Lung regions differently modulate bronchial branching development and extracellular matrix plays a role in regulating the development of chick embryo whole lung

G. Stabellini,[1] M. Calvitti,[2] E. Becchetti,[2] P. Carinci,[3] C. Calastrini,[4] C. Lilli,[2] R. Solmi,[3] L. Vizzotto,[1] T. Baroni[2]

[1]Human Morphology Department, University of Milano; [2]Experimental Medicine and Biochemistry Science Department, University of Perugia; [3]Department of Histology, Embryology and Applied Biology, Centre of Molecular Genetics, University of Bologna; [4]Morphology and Embryology Department, Section of Histology and Embryology, University of Ferrara, Italy

Normal branching development is dependent on the correlation between cells and extracellular matrix. In this interaction glycosaminoglycans, cytokines and growth factors play a fundamental role. In order to verify the distribution and influence of extracellular matrix and related enzymes on chick embryo lung development, 6 day-old whole lungs were maintained *in vitro* with testicular hyaluronidase, β-N-acetyl-D-glucosaminidase and chondrotinase ABC or in linkage with apical, medial and caudal lung regions of 6-day development before and after enzyme treatment. In a separate lung region β-N-acetyl-D-glucosaminidase and hyaluronidase were determined. Our data show that the whole lung cultures increase bronchial branching development when the medial region is admixed separately, while the separate apical or caudal regions or apical combined with caudal region do not affect bronchial branching development. The enzyme treatment of medial region prevents the branching development in associated whole lung. The bronchial branching development of whole lung cultured in medium containing the enzymes related to glycosaminoglycans turnover is significantly altered. In conclusion, these data show that the different influence of separate apical, medial, caudal lung regions on bronchial branching development is related to the extracellular matrix composition.

Key words: bronchial branching, extracellular matrix, glycosidases, glycosaminoglycan, lung.

Correspondence: Giordano Stabellini,
Dipartimento di Morfologia Umana,
Via Mangiagalli 31 20133 Milan, Italy
Tel: +39.0250315393.
Fax:+39.0250315387.
E-mail: giordano.stabellini@unimi.it

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Branching plays a fundamental role in the morphogenesis of several organs such as the salivary system, kidney and lung (Hieda and Nakanishi, 1997; Qiao *et al.*, 1999). Proliferation and cell migration are involved in these processes and are regulated by the extracellular matrix (ECM) composition. Again, the regulation of cell compartments and ECM composition is due to specific growth factors (Bush *et al.*, 2004). Various experimental data show that the lung rudiments are dependent for differentiation on mutual interaction between epithelial cells and the mesenchyme (Grobstein, 1953; McGowan, 1992). In such regulative action a critical factor is represented by ECM composition. Bronchial branching and cytodifferentiation are regulated by ECM components, such as protein, proteoglycans, sulphated- and non sulphated-glycosaminoglycans (GAG).

Particularly the synthesis of hyaluronic acid (HA), chondroitin sulphated-proteoglycans and components of basal membrane such as laminin and fibronectin (Sannes *et al.*, 1993) are modified by cytokines (Locci *et al.*, 1993; Bodo *et al.*, 1998). In turn HA and chondroitinsulphate (CS) modulate the availability and the accessibility of cytokines with respect to cell activity (Locci *et al.*, 1993). ECM remodelling is the necessary condition so that both cell adhesion and morphogenetic movements may carry on during organ development (Perris and Perissinotto, 2000; Perissinotto *et al.*, 2000). The variation in HA distribution plays a basic role in the control of directed myogenetic migration (Krenn and Brand-Saberi, 1991), while CS interferes with migration (Landolt *et al.*, 1995; Canning *et al.*, 2000).

Previous research has shown there is a different composition and distribution of CS, dermatan sulphate (DS), heparan sulphate (HS), HA along the cranio-caudal axis (apical, medial and caudal regions) in the lung mesenchyme of the chick embryo at 6-7 days of incubation (Becchetti *et al.*, 1988). Moreover HS concentrations alter bronchial branching formation, gene expression (Izvolsky *et al.*, 2003) and basal membrane formation (Calvitti *et al.*, 2004). These extracellular GAG changes are correlated to a different concentration of specific endo-and exoglycasidases acting on GAG turnover, as seen along the apical/caudal axis. Exoglycosidases such as β-N-acetyl-D-glucosaminidase (β-NAG) act on HA and KS, chondroitinase ABC (Chase) acts on CS and DS and endoglycosidases such as hyaluronidase (HAase) act on HA and CS. Organotypical cultures are an interesting experimental model to clarify the morphogenesis of the lung (Izvolsky *et al.*, 2003; Maina, 2004).

