AN ORGAN CULTURE SYSTEM FOR STUDY OF FETAL LUNG DEVELOPMENT

Jane Dawson Funkhouser, E. R. Hughes and R. D. A. Peterson

From the University of South Alabama, Mobile, Alabama, 36617

Received March 26,1976

Summary

Fetal rat lungs placed in in vitro organ culture at 15.5 days gestation grow significantly based on accumulation of DNA and protein. In the experimental system described, DNA accumulated rapidly during the first three days in culture and increased from 4.8 to 15.6 micrograms per lung culture. Protein content increased more slowly and reached a value more than double the initial value after six days in the culture system. Glycogen accumulated in the tissue during the first six days in culture and was depleted during the subsequent culture period, a pattern strikingly similar to that observed during lung development in vivo. Phospholipid accumulation was biphasic with respect to time with an inflection point at about the sixth day of culture. The phosphatidylcholine species synthesized in the culture system in vitro were similar to those produced in vivo in fetal lung at 21 days gestation.

Introduction

The following report describes an in vitro organ culture system for fetal rat lung. The system has been developed to study the regulation of the biosynthesis of pulmonary surfactant during fetal development. The major advantage of this system is the maintenance of a near normal physical relationship between the lung mesenchymal and epithelial tissues during the developmental period. Mesenchymal-epithelial interactions are known to be important during the epithelial proliferation associated with bronchial budding in the embryonic lung (1) and in the hydrocortisone effect on carbohydrate metabolism in lung (2). Mesenchymal-epithelial interactions are also involved in differentiation of a number of other organs including developing kidney (3), salivary gland (4), thymus (5), pancreas (6), thyroid (7), and mammary gland (8).

The fetal rat lung removed at 15.5 days gestation and placed in this system follows the growth pattern and exhibits biochemical changes characteristic of fetal lung differentiation in vivo.

Materials and Methods

Animals: Timed-bred Sprague Dawley rats were obtained from Charles River Laboratories, Wilmington, Mass. The fetuses were harvested under sterile conditions at 15.5 days gestation following maternal laparotomy using ether anesthesia. The fetal lungs were dissected in ice-cold phosphate buffered isotonic saline (9).

Organ culture: The lungs were cultured at the gas-liquid interface in a humidified atmosphere of 95% air and 5% CO_2 at 37°. Stainless steel wire grids (about 0.5 mm-square mesh, 5 X 10 mm area) covered with Millipore filter paper (GS 0.22 micron) were used to support the organs. The cultures were placed in 35 X 10 mm plastic dishes (Falcon Plastics, Oxnard, Calif.) containing two mls Medium MB 752/1 (Waymouth) from Grand Island Biological Company, Grand Island, N. Y., 14072. The medium contained 10% heat inactivated fetal calf serum (Grand Island Biological Co.) and 100 units per ml Penicillin-G. Each culture contained lung tissue from one fetus. Cultures were harvested by removing the lung from the filter with a cataract knife. The tissue was washed with 10 ml ice cold 0.9% NaCl and homogenized (30 strokes) in 0.2 ml ice water using 1.5 ml homogenizers with a teflon pestle. In experiments where lipid was extracted, 0.2 ml chloroform-methanol (2:1) was substituted for the ice water and the number of strokes increased to 50.

DNA analysis: DNA was estimated using the fluorometric assay of Kissane and Robbins (10) as modified by Hinegardner (11). Calf thymus DNA (Sigma Chemical Company) was used as standard.

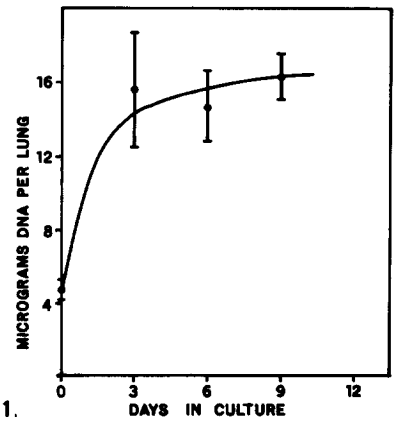
Lipid phosphorus analysis: Tissue was homogenized in chloroform:methanol (2:1) and extracted with an equal volume of distilled water. The aqueous phase was re-extracted with chloroform and the organic phase with water. The organic extracts were then pooled and evaporated to dryness. The samples were ashed and phosphorus determined by the procedure described by Chen et al (14).

Glycogen analysis: Glycogen was determined using the anthrone colorimetric assay described by Carroll et al (15). Glucose was used as standard.

