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Epithelial-mesenchymal interactions and lung branching morphogenesis. Role of polyamines and transforming growth factor ß$_1$

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SUMMARY

Lung branching morphogenesis is a result of epithelial-mesenchymal interactions, which are in turn dependent on extracellular matrix composition and cytokine regulation. Polyamines have recently been demonstrated as able to modify chick embryo skin differentiation. In this work we have examined the effects of putrescine and spermidine during chick embryo lung morphogenesis in organotypic cultures by morphological, histochemical and biochemical examination. To verify the role of polyamines, we used specific inhibitors, such as bis-cyclohexylammonium sulphate and alfa-difluoromethylornithine, and transforming growth factor ß$_1$, an ornithine decarboxylase and polyamine stimulator. Our data show that lung morphogenesis is significantly altered following the induced mesenchymal glycosaminoglycan changes. The increase of mesenchymal glycosaminoglycans is correlated with a stimulation of lung development in the presence of polyamines, and with its inhibition when transforming growth factor ß$_1$ is added to the culture medium. The morphometric data show a uniform increase of both the mesenchyme and epithelial branching with spermidine and putrescine stimulus, whereas the mesenchymal substance alone is significantly increased in apical-median lung sections with transforming growth factor ß$_1$ and transforming growth factor ß$_1$ + spermidine lung cultures. Transforming growth factor ß$_1$ and transforming growth factor ß$_1$ + spermidine confirm the blocking of epithelial branching formations and fibroblast activation, and show that polyamines are unable to prevent the blocking of epithelial cells due to the inhibitory effect of transforming growth factor ß$_1$.

INTRODUCTION

Epithelial-mesenchymal interactions are required for the development of many organs, including those of the gastrointestinal, tegumental, renal and respiratory systems (Peters *et al*., 1992; Stabellini *et al*., 1998; Wallner *et al*., 1998; Hogan, 1999; Quin *et al*., 1999). They are mediated by soluble and /or membrane-bound mesenchymal factors, which act as signalling molecules (Ohmichi *et al*.,

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1998). The activity of these factors is affected by the specific composition of the mesenchyme, which gives them easy access and masks associated receptors.

Lung morphogenesis entails a complex branching process with a specific spatial-temporal sequence (ecto-para-entobronchi) (Romanoff, 1960) dependent on epithelial-mesenchymal interactions. The composition of mesenchymal of glycosaminoglycans (GAG) varies during the various phases of the branch formation (Becchetti *et al*., 1988). Furthermore, *in vitro* (Carinci *et al*., 1986) and *in vivo* studies have shown that both the branching and differentiation of the lung epithelium are related to mesenchymal changes (Becchetti *et al*., 1988; Shannon, 1994). Experiments on mouse or transgenic mouse lung cultures have shown that factors such as fibroblasts growth factor tipe b, plateled growth factor, and epithelial growth factor play a regulatory role in embryonic lung development (Roberts *et al*., 1990; Souza *et al*., 1995; Bonström *et al*., 1996; Miettinen *et al*., 1997; Ohmichi *et al*., 1998). On the other hand, the addition of exogenous transforming growth factor ß to embryonic mouse lung cultures inhibits branching morphogenesis (Zhao *et al*., 1996, 1998).

Polyamine (PA) stimulate cell proliferation and differentiation. In particular, putrescine (PUT), spermidine (SPD) and spermine (SPM) are necessary for cell growth and differentiation (Pegg, 1986) and play a role in DNA, RNA and protein synthesis (Tabor and Tabor, 1984). PA are involved in the transduction of signals between the cellular membranes, and especially in transforming growth factor $ß_1$ ($TGFß_1$) signal transduction (Tabib and Bachrach, 1994; Blachowski *et al*., 1994; Evangelisti *et al*., 1997). A key enzyme in the PA biosynthetic pathway is ornithine decarboxylase (ODC). An important point is the PA-$TGFß_1$ ratio because it promotes or inhibits cell proliferation according to the cell type and presence of different growth factors (Frazer *et al*., 1994). The decrease in intracellular PA due to long-term treatment with α-difluoromethylornithine (DFMO), is associated with increased expression of the $TGFß_1$ gene in normal small-intestinal epithelial crypt cells, and it has been suggested that this gene can play an important role in the process of growth inhibition following PA depletion (Patel *et al*., 1998). In chick embryo fibroblasts, $TGFß_1$ stimulates proliferation, increasing PA levels and ODC activity (Evangelisti *et al*., 1997) as well as extracellular GAG synthesis (Locci *et al*., 1993). Therefore, a crucial point to be clarified in embryonic lung development regards the effects of different growth factors on extracellular matrix (ECM) modifications during bronchial branching. We have recently shown that exogenous SPD modulates the accumulation of sulphated GAG in chick embryo skin, leading to an increase of feather formation (Stabellini *et al*., 1998). The organotypic culture system offers a suitable model to study the interactions of growth factors, including PA, and ECM, and hence the resulting effects on morphogenesis. The aim of the present work was to examine the morphogenesis of the chick embryo lung in organotypic cultures in the presence of PUT and SPD, together with the spermine synthase inhibitor bis-cyclohexylammonium sulphate (BCHS) (Fuerstein *et al*., 1985), and the an irreversible inhibitor ODC DFMO, as well as $TGFß_1$. Our results show that PA promotes morphogenesis, via a mesenchymal stimulus. However PA are not able of overcoming the inhibitory effect of $TGFß_1$ on epithelial branching.