Previously, through such experimental models, the influence of mass has been demonstrated (Alescio and Colombo Piperno, 1967; Masters, 1976) but not the level of regulation. On the whole, these data show that the mesenchyme modifies its GAG composition during development and suggest that these modifications are induced by control factors, the correlations of which are important for our understanding of how the mechanisms which regulate branching work (Maina *et al.*, 2003; Calvitti *et al.*, 2004). In order to contribute to explaining lung morphogenesis, it is very interesting to verify how the changes depending on mesenchymal mass were or were not correlated to GAG composition in the various different lung areas. For this purpose we performed cultures of 6 day-old chick embryo whole lungs in a medium containing HAase which removes HA and CS or β-NAG which acts on HA and KS or chondroitinase ABC (Chase) which digests CS and DS but not HA. We also cultured whole lungs together with apical or medial or caudal regions or whole lungs in addition with medial region previously treated with HAase. In the different lung regions we determined the HAase and β-NAG levels at 6, 11 and 14 days lung development.

Our results enable us better to define the possible regulatory role of the GAG in different lung regions during bronchial branching development.

Materials and Methods

Organ cultures

Six-day-old Hubbard chick embryos, staged according to the Hamilton tables (Hamilton, 1965) provided by the Selice Incubator Company (Bubano, Imola, Italy) were removed under sterile conditions, placed in Petri dishes, decapitated and lungs were removed. 30 whole lungs (10 per culture set) were maintained in solid serum-free medium containing 280 μg/mL testicular hyaluronidase (Miles) or 100 U/mL (final concentration) β-NAG (SIGMA, Milan, Italy) or 0.32 U/mL (final concentration) chondroitinase ABC (Seikagaku Kogyo, Tokyo); 10 were maintained without any enzyme as controls. The other 30 whole lungs were cross-sectioned in the apical, medial and caudal regions according to previous studies on the spatial distribution of GAG in chick embryo lung (Becchetti *et al.*, 1988). The apical, medial or caudal lung regions were added to whole lung (10 cultures each set) and cultured in serum free medium alone; and 10 whole lung as controls. Another 10 whole lungs were added to the medial lung region previously cultured in medium containing 280 μg/mL testicular hyaluronidase for 3 days. The whole lungs, the whole lung plus enzymes, the whole lungs plus lung regions and the whole lungs plus enzyme-pretreated medial region were cultured on testacea membrane and solid serum-free media at 37°C for 3 days, according to the technique elsewhere described (Carinci *et al.*, 1986).

Morphometric investigation

After this time the cultures were fixed in buffered formalin at 4°C for 3-4 hours, and routine histological procedures were followed. The whole lung and the whole lung plus apical, medial, caudal or apical plus caudal regions were cut in 5 μ sections at intervals of 20 μ, stained with haematoxylin and eosin, and observed by light microscope. To quantify the structural differences between lung cultures, we performed morphometric analysis on the sections. We assessed the total number of branching and the ratio between the total area of branching and total area of single sections by a Zeiss Axioplane Microscope connected to a Kontron Electronic Scanner using Vidas software. Total area development of epithelial branching was evaluated by adding together

all determined branching areas. We performed two slides for sample; the values were expressed as μ^2 (bronchial branching/culture) or as bronchial branching number per whole lung and were the mean ± SD of five determinations per slide.

