Presence of N-acetylgalactosamine/galactose residues on bronchioloalveolar cells during rat postnatal development

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Abstract

In mammals, the alveolarization process develops predominantly after birth. Airway cells display a complex assemblage of glycans on their surface. These glycans, particularly terminal glycan extensions, are important effective carriers of information that change during the differentiation process. Nevertheless, few systematic data are reported about the cell surface sugar residue content during postnatal lung development. In the present work, we aimed to identify and semi-quantify N-acetylgalactosamine (GalNAc)/galactose (Gal) residues on the bronchioloalveolar cell surface in rat lung sections from 1-, 4-, 8-day-old and adult animals and link these data with the lung glycocalyx composition. Horseradish peroxidase-conjugated lectin from *Glycine max* (soybean agglutinin, SBA) was used, and light microscopy methodologies were performed. SBA labelling intensity was studied before and after sialidase pre-treatment, in 1-, 4-, and 8-day-old animals and adult animals. For semi-quantitative evaluation of SBA binding intensity, two investigators performed the analysis independently, blinded to the type of experiment. Reactivity of the lectin was assessed in bronchiolar and respiratory portion/alveolar epithelial cell surfaces. We evidenced a stronger positive reaction when lung sections were pre-treated with neuraminidase before incubation with the lectin in 1- and 4-day-old animals and adult animals. These results were not so manifest in 8-day-old animals. This binding pattern, generally points towards the presence of terminal but mainly sub-terminal GalNAc/Gal residues probably capped by sialic acids on the rat bronchiolar/respiratory tract epithelial cells. As this glycan extension is common in O- and N-glycans, our results suggest that these glycan classes can be present in bronchioloalveolar cells immediately after birth and exist during the postnatal period. The

results observed in 8-day-old rat lung sections may be due to the dramatic lung morphologic changes and the possible underlying biological mechanisms that occur during this age-moment.

Introduction

The mammalian respiratory system is a complex branching structure that arises from the ventral foregut endoderm, with endoderm/mesoderm interactions indispensable for the characteristic branching morphogenesis.[1-3] During the embryonic period, the fetal period and the postnatal period, the lung develops from an outpouching of the foregut to a tree-like system and ultimately to a gas exchange area.[4,5] Over the years, postnatal lung development has been extensively investigated in experimental animals and in humans, and it is now well known that after birth, the mammalian gas exchange apparatus undergoes very important structural modifications, being the formation of highly septated and alveolarized structures a key event.

During this phase, new septa are formed by a subdivision of the terminal air spaces, "sacculi", followed by microvasculature maturation with the onset of a single-layered capillary network and still more alveoli generation.[6-10] Unlike humans, rats and mice are born during the saccular stage, but except for the phase of development at birth, rat lung postnatal development comprises the same developmental steps as human lung in the alveolarization process.[7,10,11] With the advent of new imaging tools, it is now accepted that mammalian lung development continues throughout adulthood.[12,13] Intrapulmonary epithelial cells, including ciliated cells, club cells, type II and type I cells, share a common lineage and undergo pronounced biochemical and morphofunctional changes during pre- and postnatal lung maturation.[5,14-16]

A complex and intricate array of monosaccharides or oligosaccharides, generically named glycans, surrounds the living cells of every organism and is required for critical cellular functions. The numerous physiological functions of glycans include organizational, modulatory, protective, interactive, and recognition roles and can be placed into three categories: structural, recognition and molecular mimicry of host glycans.[17-19] Glycans located at the cell surface that define the molecular frontier of cells are closely implicated in cell-cell, cell-matrix and cell-molecule interactions and are involved in many biological events during development, such as differentiation and organogenesis.[20,21] Carbohydrate chains of cell surface glycans

can have spatial and temporal patterns of expression during development, and many glycan extensions are regulated not only during embryonic and fetal periods but also during postnatal development.[22-24]

Since the early 1950s, with the description of a pulmonary extracellular lining layer by Low[25] and Macklin[26], different histochemical methodologies, including those using lectins, have been applied in lung glycan analysis. Different studies have shown the presence of several glycoconjugate sugar residues during lung development using diverse lectins, and different morphological lung growth stages.[25-35] However, to the best of our knowledge, systematic information on the composition of mammalian postnatal lung glycocalyx is scarce.

In the present study, we continued previously published studies and sought to identify and compare the presence of N-acetylgalactosamine(GalNAc)/galactose(Gal) residues on rat bronchioloalveolar epithelial cell surfaces using lectin histochemical light microscopy (LM) method-

ologies. The binding pattern of lectin from *Glycine max* (common name soybean agglutinin, SBA) with specificity to GalNAc/Gal residues, before and after neuraminidase treatment, was systematically studied and compared in rat lung sections from 1-, 4-, 8-day-old and adult animals, and possible modifications of presence of these saccharides in all the age moments studied was surveyed.

