc. The difference in the response of mesenchymal and epithelial cells to TGFβ has been well documented (Tucker et al., 1984; Moses et al., 1985). The 5′ regulatory regions of both c-myc and N-myc contain E2F sites (Nevins, 1992) and are presumably transactivated by E2F. However, overexpression of E2F-1, E2F-2 or E2F-3 (Lee et al., 1993) have different effects on transcription of c-myc and N-myc promoters in P19 cells; N-myc is transactivated while c-myc is not (Dagino, L and Moses, H. L., unpublished data). TGFβ1 may directly regulate N-myc and c-myc expression through different pathways.

While effects of TGFβ1 on N-myc expression in the epithelium likely play a role in the inhibition of lung bud development, modulation of ECM synthesis and deposition by the stroma may also be a significant factor. Fibronectin and type I and III collagens were shown to localize at developing clefts with TGFβ protein (Heine et al., 1990). There is considerable evidence that supports a role in branching morphogenesis for localized anchoring of cells at the cleft points by specific ECM components (reviewed in McGowan, 1992). Laminin domains necessary for branching morphogenesis have been identified using domain-specific anti-laminin antibodies on lung buds in organ culture (Schuger et al., 1991). Lung organ cultures treated with RGD peptide, which inhibits specific integrin and ECM interactions, demonstrated diminished branching (Roman et al., 1991). Unlike treatment with TGFβ1, which appears to inhibit bud growth, cleft formation was diminished suggesting a role for integrin-ECM interaction in formation of the anchoring point which makes up the branching cleft (Roman et al., 1991). However, it has been suggested that remodeling of ECM is required for bud formation during branching (Bernfield and Banerjee, 1982; Ganser et al., 1991). It is possible that TGFβ1 treatment results in accumulation of extracellular matrix and inhibition of bud formation. The effects of TGFβ on ECM accumulation and adhesion have been well documented and TGFβ1 are known to stimulate connective tissue formation (Moses et al., 1990 and Massague et al., 1990). In the present study, the stroma of lung buds treated with TGFβ1 appeared more condensed and cellular than stroma in untreated lung buds. TGFβ1 also promotes the differentiation of chondrocytes under specific conditions (Chen et al., 1991; Frenz et al., 1991). The formation of cartilaginous material around the proximal airways observed in this study in lung buds treated with TGFβ1 for 96 hours may be the result of TGFβ inducing cartilage differentiation of stromal cells. Thus, TGFβ1 had demonstrable stromal effects in the lung buds and could inhibit bud formation, in part, by causing an accumulation of ECM.

Data presented in this report demonstrate that TGFβ1 treatment results in inhibition of N-myc expression and tracheobronchial development of lung buds in organ culture in a dose-dependent and reversible manner. The mechanism by which TGFβ1 inhibits N-myc expression is not clear. TGFβ1 may inhibit N-myc expression directly or indirectly as a result of alterations in extracellular matrix composition or as a consequence of growth inhibition. Proof of a causal connection between suppression of N-myc expression and inhibition of lung bud development by TGFβ1 will require additional experiments. Lung bud organ cultures from mice with targeted disruption of a specific gene such as the retinoblastoma tumor susceptibility gene or overexpression of a specific transgene such as SV40 T-antigen or N-myc could be used to elucidate the mechanisms of TGFβ1 growth inhibition. In addition, treatment of lung buds with TGFβ1 may be used to determine how TGFβ1, normally localized to proximal airways and branching clefts, acts during lung development.

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