Histochemical technique

Alcian blue staining. The histochemical study was performed on homologous sections of intraclavicular 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; Stabellini *et al.*, 2002). GAG were identified by critical electrolyte concentrations at which the polyanions changed from binding Alcian to Mg^{++} (Scott and Dorling 1965). Alcian stained polyanions increasing selectivity when the $MgCl_2$ concentration in the staining solution increased: at 0.025 $MgCl_2$, all GAG as well as nucleic acid and sulphated glycoproteins; at 0.3 $MgCl_2$, the only macromolecules stained positively were CS, DS, keratansulfate (KS), and HS. The Alcian blue technique used to distinguish different GAG has been described previously (Becchetti *et al.*,1988). We used 1% Alcian blue 8GX staining (AB) (Sigma-Aldrich; St Louis, MO) in 0.1 M acetetate buffer, pH 5.8, in the presence of 0.025 M or 0.3 M or 0.65 M $MgCl_2$ solution (Sigma-Aldrich) for 2 hr. For enzymatic digestion, the sections were incubated with testicular hyaluronidase (Merk, Darmstadt, Germany; 1 mg/mL in 0.1 M phosphate buffer, pH 7.6, 6 hr at 37°C). Control sections were incubated in buffer alone. The action of specific enzymes on the section, followed by Alcian blue staining, allowed us to determine the distribution of individual GAG. Digestion with testicular hyaluronidase, in particular, selectively removed HA and CS. GAG values were obtained by connecting a Zeiss Axioplane Microscope to a Kontron Electronic Scanner using Vidas Software which converted the blue colour into a grey scale (arrangement: black = 0, white = 1). The values are expressed as relative optical density and were a mean ± SD of five determinations per slide.

Determination of exoglycosidases

For β-NAG activity, apical, medial and caudal lung regions were pooled and 6, 11 and 14 day development, treated with Con A, SBA and WGA, homogenized in 2 mL of 0.1 M citrate/0.2 M phosphate buffer pH 4.5, then centrifuged at 25,000 g for 10 min and the supernatants used. In preliminary experiments, no enzymatic activity was detected in the sediment. β-N acetylglucosaminidase (EC 3.2.1.30) was assayed as previously described by Orlacchio *et al.,* (1984) using, respectively, as substrates 4-methyl-umbelliferyl-β-N-acetyl-glucosaminidase in 0.1 M citrate/0.2 M phosphate buffer, pH 4.5, and 50 μL of enzyme preparation. One enzyme unit is defined as the amount of enzyme that converts 1 nmol/hr of substrate into 4-methyl-umbelliferone at 37°C. Specific activities were expressed as units/mg protein. Proteins were determined according to the Bradford method (Bradford, 1976) using crystalline calf gamma globulin (Merck) as the standard.

Determination of endoglycosidases

For HAase activity various lung regions at 6, 11 and 14 day development were sonicated on ice in formate extraction buffer (0.1 M sodium formate/0.15 M NaCl/0.1% Triton X-100, pH 3.5) containing 2.5 mM saccharic acid 1,4-lactone, an inhibitor of exoglycosidase activity, according to the method of Kulyk and Kosher (1987). Aliquots of the sonicate were used for protein determination (Bradford, 1976). The sonicate was centrifuged at 10,000g for 5 min and aliquots of the supernatant were incubated at 37°C for 18 hr with 200 μg HA (Sigma-Aldrich, Milan) in 250 μL reaction mixture. HAase activity was measured by assaying the formation of reducing terminal N-acetyl-glucosamine-containing oligosaccharides by micromodification of the procedure of Reissig *et al.* (1975). Briefly, the 250 μL reaction mixture was evaporated to dryness, and the residue was dissolved in 70 μL of 0.125 M potassium tetraborate and heated in a boiling water bath for exactly 3 min. After cooling, the samples were added with 330 μL of diluted dimethylaminobenzaldehyde reagent was added to samples and incubated at 37°C for 20 min. At the end of the incubation, samples were cooled in an ice bath and centrifuged at 10,000 g for 5 min, and the adsorbance of supernatants was measured at 585 nm. The control, supernatants of cells and tissue extract were heat-inactivated by boiling before addition of HA substrate. Terminal N acetylglucosamine was not