MATERIALS AND METHODS

Organ cultures

Six-day-old Hubbard chick embryos, staged according to the Hamilton tables (1952) provided by the Selice Incubator Company (Bubano, Imola, Italy), were removed under sterile conditions, placed in Petri dishes and decapitated. Lungs were removed under sterile conditions and placed in culture dishes on membrana testacea at 37°C for 3 days. Semisolid serum-free media were used according to the technique elsewhere described (Carinci *et al*., 1986). Test cultures were administered with semisolid serum-free media incorporated with: a) $2x10^{-7}$M SPD; b) $2x10^{-7}$M PUT; c) 200 µM BCHS; d) 200 µM BCHS + $2x10^{-7}$M SPD; e)5mM DFMO; f) 5 mM DFMO + $2x10^{-7}$M PUT; g) 50 ng/ml $TGFß_1$; h) 50 ng/ml $TGFß_1$ + $2x10^{-7}$M SPD. The inhibitor of spermidine BCHS was added at a concentration of 200 µM. Five cultures for each experimental condition were performed, for a total of four independent experiments.

Morphological and morphometric investigations

Cultures were observed daily, and bronchial branching was evaluated by counting epithelial branch points under a stereomicroscope and photographed at 30X magnification. Cultures were fixed in buffered formalin at 4°C for 3-4 hours, and routine histological procedures were followed. Entire lungs were cut in 5µ sections at intervals of 100 µ (Fig. 1L), stained with hematoxylin and eosin, and observed with an optical microscope. To quantify the structural differences between lung cultures, we performed morphometric analysis on the sections stained with hematoxylin-eosin. We assessed the differences between total area of lung section and total epithelial branching development with a Zeiss Axioplane Microscope connected to a Kontron Electronic Scanner using Vidas Software. We delimited single bronchial branching and determined the single areas. Total development of epithelial branching was evaluated by adding together all determined areas. We performed two slides for samples; the values are expressed as μ^2, and are the mean ± SD.

Histochemical study

The histochemical study was perfomed on homologous sections of the interclavicular air sac, ectobronchi, parabronchi and entobronchi on the basis of previous indications of the spatial GAG distribution and tridimensional reconstruction of epithelial lung branching *in vivo* (Becchetti *et al*., 1988). GAG are tested in mesenchyme between ectobronchi and parabronchi.

For histochemical analysis, in order to distinguish different GAG, such as hyaluronic acid (HA), chondroitin 4-6 sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) we used staining with 1% Alcian blue 8 GX (AB) (Fluka) in 0.025M or 0.3M or 0.65M $MgCl_2$ (Hoechst 1% in 0.2 M phosphate buffer, pH 6, 37°C, 1h). For enzymatic digestion, the sections were incubated with testicular hyaluronidase (Merck, Darmstadt, Germany, 1 mg/ml; 0.1 M phosphate buffer, pH 7, 6 h at 37°C). Control sections were incubated in buffer alone. The GAG were identified by critical electrolyte concentrations at which the polyanions change from binding Alcian to binding Mg^{++} (Scott and Dorling, 1965). Alcian stained the polyanions with increasing selectively as the $MgCl_2$ concentration of the staining solution was raised. At 0.025 M $MgCl_2$, not only GAG but also nucleic acids and sulphated glycoproteins were stained; at 0.3M $MgCl_2$, the only macromolecules which were positively stained were the GAG (CS, DS, KS, HS); at 0.65M $MgCl_2$, the only GAG stained were heparin, HS and KS. The action of specific enzymes on the section, followed by Alcian staining, allowed us to determine the distribution of individual GAG. In particular, digestion with testicular hyaluronidase selectively removed HA and CS, which might no longer be bound to proteoglycans. Since a good relation between colour and GAG concentration has been demonstrated (Carinci *et al*., 1968), we obtained GAG values measuring optical density of 5 random areas by connecting a Zeiss Axioplane Microscope to a Kontron Electronic Scanner using Vidas Software. We examined 2 slides for each sample; the values are expressed as relative optical density (arrangement: black = 0, white = 1) and were the mean ± SD of 5 determinations per slide.

Cell cultures

Lungs were carefully removed from 6-day-old chick embryos, washed in Tyrode's solution and dissociated in 0.25% trypsin (DIFCO, 1:300; room temperature, 25-30 min). Cells were recovered by centrifugation and suspended in 199+10% calf serum (Gibco, Grand Island, NY). Ten ml cell suspension ($1X10^6$ cell/ml) was plated in Falcon flasks and placed in a humidity-saturated atmosphere (5% CO_2, 37°C). After 24 hrs, the medium was exchanged and cultures were washed. The cells were incubated with 199 alone, or medium 199 plus SPD, PUT, BCHS, BCHS+SPD, DFMO, DFMO+PUT, TGFß$_1$ and TGFß$_1$+SPD and incubated for a subsequent 24 hrs. The viability of the cells was measured by their ability to exclude trypan blue (Patterson, 1979).

Newly synthesized GAG and their identification

Pooled media (two cultures for each experiment) were exhaustively dialyzed against distilled water and lyophilised. The GAG were isolated by the method elsewhere described (Stabellini *et al*., '98). The lyophilised material was dissolved in 8 ml 0.5 M acetate buffer (pH 7.5) with 0.5 mM $CaCl_2$, digested with pronase (Calbiochem, San Diego, CA, 2 mg/ml, 24 h at 50°C), then deproteinated with ethanol containing 5% potassium acetate. The precipitate was washed with absolute ethanol, 1:1

