Cell differentiation of alveolar epithelium in the developing rat lung: ultrahistochemical studies of glycoconjugates on the epithelial cell surface

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Abstract. Glycoconjugates on the surface of pulmonary epithelial cells were ultrahistochemically examined in the fetal, neonatal and adult rat lung. Lectin and colloidal iron staining procedures were performed in combination with digestion using carbohydrate-degrading enzymes or methylation. The glycoconjugate composition of columnar cells at 16 days gestation was similar to that of cuboidal cells at 19 days gestation. Glycoconjugate differentiation on the cell surface occurred at 20 days gestation, and especially the loss of soybean agglutinin (SBA) binding sites could be detected on type II cells. The contents of *Ricinus communis* agglutinin-I (RCA-I) and *Concanavalin A* (Con A) binding sites on type II cells also began to decrease. On the contrary, the content of sulfated saccharides decreased on the surface of type I cells during development. Glycoconjugate differentiation on both type I and II cells was completed with the disappearance of hyaluronic acid and peanut agglutinin (PNA) binding sites; type I and II cells acquired a similar histochemical composition to that on adult type I and II cells at 5 days after birth. Both type I and II cells share a common early precursor cell, that is, the cuboidal epithelial cell at the canalicular stage.

Introduction

Morphological studies of lung development have led to the identification of three different developmental stages: glandular, canalicular and alveolar (for reviews see Meyrick and Reid 1977; Burri and Weibel 1977). In the glandular stage, pulmonary epithelium consists of a single layer of high columnar cells, which decrease in height to become cuboidal in profile at the beginning of the canalicular stage. In the alveolar stage, type I and type II pulmonary epithelial cells can be distinguished morphologically. The process of their differentiation during gestation has been studied, and there are two hypotheses concerning the developmental pathway. The first is that, after type II cells differentiate, some type II cells transform into type I cells (Balis and Conen 1964; Adamson and Bowden 1975; Ten Have-Opbroek 1979; Otto-Verberne et al. 1988). The other hypothesis is that both types of pulmonary epithelial cells have a common precursor cell (Kikkawa et al. 1968; Baskerville 1976). When type I cells are selectively injured, type II cells proliferate and differentiate into type I cells during lung regeneration (Adamson and Bowden 1974; Hirai et al. 1983; Rannels and Rannels 1989). Recent histochemical investigations, however, have raised questions concerning the transformation of type II cells to type I cells during gestation (Rosenkrans et al. 1983; Joyce-Brady and Brody 1990).

The glycoconjugate composition of the plasmalemmas of type I and type II cells in adult rats is quite different, making them one of the most reliable markers for the classification of pulmonary epithelial cells (Meban 1987; Iwatsuki 1992). The aim of the present study was to investigate the ultrahistochemical features of the glycoconjugates on pulmonary epithelial cells and to clarify the process of differentiation of pulmonary epithelial cells in the lung of the fetal rat.

Materials and methods

Specimen preparation

Pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Shizuoka, Japan). Seven or eight rats were processed at each of the following ages: at 16, 19, 20, and 21 days of gestation, when newborn, and at 5 days old and 8 weeks old. Pregnant mothers were anesthetized by intraperitoneal injection of sodium pentobarbital and fetuses were immediately removed. Fetal lungs were quickly removed and cut into small blocks. After washing in chilled Ringer’s solution, tissue blocks were fixed for 1 h at room temperature in 2.5% glutaraldehyde in 70 mM cacodylate buffer (pH 7.4), and cut into thin strips of 0.1–0.2 mm in thickness.

Concerning the postnatal rats, after the trachea was cannulated under anesthesia, the thoracic cavity was exposed and the airways of the lungs were washed out with Ringer’s solution through the
Histochemical procedures

Lectin staining procedures. For the histochemical detection of specific saccharide residues of the glycoconjugates, fixed lung tissues were labeled with the following four ferritin-conjugated lectins (EY Laboratories): Concanavalin agglutinin (Con A) with a binding specificity for both mannosyl and glucosyl residues (Goldstein 1974), Ricinus communis agglutinin-I (RCA-I) specific for β-galactosyl residues (Nicolson et al. 1974), soybean agglutinin (SBA) specific for N-acetyl galactosaminyl residues (Lis et al. 1970), peanut agglutinin (PNA) specific for the terminal disaccharides, galactose-N-acetyl galactosamine residues (Lotan et al. 1975). The tissues were incubated for 16 h at 25°C in 20 mM cacoodylate buffer (pH 7.4), containing each ferritin-conjugated lectin at a concentration of 0.5-0.3 mg/ml and 150 mM NaCl. CaCl2 (1 mM) and 1 mM MnCl2 were added to the experimental solution of Con A. Excessive lectins were removed by successive washing in 150 mM NaCl. Control tissue strips were incubated in lectin solutions containing inhibitory sugars (200-400 mM) for each lectin; α-methyl-D-mannose for Con A, lactose for RCA-I, N-acetyl galactosamine for SBA, and β-methyl galactose for PNA.