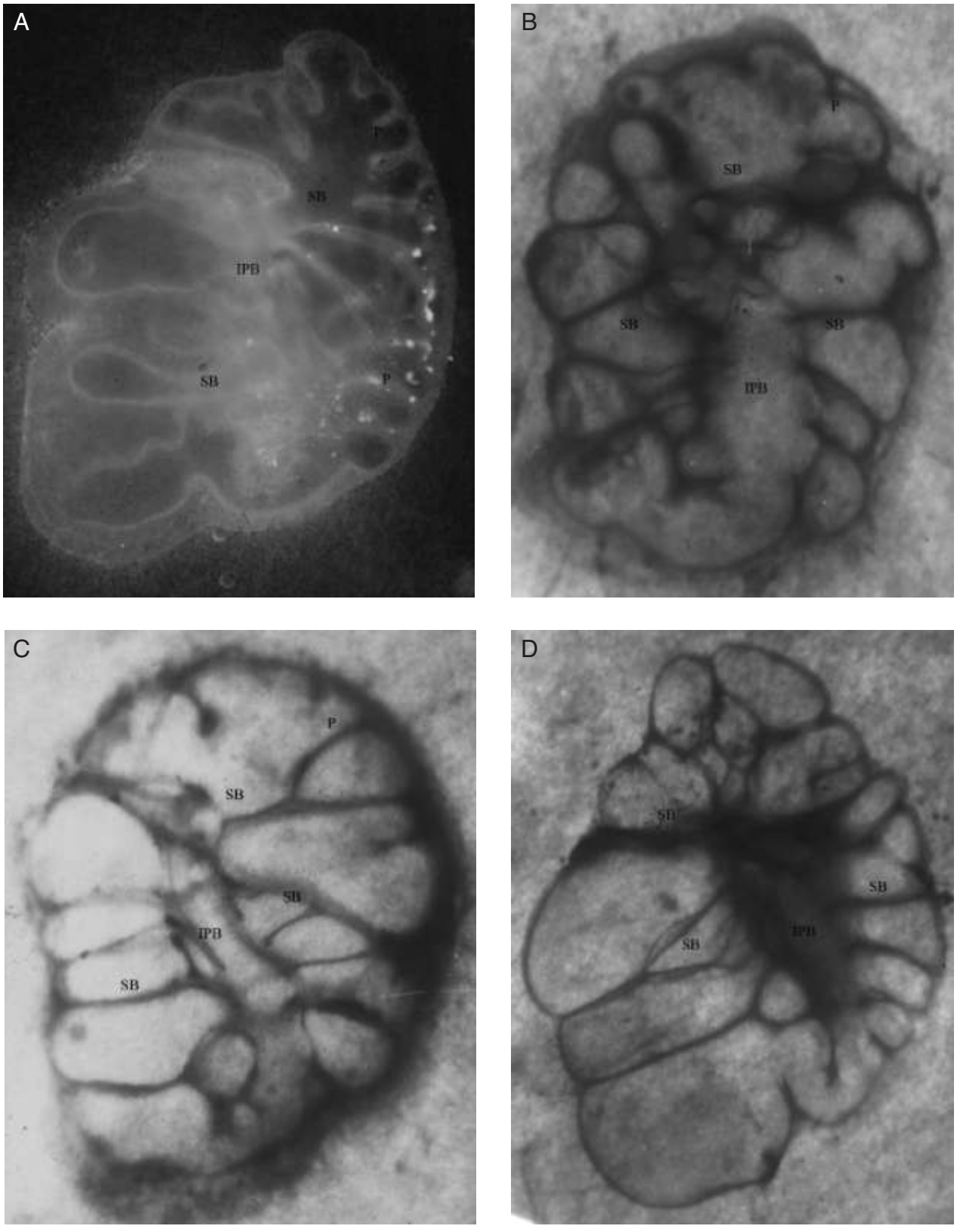


Figure 1. Stereomicroscope images of whole lung maintained *in vitro* for 3 days with HAase (B), Chase (C), b-NAG (D) and control (A). SB = secondary bronchi, IPB = intrapulmonary primary bronchus, P = parabronchi. Magnification X 25.

detectable in these control samples. Units of HAase were defined as nmol of terminal N-acetyl-glucosamine released per hr at 37°C.

Statistical analysis

The statistical analysis was made using the Student t-test for unpaired data. $p \leq 0.05$ level was considered significant.