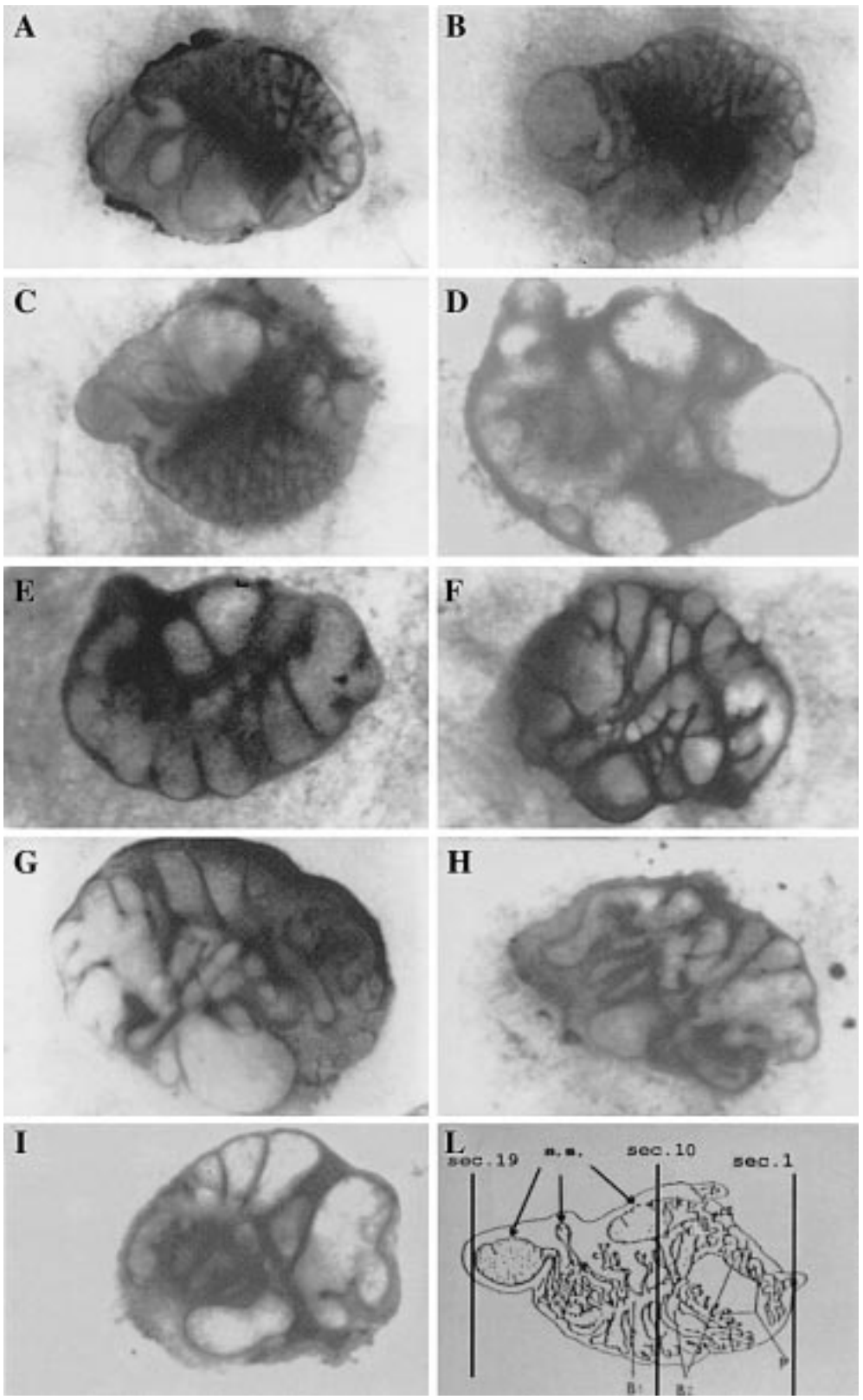

Fig. 1 (A, L) - Epithelial branching morphogenesis in chick embryo lung explant cultures maintained in a defined serum-free medium. Addition of SPD and PUT increases epithelial branching, whereas TGFß$_1$ and polyamine inhibitors (BCHS and DFMO) prevent epithelial lung morphogenesis as well as with TGFß$_1$+SPD. A) 199; B) SPD; C) PUT; D) BCHS; E) DFM; F) BCHS+SPD; G) DFMO+PUT; H) TGFß$_1$; I) TGFß$_1$+SPD. Magnifications 30X. L) 9-day lung drawing shows the position of the 1st, 10th, 19th section levels; a.s.= aerifery sacs; B1= bronchus; B2= secondary bronchus; P= parabronchus; sec= section.

ethanol/ether and ether alone. The ether was removed under *vacuum* at room temperature and GAG dissolved in 0.075 M NaCl. Individual GAG were separated by two-dimensional electrophoresis on cellulose acetate plates and identified by comparing them with the standards and their specific enzymatic susceptibility. Lyophilized samples were digested with bovine hyaluronate (EC 3.2.1.35) (Miles Italiana, Cavenago Brianza, Milano, Italy) and Streptomicetes hyaluronate lyase (EC 4.2.2.1) (Streptomycetes hyaluroniticus, Seikagaku Kogyo, Tokyo, Japan) at 37°C for 24 hrs with 30 and 5 enzyme units, respectively; or with chondroitin AC-II lyase (EC 4.2.2.5) (Arthrobacter aurescens, Seikagaku, Kogyo, Tokyo, Japan) for 24 hrs with 0.5 and 0.01 enzyme units (Conrad *et al*., 1977). They were quantified using a microcolorimetric method (Bartold and Page, 1985) and expressed as nmol/mg prot.

