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Temporal Linkage of Glycogen and Saturated Phosphatidylcholine in Fetal Lung Type II Cells

KATHLEEN S. CARLSON,[1] PAUL DAVIES, BARRY T. SMITH,[2] AND MARTIN POST[2]

Departments of Pediatrics and Pathology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT. The developmental profiles of glycogen and surfactant-associated saturated phosphatidylcholine were investigated in type II cells isolated from fetal rat lung. Incorporation of radiolabeled glucose into glycogen and type II cell unlabeled glycogen content decreased as a function of gestational age. Conversely, an increase was noted in radioactive choline incorporation into saturated phosphatidylcholine and in the content of unlabeled saturated phosphatidylcholine as a function of gestational age. Type II cells from days 19 and 21 of gestation were also studied by electron microscopy. Temporal relationships similar to those noted biochemically were observed by morphometric analysis. A decrease in glycogen content and an increase in lamellar bodies (the storage organelles for the pulmonary surfactant) were noted as gestation progressed. These studies biochemically and morphologically demonstrate a temporal relationship between glycogen degradation and saturated phosphatidylcholine synthesis in type II cells isolated from fetal rat lung. These findings provide further support for the use of such type II cell preparations for studies of development at the cellular level. (*Pediatr Res* 22: 79–82, 1987)

Abbreviations

SPC, saturated phosphatidylcholine
MEM, minimal essential medium
FCS, fetal calf serum
V_V, volume density
S_V, surface density
N_A, number per unit area

Fetal lung glycogen content has been examined in several mammalian species, including the rat, and a peak in glycogen content in the last third of gestation followed by a rapid decline has been described (1–4). In whole fetal rat lung, glycogen content appears to peak on day 20 after which it declines to low levels (1, 5). The decrease in pulmonary glycogen content is temporally related to the appearance of lamellar bodies in alveolar type II cells and to an increase in surface active phospholipid synthesis (1, 6, 7). Several investigators have postulated a link between glycogen degradation and the coincident rise in pulmonary surfactant, particularly phosphatidylcholine (8, 10). There are at least three pathways by which the disappearance of glycogen stores could be related to the increase in phosphatidylcholine during development. Glycogenolysis could provide substrate, reducing equivalents or energy which could subsequently be utilized for surfactant synthesis.

To date, biochemical and morphologic studies demonstrating the temporal relationship between glycogen degradation and phosphatidylcholine synthesis have focused on whole lung preparations. The aim of the present study was to determine if this temporal relationship is present in type II cells isolated from the fetal rat lung.

MATERIALS AND METHODS

Materials. Pregnant Sprague-Dawley rats of known gestational age were obtained from Charles River Laboratories, Wilmington, MA. Media and sera were purchased from Flow Laboratories, McLean, VA. Plasticware was from Falcon Plastics, Oxnard, CA. The Gelfoam sponges were obtained from Upjohn, Kalamazoo, MI. Trypsin, DNase, and collagenase were from Worthington Biochemical, Freehold, NJ. All remaining unlabeled biochemicals were obtained from Sigma Biochemical Company, St. Louis, MO. (Me-^{3}H)choline (spec. act. 80 Ci/mmol) was purchased from New England Nuclear and D-[U-^{14}C]glucose (spec. act. 275 mCi/mmol) was obtained from Amersham, Arlington Heights, IL.

Cell cultures. At 18 through 22 days gestation rat fetuses were removed from the mothers. The fetal lungs were dissected out and dissociated into individual cells by trypsinization (0.05% trypsin in Hanks' balanced salt solution). The cells were collected by centrifugation and resuspended in minimal essential medium containing 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and 10% fetal calf serum (MEM/10% FCS). The cells were pelleted by centrifugation, incubated as such for 1 h at 37° C to allow reaggregation (12), and then resuspended in MEM/10% FCS. The cells were then seeded onto Gelfoam sponges and incubated in Petri dishes for 20 h in MEM/10% FCS. At the end of this period, these organotypic cultures were used for isolation of type II cells or the organotypic cultures were used for 24-h incorporation studies prior to type II cell isolation. Type II cells were isolated from the Gelfoam sponges as alveolar-like structures by collagenase treatment as previously described (11). The purity of the cell population isolated as alveolar-like structures was confirmed by histochemical (glycogen, lamellar body staining) and immunocytological (cytokeratin, apoprotein, and Maclura pomifera staining) parameters and appeared to be greater than 85% type II cells. The yield of type II cells was cells approximately 10 million cells per litter, decreasing toward term. Cell viability as assessed by trypan blue exclusion was greater than 90%. Isolated type II cells not used for incorporation studies were resuspended in 0.45 M NaCl, 50 mM Tris-HCl (pH 7.4), 50 mM NaF, and 2.5 mM EDTA. The cells were ruptured with 50 strokes of a glass Dounce homogenizer and then subjected to two 1-s bursts of sonication (maximal output). Aliquots from the type II cell

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Address for reprints Kathleen S. Carlson, M.D., Division of Newborn Medicine, Department of Pediatrics, New England Medical Center, 750 Washington Street, Boston, MA 02111.
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[1] Present address Division of Newborn Medicine, Department of Pediatrics, New England Medical Center, Boston, MA 02111.
[2] Present address The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

homogenates were subsequently taken for determination of DNA, protein, glycogen, and SPC content.