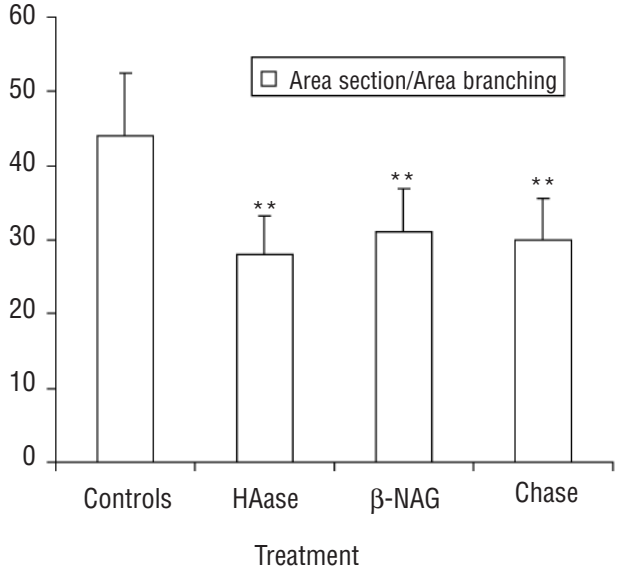
Results

Organotypical cultures

Morphological data

A single 6-day lung rudiment is formed of an intrapulmonary primary bronchus covered with pseudo-stratified epithelium from which 5 secondary bronchi emerge (Baumel *et al.*, 1979). All secondary bronchi are lined by a simple columnar epithelium. After 3 days of culturing in 199, higher growth and more numerous epithelial tubules were detectable. Lung explants grew well; epithelial tubules extended outwardly. 12-14 secondary bronchi covered with a simple isoprismatic epithelium lined by a simple squamous epithelium were present (Figure 1A). The whole lungs, maintained *in vitro* in the presence of HAase, β-NAG and Chase showed an intrapulmonary primary bronchus and very expanded secondary bronchi (Figure 1B-D) when compared to control (Figure 1A). The ratio between lung total area and bronchial branching total area was significantly reduced: HAase 0.29±0.06, β-NAG 0.34±0.05 and Chase 0.30±0.06 compared to control 0.45±0.08 (Figure 2). The whole lung maintained *in vitro* combined with apical or medial or caudal regions showed different bronchial branching development and distribution. The whole lung showed an increase in bronchial branching development (parabronchi) when associated with medial region alone (Figure 3B). By contrast, the addition of apical (Figure 3A) or caudal (Figure 3C) regions did not show any differences in whole lung bronchial branching development. Medial region previously cultured with HAase and associated with whole lung did not influence bronchial branching development in whole lung (Figure 3D).

The morphometric analysis of whole lungs associated with the single regions shows that only the addition of medial region determines a significant increase in development of the bronchial branching in whole lung (Figure 4).



***p≤as compared to control*

Figure 2. Development of lung and bronchial branching in whole lung maintained *in vitro* in the presence of Hyaluronidase (HAase), β-N-acetyl-D-glucosaminidase (β-NAG), Chondroitinase ABC(Chase) and 199 alone. The values (μ² of the ratio between total area of section and total area of bronchial branching) are a mean±SD.

Histochemistry

Table 1 shows that whole lung maintained *in vitro* with 199 alone shows that the mesenchymal ground is strongly alcian reactive at 0.025 M $MgCl_2$ and 0.3 M $MgCl_2$. The alcianophilia at 0.025 M $MgCl_2$ is greater around the intrapulmonary primary bronchus and weak around the secondary bronchi and parabronchi as compared

Table 1. Lung whole maintained *in vitro* for 3 days with 199 alone or 199+HAase. AB = Alcian Blue, IPB = Intrapulmonary primary bronchus, SB = Secondary bronchi, P = Parabronchi.

Staining	*Lung whole culture*					
	199			*199 + HAase*		
	IPB	*SB*	*P*	*IPB*	*SB*	*P*
Before AB in 0.025 MMgCl₂	105±16	13±3	16±5	-	18±5*	16±3*
After HAase	-	-	-	-	-	-
Before AB in 0.3 M MgCl₂	61±10°	115±17°	108±16°	-	15±6	15±5
After HAase	-	49±7#°	50±7#°	-	16±4*	13±2*
AB in 0.65 M MgCl₂	15±5	50±9	16±5	-	-	-