Ornithine decarboxylase activity and polyamine assay

At the end of incubation, the organ cultures were removed and lungs were detached from the membrana testacea, washed with PBS, and centrifuged at 50 g for 10 min at 4°C. The pellets were extracted with 100 µl of 50 mM TRIS-HCl, at pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.05 mM pyridoxal-5'-phosphate and 0.5% Triton X-100 (lysis buffer). Lung lysates were centrifuged at 7,000 g for 15 min at 4°C, and the resulting supernatants were used immediately for ODC assay (Stefanelli *et al*., 1992) and the determination of proteins by Bradford's method (1976). ODC activity was carried out in a 50 µl total volume, 40 µl of which were of lung extract origin. The assay mixture (final concentration) contained 50 mM Tris-HCl, at pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.05 mM pyridoxal-5'-phosphate, 0.5% Triton X-100, 0.4 mM L-ornithine and 0.1 µCi [^{14}C]-ornithine (sp. act. 58 mCi mmol^{-1}, NEN). The assays were performed in glass tubes with filter paper attached to the inside of the cap. The filter paper was moistened with 20 µl of 1 M methylbenzethonium hydroxide in methanol to trap released $^{14}CO_2$. The reaction was carried out for 1 h in a 37°C shaker bath, then stopped by injecting 200 µl of 1 M citric acid through the rubber cap. After a further 30 min, the incubation filters were cut out and the radioactivity was counted in a Kontron scintillator counter. The results were expressed as nmoles of CO_2 released h^{-1}/mg^{-1} protein.

For PA determination the lungs were washed three times with PBS and homogenized in 4 vol of 0.3 M perchloric acid. PA were extracted and derived with dansyl chloride (DNS) and a high performance liquid chromatography (HPLC) method as described by Stefanelli *et al*. (1986). The HPLC equipement was composed of two 420 pumps, a m 491 mixer, SFM 25 fluorescence detector, a Data system 450 (Kontron Instruments, Milano, Italy) and 7000 injector (Rheodyne, Inc., Cotati, CA, USA). Separations were achieved with a Spherisorb S 50DS2 5-µm column (250x4.6 mm I.D.) (Kontron). The DNS derivatives of PA were separated with Spherisorb, a solvent composed of water-acetonitrile-methanol (5:3:2) as solvent A and acetonitrile-methanol (3:2) as solvent B. The sample was eluted with a linear gradient from 28% to 90% solvent B in 15 min at a flow rate of 1.0 ml/min. The values were expressed as nmol/mg protein.

Statistical analysis

The statistical analysis was made using the student t-test for paired and unpaired data.

RESULTS

Organ cultures

Morphological data. A single 6-day lung rudiment is formed by mesobronchus covered with pseudostratified epithelium from which 4 entobronchi and 1 ectobronchus emerge (Romanoff, 1960). All secondary bronchi are lined by a simple columnar epithelium.

After 3 days of culture in 199, more growth and more epithelial tubules were detectable. Lung explants grew well; epithelial tubules extended outwardly. Four entobronchi, 3-4 parabronchi emerged from entobronchi covered with a simple isoprismatic epithelium, 3-4 ectobronchi, 4 laterobronchi, 2-3 dorsobronchi and 4-5 air sacs lined by a simple squamous epithelium were present. Altogether, 22.63±2.30 ramifications were counted under the stereomicroscope (Table I) (Fig. 1A and 2A). The lungs, maintained in the presence of SPD or PUT, showed good growth and exhibited increases in bronchial ramification ($p<0.01$) of