Incorporation studies. Organotypic cultures from days 18 through 22 of gestation were incubated for 24 h in 1 ml MEM containing 1 μCi/ml (Me-^{3}H)choline or in glucose-free Earle's medium containing 1 μCi/ml [U-^{14} C]glucose. At the end of this period, type II cells were isolated from the sponges. SPC determinations were made on the lipid extracts of the cells. Incorporation of glucose into glycogen was also determined.

SPC determinations. Type II cells previously incubated with (Me-^{3}H)choline and aliquots from the type II cell homogenates were extracted with chloroform-methanol (13). The lipid extracts of the cells were treated with osmium tetroxide (14) followed by thin-layer chromatography on precoated silica gel H plates (Eastman Kodak, Rochester, NY) with chloroform/methanol/water (65:25:4, v/v) as solvent (15). Saturated phosphatidylcholine was visualized with a bromothymol blue solution. The SPC spots from the incorporation studies were transferred to scintillation vials and radioactivity measured in a scintillation counter. The SPC spots from the type II cell homogenates were transferred to new glass test tubes and assayed for phosphorus. A standard curve was run in duplicate for each assay.

Glycogen determinations. Type II cells from the incorporation studies with [U-^{14}C]glucose and aliquots from the type II cell homogenates were digested with 30% KOH saturated with Na_2SO_4 as described by Lo *et al.* (16). Glycogen was precipitated from the alkaline digest by adding 95% ethanol (1.2 vol). The samples were placed on ice for 30 min and then were centrifuged at 12,000 × *g* for 15 min. The supernatants were aspirated. The glycogen precipitates were then dissolved in distilled water. Samples from the incorporation studies were transferred to scintillation vials and radioactivity measured in a scintillation counter. The glycogen solutions from the type II cell homogenates were analyzed with a modified phenol-sulfuric acid colorimetric technique (16). The absorbance was read on a Beckman spectrophotometer at 490 mu. Standard curves from 0 to 100 μg glycogen were run for each assay.

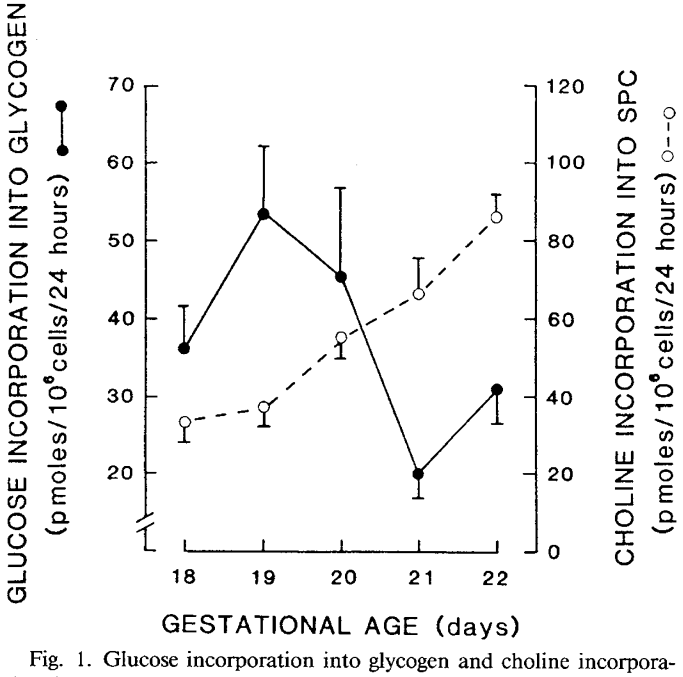

Fig. 1. Glucose incorporation into glycogen and choline incorporation into saturated phosphatidylcholine by fetal type II cells during development. Organotypic cultures were prepared with lung cells from fetal rats at 18 to 22 days gestation. The organotypic cultures were incubated with glucose-free Earle's medium supplemented with 1 μCi/ml [U-^{14}C]glucose or MEM supplemented with 1 μCi/ml (Me-^{3}H)choline. After 24 h of incubation, the type II cells were isolated from the organotypic cultures. Incorporation of radioactive glucose into glycogen and choline into saturated phosphatidylcholine were measured. The data represent the means ± SEM of three independent experiments carried out in quadruplicate.