**p≤0.01 as compared to 199; °p≤0.01 as compared to AB 0.025 $MgCl_2$; #p≤0.01 as compared to before HAase.*

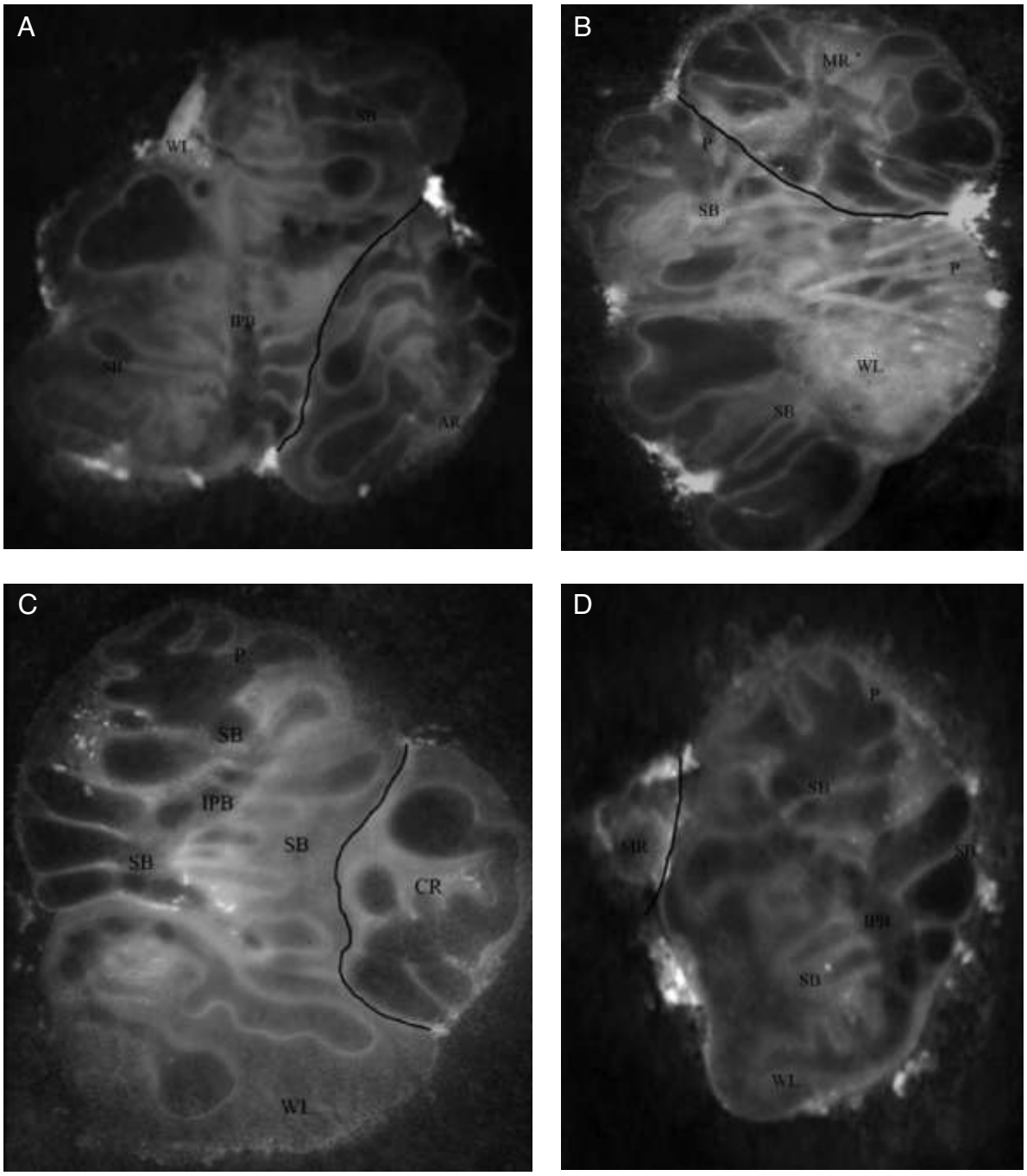
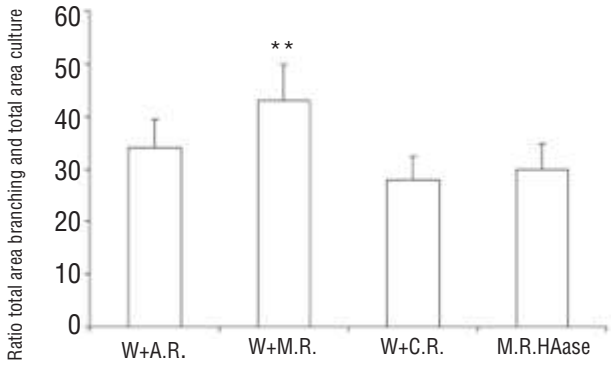