Colloidal iron (CI) staining procedure. To demonstrate acidic saccharides, the dialyzed CI staining procedure was employed because of its high specificity for them (Iwashita 1980). Fixed tissues were rinsed in 12% acetic acid and immersed in a dialyzed CI solution prepared according to the method of Mowry (1963) for 3 h at 25°C. Excessive CI was removed by successive washing in 12% acetic acid. To remove sialic acid from the glycoconjugates, fixed tissues were stained in a neuraminidase (EC 3.2.1.18, from Clostridium perfringens, Sigma type 5) solution containing 1 unit/ml enzyme, 100 mM acetate buffer (pH 5.0) and 50 mM NaCl for 4 h at 37°C. Following washing with 150 mM NaCl, the lung tissues were stained with CI.

To examine the properties of neuraminidase-resistant acidic saccharides, digestion with neuraminidase and then with hyaluronidase was carried out. Fixed tissues were incubated in the neuraminidase solution and washed in 150 mM NaCl. The tissues were then incubated in solutions containing 3000 units/ml testicular hyaluronidase (EC 3.2.1.35, from bovine testis, Sigma type 4), 50 mM NaCl, and 100 mM acetate buffer (pH 5.5) for 18 h at 37°C. After washing in 150 mM NaCl, the tissues were stained with CI. Control lung tissues were incubated in buffer solutions containing heat-inactivated enzymes or in buffer solutions without enzymes.

To differentiate sulfated saccharides from non-sulfated acidic saccharides, active and mild methylation procedures were used in combination with the CI staining procedures. For active methylation, the tissues were incubated in absolute methanol containing 0.1 N HCl for 8 h at 60°C. For mild methylation, they were incubated in the same HCl-methanol solution for 4 h at 37°C (Sperber 1960). After methylation, the tissues were washed in 150 mM NaCl and stained with CI.

Following the histochemical staining procedures, the tissue specimens were postfixed in 1% osmium tetroxide in 150 mM cacodylate buffer (pH 7.4) for 60 min at 4°C. After thorough washes in 150 mM NaCl, the tissues were stained en bloc for 20 min at 4°C with 0.5% uranyl acetate containing 150 mM NaCl (Iwatsuki 1980). Then they were rinsed in 150 mM NaCl, dehydrated and embedded in Epon 812. Ultrathin sections (80 nm thick) were cut on a Sorvall MT-2B ultramicrotome and examined using a Hitachi H-500 electron microscope. For electron microscopy, some of the glutaraldehyde-fixed lung tissues were conventionally postfixed with osmium tetroxide, stained en bloc with uranyl acetate, dehydrated and embedded in Epon 812.

Results

Glandular stage: day 16 of gestation

The glandular buds consisted of simple high columnar epithelium. All of the epithelial cells measured from 15 to 20 μm in height and exhibited numerous short microvilli on their surface (Fig. 1a). When stained with dia-
yzed CI, electron-dense CI particles were bound to the entire surface of the columnar epithelium, forming a continuous layer about 25 nm thick (Fig. 1b). Clumps of CI particles, 50-70 nm in diameter, were patchily distributed in the CI positive layer. As shown in Fig. 1c, predigestion with neuraminidase markedly decreased the number of CI particles bound to the surface of the epithelial cells. A small number of CI particles could be visualized on the surface of microvilli of the cells and clumps of CI particles occasionally remained on the surface of some epithelial cells. Following the predigestion, the epithelium contained a small number of epithelial cells with no CI binding (Fig. 1d).

In lung tissues digested with hyaluronidase following neuraminidase digestion, the clumps of CI particles disappeared but weak CI binding remained on the microvilli of most epithelial cells (Fig. 1e). No detectable alterations in CI binding were noted following incubation of tissues in an inactivated enzyme solution or in a buffer solution without the enzyme. As shown in Fig. 1f, mild methylation markedly decreased the intensity of CI binding. Weak CI reactivity was detected on the surface of the microvilli of most cells; the pattern of CI binding was similar to that after digestion with hyaluronidase in combination with predigestion with neuraminidase. In contrast, active methylation completely inhibited the CI binding on the surface of the cells.

When labeled with ferritin-conjugated lectins, all of the epithelial cells exhibited a similar lectin binding pattern. They heavily bound RCA-I (Fig. 1h), moderately bound Con A (Fig. 1g) and faintly bound SBA and PNA (Fig. 1i, j). Incubation of tissues in a mixture of lectins and their respective inhibitor sugars interfered with lectin binding to the surface of the epithelial cells.

Fig. 1a-j. Fetal lung at 16 days gestation. a Columnar epithelial cells; glycogen aggregates (G) can be recognized as clear areas. × 4000. b Colloidal iron (CI) binding on the luminal surface of epithelial cells. Clumps of CI particles (arrowheads) are distributed in the CI positive layer. × 40000. c, d Effects of neuraminidase digestion on CI binding. c One cell has both CI clumps (arrow) and is recognizable, x 50000. d Epithelium contains a small number of CI-binding negative cells. × 40000. e Effects of hyaluronidase digestion on CI binding after neuraminidase digestion. CI clumps disappear, and a small amount of CI (arrow) can be observed. × 40000. f Effects of mild methylation on CI binding; some CI binding (arrow) is recognizable. × 40000. g-j Lectin binding sites: Con A (g) is moderately bound, Ricinus communis agglutinin-I (RCA-I; h) heavily bound, soybean agglutinin (SBA; i) and peanut agglutinin (PNA; j) faintly bound. × 50000