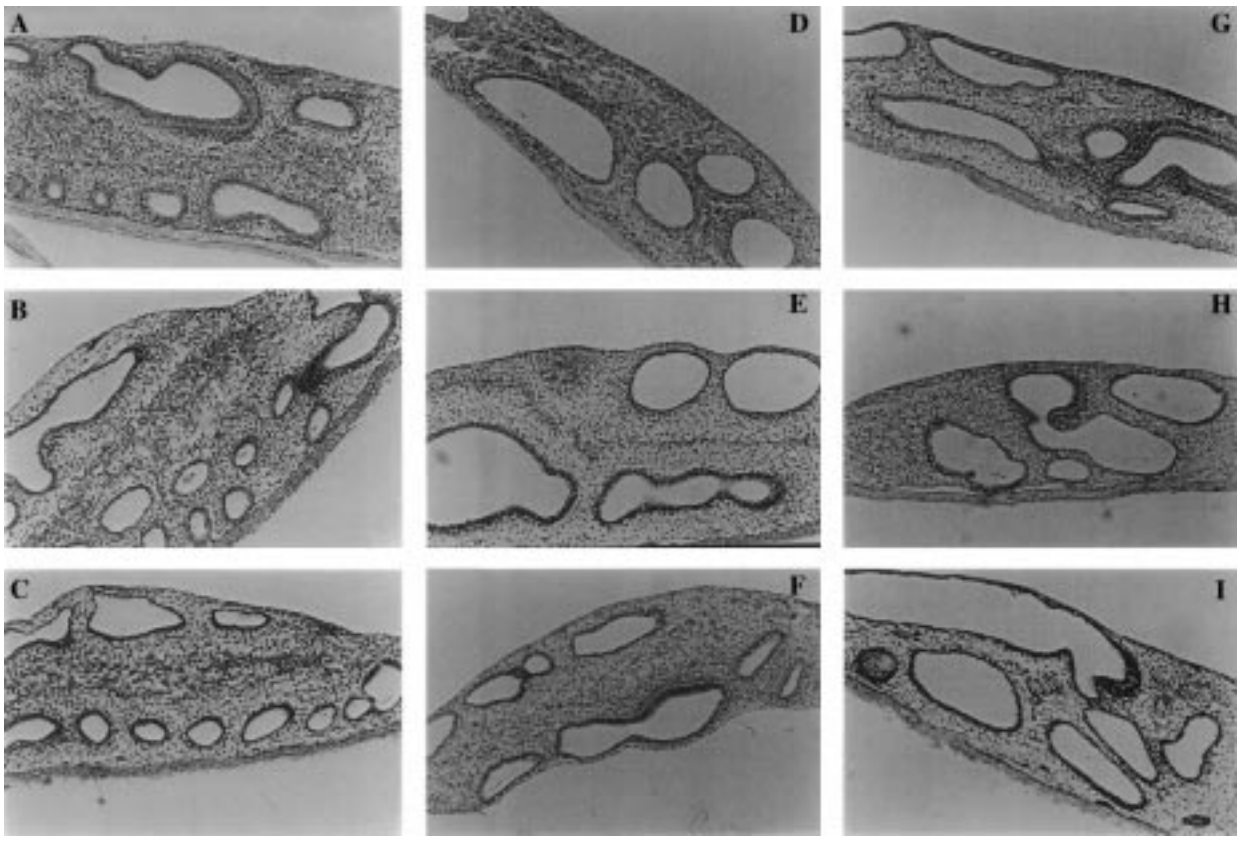

Fig. 2 (A,I) - Epithelial branching morphogenesis in chick embryo lung explant cultures maintained in a defined serum-free medium. Increased numbers of normal ramifications can be observed with SPD and PUT; a decreased number of dilated ramifications can be seen with TGFß$_1$ and polyamine inhibitors as well as with TGFß$_1$+SPD. A) 199; B) SPD; C) PUT; D) BCHS; E) DFMO; F) BCHS+SPD; G) DFMO+PUT; H) TGFß$_1$; I) TGFß$_1$+SPD. Ematoxylin and Eosin Magnifications 10X.

28.53±2.54 and 30.18±3.0 respectively (Table I) (Figs. 1B-C and 2B-C). Explants showed decreased growth ($p<0.01$) when BCHS (12.70±2.00) or DFMO (13.87±1.98) were added, as compared to controls. The ramifications were poorly developed and presented enormously dilated air sacs, covered with isoprismatic epithelium (Table I) (Figs. 1D-E and 2D-E). When BCHS was added to SPD or DFMO to PUT, lung cultures showed 19.20±2.13 and 20.10±2.48 more dilated epithelial branchings than those of controls (Table I) (Figs. 1F-G and 2F-G). Epithelial branching of organotypic lung cultures maintained in the presence of TGFß$_1$ (13.42±1.67) or TGFß$_1$+SPD (15.3±2.20), was decreased ($p<0.01$) (Table I) (Figs. 1H-I and 2H-I). At morphometric analysis (Table II) the lung cultures, treated with PUT or SPD showed a larger number of sections than controls (21 vs. 19), whereas in the presence of BCHS, DFMO or TGFß$_1$, all the lungs presented a similar decrease (14) in the number of obtained sections. When BCHS was added to SPD, or DFMO to PUT, the number of sections obtained rose again (17 vs. 14), but did not reach that of the controls (19). When TGFß$_1$ was added to SPD, the number of sections decreased (15) as compared to controls and was similar to those obtained in TGFß$_1$, BCHS and DFMO cultures (14).

Mesenchymal mass, which was obtained by subtracting the total developmental area of bronchial branching (calculated section by section) from the total section area, did not show significant differences in PUT or SPD cultures with respect to controls. Section 3, obtained from the apical region, showed an increase of mesenchymal mass ($p<0.01$) with TGFß$_1$ or TGFß$_1$+SPD. Section 4 showed a decrease ($p<0.01$) with SPD and an increase ($p<0.01$) with BCHS, BCHS+SPD,

Table I

Number of chicken embryo bronchis developed after 3-day cultures with SPD, PUT, BCHS, DFMO (polyamine inhibitor) TGFß$_1$ and TGFß$_1$+SPD: developed bronchi were counted under a stereomicroscope (means ± SD). SPD = Spermidine; PUT = Putrescine; BCHS = bis-cyclohexylammonium sulfate; DFMO = α-difluoromethylornithine; TGFß$_1$ = transforming growth factor ß$_1$

Treatment	Culture number	Bronchi/culture
Control	20	22.63±2.30
SPD	20	28.53±2.54*°
PUT	20	30.18±3.0*°
BCHS	20	12.70±2.00*
DFMO	20	13.87±1.98*
BCHS+SPD	20	19.20±2.13
DFMO+PUT	20	20.10±2.48
TGFß$_1$	20	13.42±1.67*
TGFß$_1$+SPD	20	15.35±2.2*

*P<0.01 as compared to controls, °P<0.01 as compared to TGFß$_1$.

DFMO, DFMO+PUT, TGFß$_1$ or TGFß$_1$+SPD. Levels 5 and 6, obtained from the apical region, showed an increase of mesenchymal mass ($p<0.01$) in the presence of BCHS, BCHS+SPD, DFMO, DFMO+PUT, TGFß$_1$ or TGFß$_1$+SPD. In sections 7 and 8, the mesenchymal mass significantly increased in the presence of BCHS, DFMO, TGFß$_1$ and TGFß$_1$+SPD. In sections 9 and 10, only TGFß$_1$ or TGFß$_1$+SPD produced an increase in mesenchymal mass ($p<0.01$). In medial sections (11 and 12), the mesenchymal mass decreased ($p<0.01$) in the presence of DFMO, TGFß$_1$, BCHS+SPD, DFMO+PUT, whereas in the presence of BCHS, it increased (section 11) or decreased (section 12). In sections 13 and 14, it decreased ($p<0.01$) in the presence of BCHS, DFMO, TGFß$_1$, BCHS+SPD or DFMO+PUT. Section 15 showed a significant decrease in the presence of BCHS+SPD, DFMO+PUT or TGFß$_1$+SPD. In the caudal region (sections 16 and 17), the mesenchymal mass showed a significant decrease with SPD, PUT, BCHS+SPD and DFMO+PUT. In section 18, only SPD showed the same trend ($p<0.01$) as above.