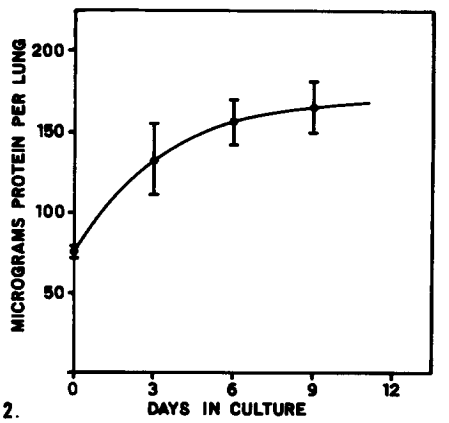
Gas chromatography: Thin-layer chromatography was used to separate the phosphatidylcholine fraction from other lipids in the chloroform:methanol extracts. The chromatography was performed on Silica Gel G (250 microns) with a solvent system of chloroform:methanol:water (65:25:4 v/v/v) (16). Precoated thin layer plates were obtained from Analtech, Inc., Newark, Delaware, 19711. Dipalmitoyl phosphatidylcholine (Sigma Chemical Co.) was used as standard. The area corresponding to phosphatidylcholine was scraped from the plate and eluted using chloroform:methanol:acetic acid:water (25:15:4:2, v/v) (17). Hydrolysis and esterification with sodium methoxide (18) was used to obtain methyl esters of the acyl substituents of the phosphatidylcholine. The derivatives were measured using a Hewlett Packard 5830A gas chromatograph with a flame ionization detector and 6' long, 6 mm O. D. glass column packed with 10% ethylene glycol succinate on high performance Chrom W (AW-DMCS) 80/100 mesh. The column was used isothermally at 180° and calibrated with methyl ester derivatives (Sigma Chemical Co.) using heptadecanoic methyl ester as the internal standard.

Results and Discussion

The fetal rat lung removed from the fetus at 15.5 days gestation and cultured in the *in vitro* organ culture system showed a period of growth which continued for approximately six days. DNA and protein content of the



1.



2.

Figures 1 and 2: DNA and protein content of the lung cultures at different times during the culture period. Each point represents the mean value from six different cultures. Error bars represent the standard error of the mean. The first point represents the value at the time the lungs were placed in culture (15.5 days gestational age). Methods of culture and analysis are described in Materials and Methods.

individual lung cultures was used as an index of growth (19). During the following six days in culture, phospholipid synthesis increased and glycogen which had accumulated during the period of tissue growth disappeared. The DNA content increased rapidly during the first three days in culture (Fig. 1) and reached a maximum at day three with approximately a three-fold increase compared to the zero time explant. Protein content (Fig. 2) increased more slowly over about six days in culture. The maximum protein attained during the culture period was slightly more than double the initial value and several fold less than expected growth *in vivo*. This maximum is characteristic of a number of fetal organs *in vitro* which grow less rapidly and fail to approach *in vivo* growth rates and total mass (20). Change of the medium after 3 days in culture resulted in no appreciable further growth. Tissue explants which exceed 1-2 mm in any dimension show rapid necrosis in the center (21) suggesting a limitation on mass imposed by nutrient and oxygen diffusion. Nevertheless, the pattern of accumulation of DNA and protein observed in Figures 1 and 2 resembles the growth pattern observed *in vivo*. The fetal rat lung *in vivo* is growing rapidly

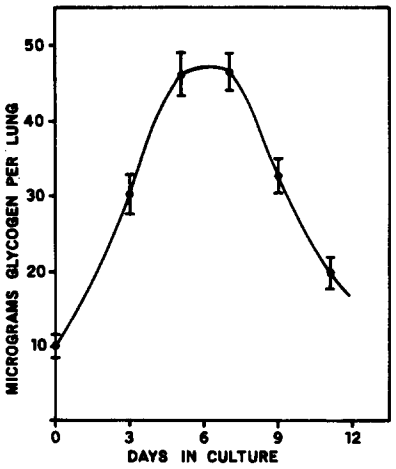


Figure 3: Glycogen content of the lung cultures. Each point on the graph represents the mean value from 12 different cultures. The error bars represent the standard error of the mean. The initial point represents the value at 15.5 days gestational age, the time the lungs were placed in culture. Glycogen assay and culture methods are described in Materials and Methods.

from gestational day 16 to 20. On day 20, cell division decreases and cellular differentiation rapidly ensues prior to birth by day 22 (22).

The pattern of fetal lung glycogen accumulation and depletion in vivo is well documented in a number of mammalian species (1, 2, 23-26) and lung glycogen levels have been used as an index of differentiation and functional maturity of lung (25-27). Glycogen accumulates during the period of rapid growth associated with epithelial proliferation (gestational days 16-20), reaches a peak at gestational day 20, and rapidly disappears during further differentiation (23). A pattern of glycogen accumulation similar to that described above was found in the fetal lungs grown in organ culture (Fig. 3). The rate of glycogen accumulation is less than the rate of DNA accumulation but greater than that for protein accumulation. The net increase in glycogen is also greater than the increase in either DNA or protein. The increased glycogen:protein ratio seen here has been reported in developing mouse lung in vivo as well as in vitro (2).