Materials and Methods

Animals and tissue collection

Wistar rats, including adult rats, animals older than a week and pregnant females, were supplied by CIFRA, S.A. (Barcelona, Spain).

An intraperitoneal anaesthesia administration was performed using sodium pentobarbital (25 mg/kg), and the lungs were collected from 1-, 4-, and 8-day-old rat pups and from nine-week-old adult animals, for a total of 20 sacrificed animals.

After the thorax and abdomen were opened and an incision in the abdominal aorta was performed, the anaesthetized animals were submitted to lung perfusion *via* the right ventricle with a 0.1 M phosphate-buffered saline (PBS, pH 7.4) solution at a flow rate of 1 mL/min to 6 mL/min for 90 sec, according to the age. The fixation process followed this perfusion and began with a 5-min perfusion with 4% formaldehyde in PBS (pH 7.4) containing 0.5% glutaraldehyde. Collapsed lungs were excised and immersed in the same fixative solution for 16 h at 4°C. Samples from different pulmonary lobes were studied in each age group. Lung pieces were rinsed in 0.5 M ammonium chloride (NH_4Cl) in PBS for 1 h at room temperature (RT) to block free aldehyde groups, then washed in PBS for one hour at RT, and finally processed for embedding in paraffin wax; 5 μm sections were made.

All the procedures were performed under the protocols established by the Ethics Committee at the Faculty of Medicine in Coimbra, Portugal.

Lectin histochemistry

Endogenous peroxidase was inhibited by treating sections after being routinely dewaxed with methanol containing 0.6% hydrogen peroxide (H_2O_2) for 30 min at room temperature (RT). Subsequently, tissue sections were incubated for one hour in a moist chamber with horseradish peroxidase-conjugated SBA (Sigma; Saint Louis, MO, USA, L-2650) diluted in 0.05 M Tris-buffered saline (TBS, pH 7.5) at the appropriate concentration, as shown in Table 1. Different lectin concentrations were studied to check reproducibility and reach the final concentration. After lectin incubation, the slides were rinsed in TBS and developed by soaking in diaminobenzidine (DAB)-H_2O_2 medium (TBS containing 0.05 mg DAB/mL and 0.015% v/v H_2O_2 at 30%) for 10 min at RT (Sigma; D-5905; 48H8202). After rinsing the sections in distilled water, the nuclei were counterstained with haematoxylin, cleared in xylene and mounted.

The stained sections were observed under a microscope (Nikon Eclipse Ci-L, Konan, Minato-ku, Tokyo, Japan) equipped with a XFCAM1080PHB/PHD CMOS digital camera (Toup Tek, Zhejiang, China) and programme ToupView. Digitalized images were captured under identical conditions.

Sialidase digestion

It is well known that sialic acids mainly occur as terminal components of cell surface glycans, acting as "caps" of underlying glycans. Bearing that in mind, neuraminidase digestion was performed in some experiments to remove terminal sialic acid residues prior to lectin staining. LM sections were incubated for 18 h at 37°C in a 0.1 M acetate buffer solution (pH 5.5) containing 0.15 M NaCl, 40 mM $CaCl_2$ and 0.8 U/mL neuraminidase Type X from *Clostridium perfringens* (Sigma; N-2133).

Semi-quantitative evaluation of SBA binding intensity

For semi-quantitative evaluation of SBA binding intensity, two investigators performed the analysis independently, blinded to the type of experiment (age-moment and pre-treated or non-pre-treated sections with sialidase). Reactivity of the lectin was assessed in bronchiolar and respiratory portion/alveolar epithelial cell surfaces. Binding intensity was evaluated using a semi-quantitative scale as follows: +++ strong; ++ positive; + faint, and – negative labelling.

Control for lectin staining

The lectin staining specificity was evaluated by pre-incubation of the horseradish peroxidase-conjugated SBA with the corresponding inhibitory sugar (0.2 M; Table 1) for 50 min before staining.

Results

The cytochemical reaction observed after incubating rat lung sections with horseradish peroxidase-conjugated SBA was present on the surface of bronchiolar and respiratory tract epithelial cells, but it was generally faint. However, when tissue sections were pre-incubated with neuraminidase before SBA staining, the intensity of the reaction markedly increased, in most cases.