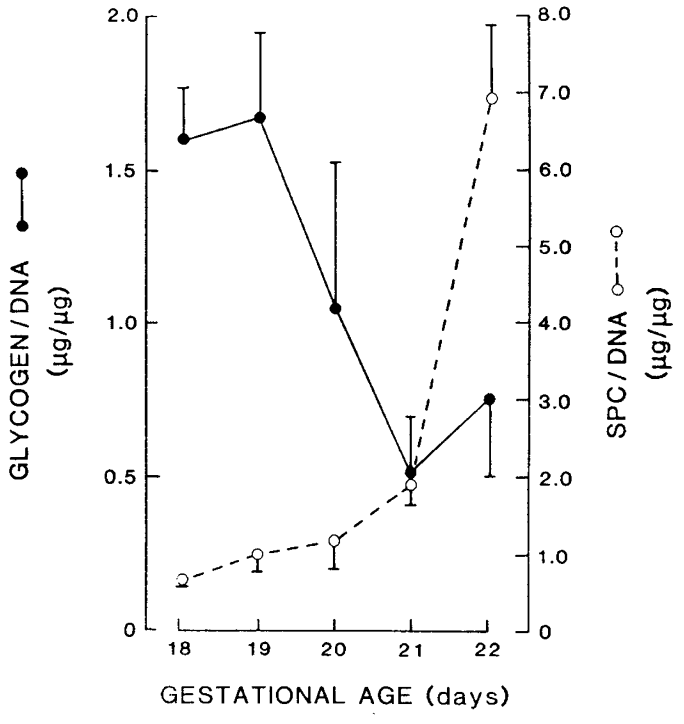

Fig. 2. Developmental profile for fetal type II cell glycogen and saturated phosphatidylcholine content. Organotypic cultures were prepared with lung cells from fetal rats at 18 to 22 days gestation. After 20 h of incubation, the type II cells were isolated from the organotypic cultures. Determinations of glycogen, saturated phosphatidylcholine, and DNA were made. The data represent means ± SEM for three independent experiments performed in quadruplicate.

Other biochemical determinations. DNA was assayed by the modified method of Burton (17). Protein was assayed by the technique of Bradford (18) and Spector (19). Phospholipid phosphorus was measured according to the method of Bartlett (20).

Electron microscopy and morphometry. Type II cells isolated at days 19 and 21 of gestation were fixed in suspension in a solution of 2% glutaraldehyde in 100 mM sodium cacodylate, pH 7.2, for 60 min at room temperature. After three washes in buffer, they were postfixed in 1% OsO_4 in cacodylate for 90 min. The cell pellets were embedded in Spurr's resin. Ultra thin sections were collected on 200 mesh copper grids, stained in uranyl acetate and lead citrate, and viewed in a Philips 300 electron microscope operating at 60 kV. For morphometry, cell profiles were randomly photographed on 3 × 4 inch film sheets at a magnification of 6800×. Each image was printed on 10 × 8 inch photographic paper at a final magnification of 19,440×. There were a total of 51 cell profiles from the 19-day sample and 59 from the 21-day sample. Morphometry was performed on each electron micrograph by the method of Weibel (21).

Statistical analysis was performed using the Student's *t* test.

RESULTS

Incorporation studies. Incorporation of [U-^{14}C]glucose into glycogen peaks on day 19 followed by a decrease toward term (Fig. 1). Figure 1 shows that (Me-^{3}H)choline incorporation into saturated phosphatidylcholine increases as a function of gestational age.

Chemical measurements. Similar developmental patterns were noted for measurements of type II cell glycogen and SPC. Figure

2 shows that the glycogen content of type II cells peaks on day 19 and subsequently decreases as gestation progresses. On the other hand, type II cell SPC content increases at the end of gestation as noted in Figure 2.

Table 1. *Morphometric assessment of isolated fetal type II cells by electron microscopy**

Ratio	Day 19 ($n = 51$)	Day 21 ($n = 59$)
V_V in total cytoplasm		
Lamellar bodies	5 ± 0.2	55 ± 0.9†
Glycogen	74 ± 2	12 ± 0.6†
Multivesicular bodies	23 ± 0.5	54 ± 0.8†
Mitochondria	40 ± 1	43 ± 0.6
Lipid	4 ± 0.2	8 ± 0.5
Cytoplasm	859 ± 2	842 ± 2
Nucleus	615 ± 7	557 ± 6
V_V in total cell		
Nucleus	357 ± 3	325 ± 3
S_V in total cytoplasm (μ^{-1})		
Lamellar bodies	21 ± 1	181 ± 4†
Glycogen	552 ± 13	61 ± 4†
Endoplasmic reticulum	118 ± 4	374 ± 10‡
S_V in total cell (μ^{-1})		
Cell membrane	789 ± 6	1496 ± 11†
N_A in total cytoplasm (μ^{-2})		
Lamellar bodies	12 ± 0.5	72 ± 1†
Glycogen	147 ± 3	24 ± 1†

* Organotypic cultures were prepared with lung cells from fetal rats at 19 and 21 days gestation. After 20 h of incubation, the type II were isolated from the organotypic cultures and fixed for electron microscopy. The data are presented as means ± SEM × 10^3.