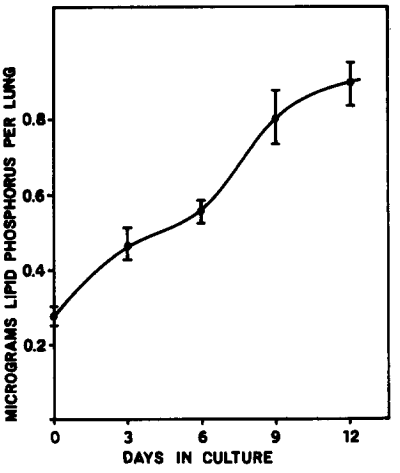


Figure 4: Phospholipid accumulation in fetal rat lung cultured in vitro. The data points represent the mean value from six different cultures and the error bars the standard error of the mean. Lipid phosphorus was determined by analysis of the phosphorus in a chloroform:methanol extract from each culture. Details of the extraction procedure and phosphorus analysis are described in Materials and Methods.

A parameter of primary importance in evaluating lung development is surfactant synthesis. Pulmonary surfactant, which is the surface active component at the air-liquid interface in alveolar spaces, is composed of about 75% phospholipid, mostly phosphatidylcholine (about 90%) (28). Significant increases in phospholipid content of fetal rat lung tissue occur in vivo on the 21 and 22 gestational days, simultaneous with differentiation (30).

The phospholipid content of the fetal lungs in culture was estimated by analysis of phosphorus in a chloroform:methanol (2:1) extract. When phospholipid content per lung is plotted as a function of time in culture (Fig. 4), the curve appears biphasic with an inflection point at about the sixth day in culture. This inflection point occurs at approximately the same time as maximum glycogen accumulation and immediately following the maximal growth period estimated from the DNA and protein data (Figures 1 and 2). A similar shaped curve was obtained by Weinhold and Villee (30) when micromoles lipid/gm tissue from fetal rat lung developing in vivo was plotted as a function of

gestational age. In vivo the inflection point occurred at 21 days gestation. In our system, the inflection occurred at 6 days in vitro which represents a 21.5 day total gestational period. This demonstrates a similarity between the time course of phospholipid accumulation of the fetal rat lung in vitro and in vivo. One interpretation of the biphasic lipid phosphorus curve is that the first phase represents phospholipid accumulation in the cell membranes during growth and the second phase the increased phospholipid synthesis associated with surfactant production.

During the second phase of the phospholipid curve, (Fig. 4) the pattern of fatty acyl substitution in the phosphatidylcholine fraction was determined and compared to that from 21 day gestational lung developing in vivo. Characterization of the phosphatidylcholine fraction from lung is of interest because increased tissue content of disaturated species particularly dipalmitoyl phosphatidylcholine parallels increased pulmonary maturity (30, 31). Palmitate is the major fatty acyl substituent of phosphatidylcholine in lung from rabbit (32), pig (33), rat (25), and rhesus monkey (34). As shown in Table 1 the phosphatidylcholine in the culture system is qualitatively similar to that present at 21 days gestational age in vivo.

The data presented here have demonstrated that fetal lung tissue removed at 15.5 days gestational age can be maintained in organ culture for a sufficient period of time for studies of biochemical and morphological differentiation. The presence of a potential for differentiation at early developmental stages was suggested earlier by Sorkin (21) based on morphological and histochemical studies of fetal rat lung. These data provide biochemical evidence that a certain potential for differentiation independent of known external influences already exists in the fetal lung removed at 15.5 days gestation. The system described has advantages other than those described above. These include ease of manipulation and precise control of hormone concentration, oxygen tension, pH, etc. In vitro organ culture also provides a definitive way to distinguish between direct effects of hormones on the lung and secondary effects due to other physiological changes.

Table 1

Comparison of Acyl Substituents in Phosphatidylcholine Fractions from *In Vitro* Lung Culture and Fetal Lung *In Vivo*

Lung Specimen	Phosphatidylcholine Acyl Substituents (% of total)						
	<16:0	16:0	16:1	18:0	18:1	18:2	18:3
15.5 days gest. cultured 14 days	2.4	45.6	14.0	7.6	30.4	ND	ND
21 day gest. not cultured	5.6	50.7	13.2	6.0	19.6	4.7	ND

ND is not detected.

Ten lung cultures remaining at the end of the phospholipid experiment shown in Fig. 4 were pooled after 14 days in culture for qualitative determination of the fatty acyl substituents. Twenty-one day gestation fetal lung was analyzed for comparison. The phosphatidylcholine was isolated by thin layer chromatography (16) and methyl ester derivatives were prepared by hydrolysis with sodium methoxide (18). Gas chromatography was performed as described in Materials and Methods using heptadecanoic methyl ester (Sigma Chemical Co.) as internal standard. Approximately 20 microgram samples of the methyl ester derivatives were used for analysis.