Therefore, we observed a weak reaction on the luminal surface of bronchiolar cells from 1-day-old rat lungs (Figure 1A). However, the apical cell surface of bronchioles and the respiratory tract from one-day-old rats are intensely stained when SBA was incubated after neuraminidase pre-treatment (Figure 1B and Table 2). When we compare the non-pre-treated sections with those for lung sections pre-treated with sialidase, the same labelling pattern was evident in 4-day-old (Figure 1 C,D; Table 2). Accordingly, in four-day-old rat lungs, we could also see an evident positive reaction on the luminal surface of bronchiolar cells and in the respiratory portion when the sections were pre-treated with the enzyme (Figure 1D). Comparatively, a weak reaction is seen in bronchiole and respiratory cell surfaces when non-pre-treated lung sections were incubated with SBA (Figure 1C).

In 8-day-old rat lung sections, the difference in reaction intensity between non-pre-treated and pre-treated sections was not so evident (Figure 1 E,F; Table 2).

In adult animals, SBA reactivity was detected on the luminal surface of ciliated and non-ciliated bronchiolar cells (Figure 2A). The alveolar epithelium of an adult animal was weakly stained by SBA, as shown in Figure 2C.

However, when the sections were pre-treated with neuraminidase, the staining intensity increased. Thus, the luminal sur-

Table 1. Lectin characteristics.

Botanical name	Common name	Acronym	SBA concentration (μg/mL)	Saccharide affinity	Inhibitory saccharide
Glycine max	Soybean	SBA	20	GalNAc>>Gal	GalNAc

face of club and ciliated cells is heavily stained with SBA in enzyme pre-treated sections (Figure 2B). The alveoli epithelial cells also showed a stronger affinity for SBA in pre-treated than in non-pre-treated sections (Figure 2 D,C).

The semi-quantitative binding evaluation (Table 2) reflects the evident increase in SBA binding when lung sections were pre-treated with sialidase, which can be observed in all age-moments studied except for the 8-day-old age.

The lectin staining was entirely absent when one-day-old rat lung sections were pre-incubated with SBA and the corresponding inhibitory sugar (Figure 3).

Discussion

The rat lung is not completely mature at birth, and the gas-exchange surface area expands during the postnatal period.[9,36,37] The cytodifferentiation of the epithelial cells that line the bronchioalveolar airway is a continuous process occurring mostly after birth.[38] A dynamic expression pattern of molecular mediators regulating cell differentiation was demonstrated in rat lung development during pre- and also postnatal periods.[39-41]

In addition to generating energy in the cell, carbohydrates act as signalling effectors and recognition markers and are key elements in post-translational modifications of proteins. It is now clear that the expression of certain glycans changes in different stages of development, which implies different roles for these glycoconjugates.[19,42] It was proven that N-glycans are important for airway epithelium organization during development. On the other hand, animals lacking O-fucose glycans in the lung have deficits in pulmonary development, particularly due to a lack of secretory cells.[43-45] Therefore, the study of the bronchioloalveolar cell surface glycan composition during postnatal rat lung development can contribute to a better understanding of lung development.

Since the middle of the 20th century, lectin histochemistry is one of the major

approaches to characterize glycoconjugates, and the binding patterns of different lectins have been studied in different organs of humans and experimental animals, including rats.[34,46-49] Concerning lung histochemical investigations, authors generally used different lectins, pointing out the most obvious results for each lectin.[50-53]

Our LM lectin histochemistry data, although not covering the second phase of alveolarization, showed that SBA binds to bronchiolar and respiratory tract epithelial