† $p < 0.0001$.

‡ $p < 0.01$.

Morphologic studies. Table 1 lists the parameters evaluated by morphometric analysis of day 19 and 21 type II cells. Student's *t* test was used for statistical analysis. V_V, S_V, and N_A of glycogen deposits decreases and of lamellar bodies increases from day 19 to 21. Similarly the volume density of multivesicular bodies increases from days 19 to 21. The surface density of endoplasmic reticulum and cell membrane exhibit developmental increases. Representative electron micrographs are shown in Figure 3.

DISCUSSION

We have biochemically demonstrated that the disappearance of glycogen is temporally associated with the appearance of saturated phosphatidylcholine in isolated fetal type II cells. This temporal relationship has been noted previously for whole lung (1, 6, 7). Findings from the present study are consistent with a link between glycogen breakdown and the synthesis of surfactant phospholipids that has been postulated by several authors (8–10).

Ultrastructural study of isolated type II cells on days 19 and 21 of gestation also revealed an inverse relationship between the presence of glycogen and lamellar bodies as a function of gestational age. These findings are consistent with previous whole lung morphometric studies which have shown a temporal relationship between the disappearance of glycogen deposits and the appearance of lamellar bodies in the type II cell during fetal lung development (6, 7). In adult lungs, morphologic studies clearly demonstrate that multivesicular bodies participate in lamellar body formation (22). Consequently, the developmental increase in volume density of multivesicular bodies observed herein (Table 1) is not surprising. Increased type II cell microvillus formation during late gestation may explain the increase in surface density of the cell membrane noted between days 19 and 21.

In summary, these studies biochemically demonstrate a temporal relationship between glycogen degradation and saturated phosphatidylcholine synthesis within the type II cell. Type II cell electron microscopic studies reveal a similar temporal relationship. These investigations support the use of freshly isolated type II cells from organotypic cultures as a good system for the study of biochemical events during development.

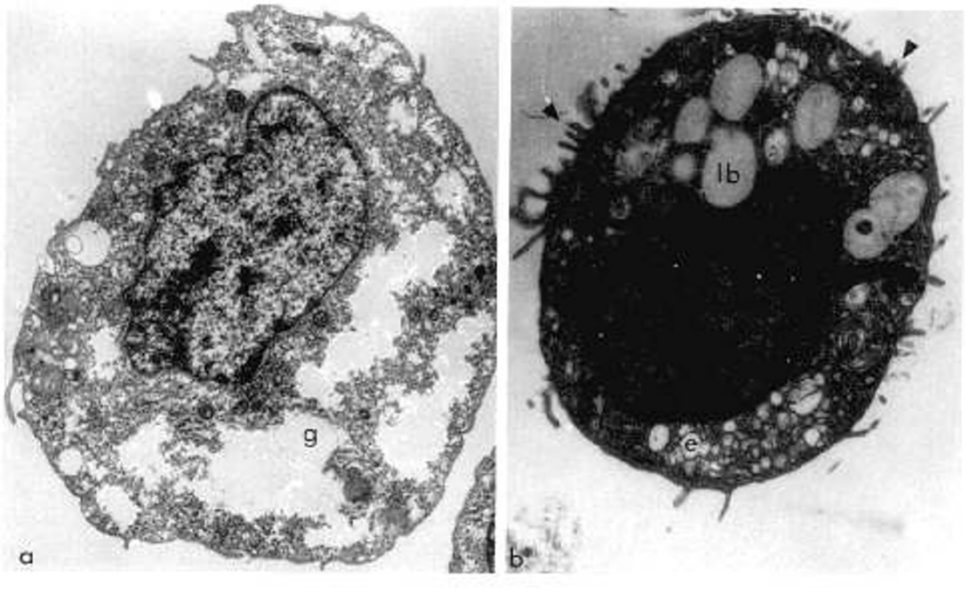

Fig. 3. Electron micrographs of rat fetal lung epithelial cells fixed in suspension after *in vitro* culture. Both are at a magnification of ×16,0000. *A*, cell from a 19-day fetus. Much of the cytoplasm is filled with moderately electron-dense glycogen (*g*). *B*, cell from a 21-day fetus. The cytoplasm is filled with large lamellar bodies (*lb*) and dilated cisternae of the endoplasmic reticulum (*e*). Microvilli (*arrows*) occur at the cell boundary.

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