Acknowledgement

Jane Dawson Funkhouser was supported by National Institutes of Health Postdoctoral Fellowship 5 F22 HD00502. The authors express appreciation to Ms. Judy Grafton for typing the manuscript and Ms. Jennifer Johnson for assistance with the animal surgery.

REFERENCES

1. Alescio, T. and Dani, A. M. (1971) J. Embryol. Exp. Morphol. 25, 131-140.
2. Alescio, T. and Dani, A. M. (1972) J. Embryol, Exp. Morphol. 27, 155-162.
3. Lombard, M. N. and Grobstein, C. (1969) Develop. Biol. 19, 41-51.
4. Grobstein, C. (1953) Nature 171, 869-871.
5. Auerback, R. (1960) Develop. Biol. 2, 271-284.
6. Golosow, N. and Grobstein, C. (1962) Develop. Biol. 4, 242-255.
7. Hilfer, S. R. (1962) Develop. Biol. 4, 1-21.
8. Kratochwil, K. (1969) Develop. Biol. 20, 46-71.
9. Merchant, D. J., Kahn, R. H. and Murphy, Wm. H., Jr. (1964) Handbook of Cell and Organ Culture, pp. 240, Burgess, Minneapolis.
10. Kissane, J. M. and Robbins, E. (1958) J. Biol. Chem. 233, 184-188.
11. Hinegardner, R. T. (1971) Anal. Biochem. 39, 197-201.

12. Williams, C. A. and Chase, M. W. (1968) Methods in Immunology and Immunochemistry, Vol. II, Physical and Chemical Methods, pp. 273-275, Academic Press, New York.
13. Lowry, O. H., Rosebrough, N. J. Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
14. Chen, P. S., Jr., Toribara, T. Y. and Warner, H. (1956) Anal. Chem. 28, 1756-1758.
15. Carroll, N. V., Longley, R. W. and Roe, J. H. (1956) J. Biol. Chem. 220, 583-593.
16. Kuksis, A. Breckenridge, W. C., Marai, L. and Stachnyk, O. (1963) J. Amer. Oil Chem. Soc. 45, 537-546.
17. Dittmer, J. C. and Wells, M. A. (1969) Methods in Enzymology XIV (Lowenstein, J. M., ed.) pp. 482-530, Academic Press, New York.
18. Marinetti, G. V. (1962) Biochem. 1, 350-353.
19. Carson, S. H., Taeusch, Wm. H., Jr. and Avery, M. E. (1973) J. of Appl. Physiol. 34, 660-663.
20. Sorokin, S. (1961) Develop. Biol. 3, 60-83.
21. Wessells, N. K. (1967) Methods in Developmental Biology, pp. 445-456, Thomas Y. Crowell Co., New York.
22. O'Hare, K. H. and Townes, P. L. (1970) J. Morph. 132, 69-87.
23. Blackburn, W. R. Travers, H. and Potter, D. M. (1972) Lab. Invest. 26, 306-318.
24. Brandstrup, N. and Kretchmer, N. (1965) Develop. Biol. 11, 202-216.
25. Blackburn, W. R. Kelly, J. S. Dickman, P. S. Travers, H. Lopata, M. A. and Rhoades, R. A. (1973) Lab. Invest. 28, 352-360.
26. Kikkaway, Y. Kaibara, M. Motoyama, E. K. Orzalesi, M. M. and Cook, C. D. (1971) Am. J. Pathol. 64, 423-442.
27. Wu, B. Kikkawa, Y., Orzaleski, M. M. Motoyama, E. K., Kaibara, M. Zigas, C. J. and Cook, C. D. (1973) Biol. Neonate 22, 161-168.
28. King, R. J. and Clements, J. A. (1972) Amer. J. of Physiol. 223, 715-726.
29. Blackburn, W. R. and Potter, D. M. Travers, H., Gassenheimer, L. L. and Rhoades, R. A. (1972) Proc. Soc. Exp. Biol. Med. 140, 885-889.
30. Weinhold, P. A. and Villee, C. A. (1965) Biochem. Biophys. Acta 106, 540-550.
31. Brumley, G. W. Chernick, V. Hodson, W. A. Normand, C., Fenner, A. and Avery, M. E. (1967) J. Clin. Invest. 46, 863-873.
32. Acebal, C. Archie, R. Castro, J. and Municio, A. M. (1974) Steroids Lipids Res. 5, 65-71.
33. Gray, G. M. and MacFarlane, M. G. (1961) Biochem. J. 81, 480-488.
34. Kerr, G. R. and Helmuth, A. C. (1975) Biol. Neonate 25, 10-22.