Figure 3. Stereomicroscope images of whole lung maintained *in vitro* for 3 days associated with apical or medial or caudal lung regions and whole lung associated with medial region precultured with HAase. (A) Whole lung + apical region; (B) Whole lung + medial region; (C) Whole lung + caudal region; (D) Whole lung + medial region previously treated with HAase. WL = whole lung, AR = apical region, MR = medial region, CR = caudal region, SB = secondary bronchi, IPB = intrapulmonary primary bronchus, P = parabronchi. Magnification X 25.

to 0.3 M $MgCl_2$ (showing a greater quantity of HA and CS respectively). After testicular HAase treatment the alcianophilic staining completely vanishes at 0.025 M $MgCl_2$ (HA, CS) and largely vanishes at 0.3 M $MgCl_2$ (CS). After HAase digestion the alcianophilia at 0.3 M $MgCl_2$ has a clearly different distribution in relation to the various sites and is dectectable around the secondary bronchi and parabronchi. At 0.65 M $MgCl_2$ the alcianophilia is poor but detectable (HS) around the intrapulmonary primary bronchus and parabronchi and clearly evident



***p≤0.01 as compared to W+A.R., W+C.R., W+M.R.HAase*

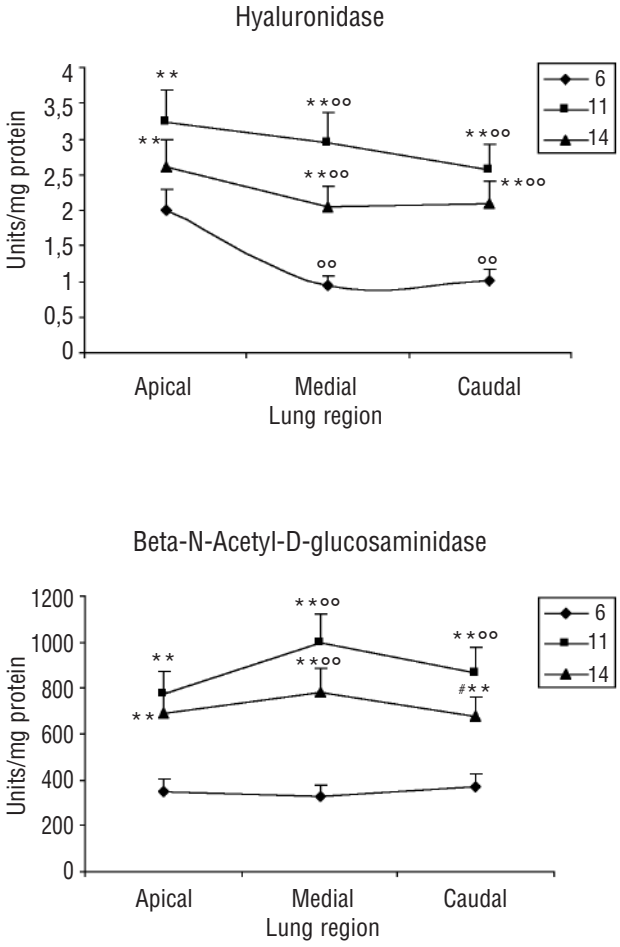
Figure 4. Whole lung at 6 days. 2nd + 3rd bronchial branching development/culture after 3 days of *in vitro* maintenance with apical or medial or caudal regions added. The values are a mean ± SD. W + A.R. = Whole + Apical Region; W + M.R. = Whole + Medial Region; W + C.R. = Whole + Caudal Region; W + M.R.HAase = Whole + Medial Region added after HAase treatment.

around the secondary bronchi. In lung explants maintained *in vitro* for 72 hours in the presence of HAase the mesenchymal ground substance shows a decrease ($p \leq 0.01$) in alcianophilia at 0.025 M $MgCl_2$ and 0.3 M $MgCl_2$ (less HA and CS) around the intrapulmonary primary bronchus, secondary bronchi and parabronchi as compared to control. After HAase treatment the alcianophilia completely vanishes at 0.025 M $MgCl_2$ (HA, CS) and is poorly detectable at 0.3 M $MgCl_2$ (CS). After HAase digestion the alcianophilia at 0.3 M $MgCl_2$ has a clearly different distribution in relation to the various sites and is scarcely dectectable around the secondary bronchi and parabronchi. At 0.65 M $MgCl_2$ alcian reactivity is negative (absence of HS).