cells in rat lung sections form 1-, 4-, 8-day-old and adult animals. However, although present, the binding sites are generally much more evident after neuraminidase treatment. These results are in accordance with Iwatsuki *et al.*,[31] who revealed "a small number of SBA binding sites" on adult rat alveolar cells. Shimizu *et al.*[54] also reported that only a small percentage of rat tracheo-bronchial cells reacted with SBA as well as with other lectins studied. Castells *et al.*,[55] studying the pre- and postnatal developing
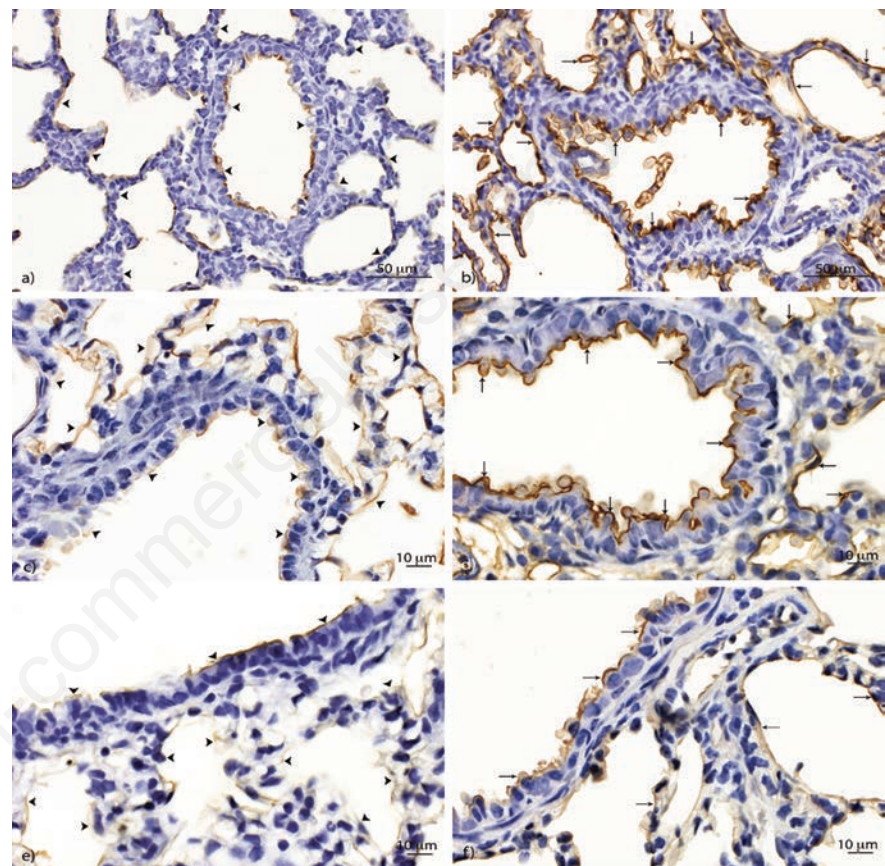

Figure 1. An increase in SBA binding is evident in sialidase pre-treated sections (arrows in B and D) when compared with non-pre-treated sections (arrowheads in A and C) from 1-day-old (A and B) and from 4-day-old (B and C). This increase is not so evident in sections from 8-day-old rat tissue sections (arrowheads in E and arrows in F).

Table 2. Semi-quantitative binding intensity of SBA to bronchiolar and respiratory portion/alveolar cell surfaces in four different postnatal age moments.

	AGE MOMENT							
	One-day-old		Four-day-old		Eight-day-old		Adult	
	NPT	PT	NPT	PT	NPT	PT	NPT	PT
SBA staining intensity								
Bronchiolar cell surfaces	+	+++	+	+++	+	++	++	+++
Respiratory portion/alveolar cell surfaces	+	+++	+	++	+	+	+	+++

NPT, non-pre-treated; PT, pre-treated; +++, strongly positive; ++, positive; +, faint; –, negative labeling.

rat respiratory system by means of lectin histochemistry, reported a positive reaction of SBA on the goblet cells of adult rat airway epithelium. After neuraminidase treatment, the same authors did not describe the SBA results but reported an increase in the peanut agglutinin (PNA) binding sites, whose carbohydrate sequence binding specificity is Gal-GalNAc.

Martins *et al.*[33] demonstrated that at the ultrastructural level, the SBA labelling pattern is faint and present on both adult rat alveolar and endothelial cells. SBA staining is much stronger after sialidase treatment than before treatment and predominantly located on the surface of the alveolar epithelial cells. Our present results systematically point towards the presence of

GalNAc/Gal residues on the bronchiolar/respiratory tract epithelial cells in the age-moments studied. In all the ages studied, these residues are also much more available for lectin binding after neuraminidase treatment, suggesting that sialic acid moieties are capping GalNAc/Gal residues. These labelling patterns were consistent in 1- to 4-day-old and adult rat lungs. In 8-day-old animals the less evident difference between non-pre-treated and pre-treated sections suggests a lower availability of sub-terminal GalNAc/Gal residues for the lectin. This could be due to the dramatic morpho-biological transformations that the lung experiences at this age. A massive number of alveoli are formed in a period that in rats occurs on postnatal (P) day 3 to P14. After this period, alveoli continue to develop although in a slower pace.[56,57]

These relevant transformations, which are notably present around P8 may underlie molecular changes that could lead to modifications in the complex structure of surface glycans. Therefore, a transitional period where N-acetylgalactosamine/ galactose residues are less available to SBA in sub-terminal locations could be present. In adult rat lung sections, the binding patterns indicate the presence of GalNAc/Gal residues in terminal, but mainly in sub-terminal positions.