Histochemical data

Six-day lung rudiments showed loose mesenchyme, and the ground substance exhibited greater alcianophilia in the entobronchi and ectobronchi staining at 0.025, 0.3, 0.65 M $MgCl_2$, partially removable by testicular hyluronidase treatment (HS, CS, DS, HA).

After 3 days of culture, the mesenchymal ground substance exibited alcianophilic histochemical staining as above.

In 199 alone, the alcianophilia at 0.3 M $MgCl_2$ was largely abolished by hyaluronidase (CS). It showed different intensities in relation to the various sites: maximum in the outer region of the growth, clear around the ento- and ecto-, poor in the latero- and para-bronchi, and scarce around the air sacs. In SPD- and PUT- treated lungs, the ground substance showed alcianophilia, which was hyaluronidase-sensitive at 0.3M $MgCl_2$, stronger (higher CS content) than controls. The basement membrane of lung cultures added with SPD or PUT showed clear alcianophilia in 0.025, 0.3 and 0.65 M $MgCl_2$, which was partially removable by hyaluronidase. In BCHS- and DFMO-treated lungs, at 0.3 M $MgCl_2$, alcianophilia was strongly reduced (less CS content), and basement membranes showed poor reactivity, which was partially abolished by hyaluronidase. In BCHS+SPD and DFMO+PUT cultures, alcianophilia increased at 0.3 M (CS increase). In TGFß$_1$ or TGFß$_1$+SPD cultures, the mesenchyme showed poor cellular condensation and a rather fine intercellular network, particularly evident between the parabronchi and at the periph-

Table II

In vitro chick embryo lung development with polyamines, polyamine inhibitor and cytokine. Values are expressed as $1X10^{-4}\ \mu^2$ and represent the difference between the total area section and total area bronchial branching. CONTR = Control. SEC = Section

SEC	CONTR	SPD	PUT	BCHS	BCHS+SPD	DFMO	DFMO+PUT	TGFß$_1$	TGFß$_1$+SPD
1	-	-	-	-	-	-	-	-	-
2	17.3±1.9	18.1±2.2	19.1±2.1	20.9±2.5	16.2±1.9	14.7±1.6	15.9±1.7	14.6±2.0	13.6±3.1
3	16.7±2.1	15.1±1.5	16.5±2.4	30.9±4.1	23.5±3.6	25.6±2.8	20.3±3.1	*26.3±3.8**	*24.5±2.9**
4	19.6±3.0	*12.7±1.7**	16.6±2.3	38.9±5.1	29.9±4.0	34.1±4.1	30.5±3.3	*31.5±4.0**	*28.8±3.0**
5	23.8±2.9	25.0±3.0	26.0±4.2	*53.2±6.3**	*39.9±5.4**	*39.1±3.9**	*37.9±5.5**	*34.0±3.9**	*32.1±4.2**
6	24.8±3.9	21.9±2.9	23.2±3.0	*56.5±7.7**	*42.6±6.3**	*41.0±5.6**	*35.1±4.6**	*35.1±4.7**	*36.3±5.3**
7	32.6±4.4	*26.8±3.9**	27.9±3.3	*49.5±5.4**	38.2±4.6	40.2±4.5	35.4±4.8	*42.2±5.5**	*40.7±3.9**
8	29.9±3.9	29.9±4.2	28.8±3.6	*46.4±5.8**	34.0±5.4	*38.9±4.9**	30.3±3.7	*48.5±6.2**	*45.5±4.4**
9	40.1±5.3	34.5±4.7	35.5±4.3	42.4±5.1	30.2±4.6	37.6±4.9	33.3±3.9	*51.6±6.9**	*49.3±4.1***
10	34.9±5.2	27.2±3.3	30.3±4.2	36.4±4.9	26.8±3.5	35.5±4.3	28.7±4.0	*47.7±5.7**	*48.0±5.0**
11	42.8±5.6	39.1±4.6	38.1±5.3	44.0±5.7	*25.4±3.6**	34.1±4.9	*26.4±3.9**	*34.1±4.0***	36.2±5.5
12	43.4±5.5	34.0±3.9	33.5±5.0	*32.4±4.2**	*32.6±4.4**	*32.7±4.6**	*27.2±3.5**	35.6±4.9	36.1±4.7
13	41.4±5.3	39.1±4.6	38.8±4.5	*23.7±3.1**	*28.8±4.1**	*26.3±3.4**	*25.3±3.5**	*31.0±5.3**	33.5±3.5
14	36.2±5.0	30.7±4.0	33.4±5.1	*24.8±5.5**	*27.8±3.6**	*18.9±2.8**	*23.8±3.5**	*23.0±4.2**	28.8±5.7
15	33.7±4.1	29.4±3.3	28.6±3.4	-	*23.1±3.5**	-	*23.7±3.5***	-	21.4±4.9
16	34.5±4.4	27.1±4.2	27.7±4.1	-	*20.6±3.7**	-	*18.9±2.8**	-	-
17	32.7±4.9	18.5±2.6*	20.4±2.8*	-	17.6±2.9*	-	14.3±2.5*	-	-
18	21.7±3.2	15.6±2.2*	18.5±2.7	-	-	-	-	-	-
19	10.4±1.7	10.6±2.0	13.1±2.4	-	-	-	-	-	-
20	-	8.2±2.4	10.4±1.9	-	-	-	-	-	-
21	-	5.6±0.9	7.7±1.1	-	-	-	-	-	-

*P<0.01, **P<0.05 as compared to controls.

ery. At 0.3 M $MgCl_2$, the positivity increased (high CS content) in TGFß$_1$-and TGFß$_1$+SPD-treated lungs, as compared to controls. At this molarity, the basement membranes showed strong reactivity which was partially abolished by hyaluronidase. At 0.65 M $MgCl_2$, the alcianophilia significantly decreased in BCHS- and DFMO-treated lung cultures.