Exoglycosidases and endoglycosidases in lung regions

In apical, medial and caudal regions (Figure 5) in 6 day chick embryo lungs, HAase showed activity in the apical region (2.00±0.20 units/mg proteins) which decreased ($p \leq 0.01$) in the medial region (0.94±0.12 units/mg proteins) and caudal region (1.02±0.14), while the differences in β-NAG activity are not significant.

In 11 day chick embryo lung, HAase decreased constantly ($p \leq 0.01$) in the medial (2.56±0.21) and caudal (2.95±0.28) regions compared to the apical region (3.24±0.29). β-NAG increased ($p \leq 0.01$; $p \leq 0.05$) in the medial (996.5±94.8)



***p≤0.01 as compared to day 6; °°p≤0.01 as compared to Apical region; #p≤0.05 as compared to Medial region*

Figure 5. Levels of HAase and β-NAG activity in apical, medial and caudal regions in 6, 11 and 14 day chick embryo lungs. The values are specific activity as units/mg proteins.

and caudal (867.3±95.8) region without significant differences between the medial or caudal regions. In 14 chick embryo lung, in the apical region (2.60±0.26) the HAase activity decreased ($p \leq 0.01$) in the medial (2.04±0.22) and caudal (2.09±0.20) regions. β-NAG increased ($p \leq 0.01$) in the medial (783.0±95.2) but decreased in the caudal region (675.0±94.2) as compared to the apical region (690.2±88.0)

In apical, medial and caudal lung regions the HAase and β-NAG activity showed an increase ($p \leq 0.01$) at 11 days as compared to day 6. At 14 days the two enzymes decreased as compared to day 11, though their levels were higher ($p \leq 0.01$) than on day 6.

Discussion

In whole lung the normal bronchial branching development is related to the GAG composition of ECM and to the continuity of mesenchymal mass. In fact altered development of bronchial branching corresponds to the modifications to HA and sulphated GAG caused by HAase, β-NAG and Chase enzymes in the mesenchyme of whole lung at 6 days' development. Moreover the apical, medial and caudal regions, when added to whole lung, have a different effect on the normal bronchial branching in the whole lung, depending on the lung region involved. Only the medial region is able to increase the branching development, while the apical and caudal regions do not increase the branching development even when added together to whole lung or when added separately. It must be remembered that the various added regions have a different GAG composition of the ECM (Becchetti *et al.*, 1988). HAase is able to modify the ECM composition in whole lung cultures (Carinci *et al.*, 1986) and the medial lung region pre-digested with this enzyme does not increase branching in whole lung. The data thus confirm that the morphogenetic stimulus depending on the addition of fragments to the whole lung is correlated to the quality of ECM mesenchymal composition (Carinci *et al.*,1986; Calvitti *et al.*, 2004). The bronchial branching development in whole lung shows that the relationship between branch development and branch diameter is altered. Since during lung development a relation exists between bronchial branching and branch diameter (Weibel, 1987) these observations indicate that the bronchial branching in lung regions with GAG classes alteration is able to develop, but it occurs in an anomalous way. The epithelial-mesenchymal interaction in the branching development pattern occurs through stimulating and inhibiting factors that regulate the gene expression. Other authors show that fibroblast growth factor-2 is diffusely expressed in the epithelial and mesenchymal cells (Maina *et al.*, 2003); this could be an indirect confirmation of the regulatory role of HA and CS on cytokine activity (Locci *et al.* 1993, Canning *et al.*, 2000). In fact our data show that the composition of ECM and the continuous regulation of these signals in the apical, medial and caudal regions play a crucial role in gene expression which regulates branch elongation and establishes the pattern of branching as happens in the *in vitro* model of the developing kidney (Qiao *et al.*, 1999). Moreover, since during development ECM transduces signals from the microenvironment, regulates cell function and growth factor release and modulates growth factor activity and distribution (Kresse and Schonherr, 2001), our observations on the different enzyme composition related to GAG turnover of the lung region show that ECM remodelling plays an important role in the coordination of normal branching development. In fact the alteration in ECM composition during development leads to dynamic changes in its signalling properties according to Ivkovic *et al.* (2003). Besides, the coordination failure in extracellular GAG turnover in various different lung regions could affect of gene expression, leading to anomalous development of branching (Muruoka *et al.* 2000). All in all our data show that the different lung regions have different ECM composition and differently act on branching morphogenesis.

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