In conclusion, data presented here indicates a systematic increase in the positive reaction of SBA after neuraminidase treatment in 1- and 4-day-old and adult rat lung sections, which advocates a prevailing sub-terminal presence of GalNAc/Gal residues mainly with terminal sialic acids. These glycan compositions are present in glycoproteins, indicating the existence of O- and N-glycans on rat bronchiole/respiratory portion cells immediately after birth and during the postnatal period. In 8-day-old rats, the less obvious difference in reaction intensity between pre-treated and non-pre-treated sections may be due to dramatic changes that occur during the "bulk alveolarization" process on this age-moment.

Nevertheless, further studies are required to more clearly understand the lung glycocalyx composition during postnatal lung development and a great deal of research persists to be done before the composition and role of lung surface glycans can be fully understood.
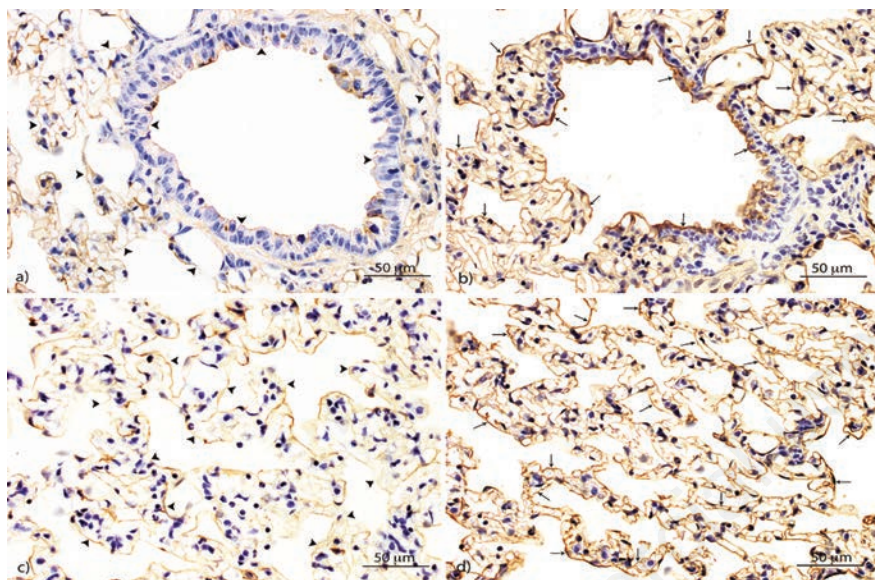

Figure 2. Adult rat lung sections show a stronger positive reaction on the surface of ciliated and non-ciliated cells when we compare sections pre-treated with neuraminidase (arrows in B), with the ones non-pre-treated (arrowheads in A). The surface of the alveoli shows the same labelling pattern (arrowheads in C and arrows in D).
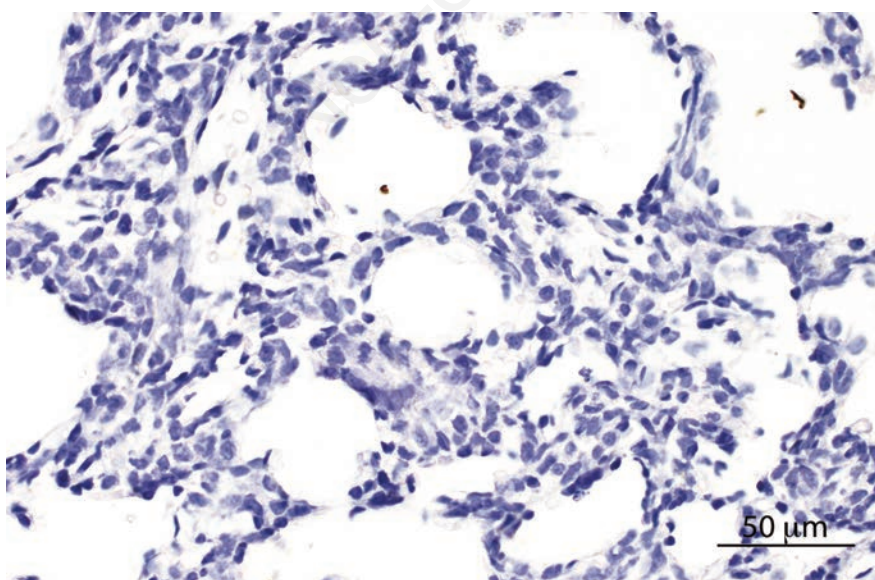

Figure 3. No staining is present when the lectin was pre-incubated with GalNAc. 1-day-old rat lung section.

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