The histochemical reactivity of the mesenchymal ground substance around the ectobronchi (where laterobronchi emerge) exhibited quantitative differences according to the experimental treatments (Table III). The staining pattern, produced by treatment with Alcian blue in 0.025 M $MgCl_2$, revealed strong alcianophilia, which drastically decreased after testicular hyaluronidase digestion. However, the reduction observed in the controls was significantly greater ($p<0.01$) than the decrease in lung cultures administered with SPD, PUT, BCHS+SPD, DFMO+PUT, TGFß$_1$ or TGFß$_1$+SPD. The reactivity to the enzyme proved that the decrease was due to the digestion of hyaluronic acid. The pattern of residual staining denotes that sulphated GAG were abundant. This finding was confirmed by reactivity with Alcian blue at 0.3 M $MgCl_2$ (Table III). Since chondroitin sulfates and hyaluronic acid are sensitive to the enzyme, it may be deduced that the higher amounts of sulphated GAG, observed in SPD, PUT, TGFß$_1$ and TGFß$_1$+SPD-treated cultures, are partly due to the CS themselves, and partly due to DS, KS and HS. At 0.65 M $MgCl_2$, the alcianophilia significantly decreased in BCHS-and DFMO-treated lung cultures.

Table III

Histochemical GAG evaluation in mesenchyme of 6-day chick embryo lung. Development after 3-day culture. After Jal = after hyaluronidase treatment. SPD = Spermidine; PUT = Putrescine; BCHS = bis-cyclohexylammonium sulfate; DFMO = α-difluoromethylornithine; TGFß$_1$ = transforming growth factor ß$_1$

	ALCIAN BLUE IN $MgCl_2$				
	0.025 M		0.3 M		0.65 M
	Before	*After jal*	*Before*	*After jal*	
CONTROL	182.0±12.7	0	110.6±7.7	18.9±1.3	59.8±4.2
SPD	231.6±16.2 *	67.9±4.1 *	130.1±10.8 *	37.9±1.5 *	61.8±4.3
PUT	229.4±20.5 *	65.8±6.0 *	120.3±9.2	39.4±2.9 *	55.2±5.1
BCHS	186.7±13.1	0	109.9±7.1	0	25.6±1.8 *
BCHS+SPD	181.7±13.0	20.5±1.2 *	128.5±11.6 **	28.8±1.5 *	65.1±4.6
DFMO	178.3±14.1	0	102.4±10.7	0	30.7±5.1 *
DFMO+PUT	181.6±14.8	22.6±2.4	104.3±11.0	20.9±2.7	58.6±3.9
TGFß$_1$	224.6±15.7 *	62.3±1.6 *	133.2±12.8 *	39.3±1.6 *	64.3±4.5
TGFß$_1$+SPD	230.5±17.4 *	64.1±3.1 *	140.2±13.6 *	40.7±2.8 *	63.2±4.0

*P<0.01, **P<0.05 as compared to controls.

Ornithindecarboxylase activity and polyamine values

In Table IV, ODC and PA values of 6-day chick embryo lungs are reported after 3-day cultures. ODC increased (p<0.01) in TGFß$_1$- and BCHS-treated cultures, whereas it decreased in DFMO-treated ones. PUT significantly increased in TGFß$_1$-and BCHS-treated cultures, and decreased in DFMO- treated ones. SPD increased (p<0.01) in TGFß$_1$- treated cultures and decreased (p<0.01) in BCHS- or DFMO-treated ones. The SPM variation was not significant.

Table IV

Ornithinedecarboxylase activity and polyamine levels in organotypic cultures of chick embryo lung after 3 days in vitro growth. The data are means ± SD; ODC is expressed as nmolCO_2/mg proteins x hour and PA as nmol/mg prot. TGFß$_1$ = transforming growth factor ß1, BCHS = bis-cyclohexylammonium sulfate; DFMO = α-difluoromethylornithine

	ODC	PUT	SPD	SPM
CONTROL	4.87±0.58	2.65±0.29	5.02±0.49	9.67±1.2
TGFß$_1$	6.01±0.78*	5.15±0.57*	10.55±1.16*	8.93±0.98
BCHS	10.34±1.13*	33.49±4.21*	1.93±0.21*	10.92±1.42
DFMO	1.26±0.18*	0.0	1.58±0.19*	8.55±1.11

*P<0.01 as compared to controls.

Table V

Extracellular GAG values of chick embryo lung fibroblasts. The values are the means ± SD. T.GAG = Total Glycosaminoglycans; HA = hyaluronic acid; HS = heparan sulfate; CS+DS = condrotin sulfate + dermatansulfate; S.GAG = Sulphated Glycosaminoglycans; SPD = Spermidine; PUT = Putrescine; BCHS = bis-cyclohexylammonium sulfate; DFMO = α-difluoromethylornithine; TGFß$_1$ = transforming growth factor ß$_1$

	EXTRACELLULAR GAG				
	T.GAG	HA	HS	CS+DS	HA/S.GAG
CONTROL	7.35±0.7	1.50±0.2 (20.4)	1.37±0.5 (18.6)	4.48±0.4 (60.9)	0.26±0.03
SPD	11.25±0.8*	1.47±0.3 (13.1)	1.50±0.3 (13.3)	8.28±0.4* (73.6)	0.15±0.02*
PUT	12.00±1.0*	1.68±0.5 (14.0)	1.74±0.4 (14.5)	8.58±0.6* (71.5)	0.16±0.3*
BCHS	10.08±0.8*	2.24±0.4* (22.2)	2.19±0.3* (21.7)	5.65±0.5 (56.0)	0.29±0.04
BCHS+SPD	9.17±1.0*	1.92±0.3* (21.0)	1.83±0.4 (20.0)	5.41±0.4 (59.0)	0.26±0.04
DFMO	5.56±0.9*	1.12±0.2* (20.1)	1.11±0.2 (20.0)	3.33±0.5* (59.9)	0.25±0.04
DFMO+PUT	8.00±1.6	1.67±0.4* (20.9)	1.72±0.5 (21.5)	4.61±0.3 (57.6)	0.26±0.2
TGFß$_1$	12.49±0.9*	2.37±0.4* (19.0)	2.08±0.4* (16.7)	8.03±0.5* (64.3)	0.23±0.03
TGFß$_1$+SPD	11.1±0.7*	2.23±0.5* (20.1)	2.15±0.3* (19.4)	6.71±0.6* (60.5)	0.25±0.04

*P<0.01 as compared to controls.

Cell cultures

Newly synthetised GAG. The amount of total extracellular GAG (Table V) increased ($p<0.01$) in 6-day lung fibroblast cultures in all experimental conditions except under DFMO-treatment. Regarding the single classes of GAG, HA increased ($p<0.01$) with BCHS, BCHS+SPD, DFMO+PUT, TGFß$_1$ and TGFß$_1$+SPD lung cultures, whereas it decreased with DFMO. CS+DS increased ($p<0.01$) in SPD-, PUT-, TGFß$_1$-, TGFß$_1$+SPD-treated cultures and decreased with DFMO. HS increased ($p<0.01$) only in BCHS-, TGFß$_1$- or TGFß$_1$+SPD-treated ones. The HA/sulphated GAG ratio was significantly lowered in SPD-and PUT-treated cultures, while the changes obtained with BCHS, DFMO, TGFß$_1$ and TGFß$_1$+SPD were not significant.

DISCUSSION

We have previously established a clear relationship between accumulated mesenchymal GAG and epithelial morphogenesis in the course of lung development *in vivo* (Becchetti *et al.*, 1988). The present study reveals the presence of similar epithelial branching features *in vitro.* In particular**,** the mesenchymal ground substance exhibits analogous GAG pattern heterogeneity.

Our results indicate that PA treatment stimulates lung explant growth as seen in Table I and GAG accumulation as seen Table III. Inhibitor administration corroborated these observations : indeed, both inhibition of SPD synthesis by BCHS and PUT production by DFMO arrested lung growth. The arrested lung growth was in turn almost reversible by the simultaneous administration of PA (Table I).

After a 3-day culture, lung histochemistry showed alcianophilia at 0.3M $MgCl_2$. Testicular hyaluronidase digestion (an index of sulphated GAG) was observable around the secondary bronchi and parabronchi, but was strongest around the parabronchi growing in the laterodorsal region

in the presence of SPD and PUT. By contrast, it decreased in the presence of BCHS and DFMO. These observations underline the fundamental role of SPD and PUT on organ differentiation. Treatment with TGFß$_1$ or TGFß$_1$+SPD (Table III) increased the production of sulphated GAG (CS, DS and HS) in fibroblasts and lung cultures. TGFß$_1$ also determined an increase in PA levels following ODC activation (Table IV), which however was accompanied by decreased epithelial branching. Taken together, our histochemical and biochemical data show an increase of sulphated GAG in both PA- and TGFß$_1$-treated lungs. Neverthles, these two growth factors exerted a different effect on lung chick embryo development. Indeed, whereas PA stimulate epithelial banching (Table II), TGFß$_1$ inhibits branching, and stimulated mesenchymal cell function. We have previously demonstrated that epithelial branching stops when sulphated GAG are removed from organotypic lung cultures, and starts again when GAG accumulation takes place (Carinci *et al*., 1986). The present *in vitro* study shows that a significant decrease in the HA/sulphated GAG ratio is associated with increased lung differentiation. This finding is in agreement with Caniggia and Post (1992), who demonstrated that in rat lungs fibroblasts produce HA in proximity to the epithelium, and HS or CS in more distant regions, and that the shift to HA occurs with the thinning of the alveolar septum.

PA are known stimulate GAG synthesis and cell proliferation in both fibroblasts and epithelial cells whereas TGFß$_1$ induces PA and GAG synthesis in fibroblasts and growth arrest in epithelial cells in G1 phase (Moses *et al*., 1985). Our morphometric data show increases of both mesenchymal and epithelial branching in the presence of PA stimulation, whereas TGFß$_1$ and TGFß$_1$+SPD stimulate mesenchymal growth but inhibit branching. Zhao *et al*. (1998) have demonstrated that the abolition of the anti-proliferative effect by TGFß$_1$ inactivation in culture media restores epithelial branching morphogenesis, probably due to the inductive effects of other endogenous peptide growth factors such as EGF and PDGF-AA. Furthermore, Lyon *et al*. (1997) have shown that the biological activity of TGFß$_1$ is potentiated by interaction with heparin sulfate: this implies that an increase in TGFß$_1$-induced HS should contribute to block epithelial cell proliferation in the G1 phase.

In conclusion, the present study provides an *in vitro* demonstration that TGFß$_1$ stimulates mesenchymal cells and modifies ECM, but these changes cannot prevent the blocking of epithelial cells in the G1 phase of the cell cycle, due to the inhibitory effect of TGFß$_1$. Moreover, our data provide important confirmation that the ECM is important for lung development, and suggest that the coordination of TGFß$_1$ and other growth signals are essential for differentiation.

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