EFFECTS OF PREDNISOLONE ON RAT LUNG DEVELOPMENT IN ORGAN CULTURE

A THESIS
Presented to the Faculty of Graduate Studies
University of Manitoba

In partial fulfillment
of the
requirements for the degree of
Master of Science

by

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April, 1978

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To My Parents

ACKNOWLEDGEMENTS

I wish to express my appreciation to my advisor, Dr. I. Y. R. Adamson, for his direction and support throughout this investigation. Thanks are also due to Drs. D. H. Bowden and J. S. Henderson for their helpful advice.

To the technical staff of the Department of Pathology, my sincere thanks for their many services. I also like to thank Ms. J. Moore for her co-operation in typing this manuscript.

I am particularly grateful to the University of Manitoba for a Graduate Fellowship which was awarded to me during the tenure of this research.

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ABSTRACT

The administration of glucocorticoids to fetuses is known to accelerate lung maturation and to produce a functional blood-air barrier at birth. However, questions remain as to the optimum time for drug administration, the precise cellular localization of the glucocorticoid receptors in fetal lung and the effects of steroid on epithelial cells and other lung cells such as the alveolar macrophages, which are newly formed just before birth. In the present investigation, two variations of an organ culture system are used to study these problems.

Lung explants from rats (Term: 22 days) of each gestation day 17 to 22 and of postnatal days 1,2,5,7, and 10 are cultured for three days in normal and prednisolone-treated growth media; the morphology is compared by light and electron microscopy. The timing of maximal tritiated prednisolone incorporation by the lung explants is studied by liquid scintillation counting. Autoradiographic techniques are used to identify the particular cell type which is predominantly labeled. The influence of prednisolone on the production of alveolar macrophages is studied by culturing lung explants on cover glasses and counting the number of cells that migrate outward from the tissue after a 7 day culture period.

The gestation day when alveolar macrophages first appear is noted, and the morphology of alveolar macrophages from prenatal and postnatal rats is compared.

Developmental processes in the lung explants are found to be accelerated by prednisolone beginning around day 19 of gestation. Lamellar bodies are present at an earlier time and in larger amounts in the prednisolone-treated explants compared to the control. The production of lamellar bodies in the type II cells is accompanied by decreasing amounts of intra-cytoplasmic glycogen. Prednisolone also appears to inhibit the epithelial and interstitial cell proliferation of the explants. Results from the liquid scintillation counting show peaks of ^{3}H prednisolone incorporation on gestation day 20 and postnatal day 1. The autoradiographs indicate that ^{3}H prednisolone is preferentially incorporated by the cuboidal epithelial cells lining the lumens on gestation day 18. As cytodifferentiation continues, type II cells are found to be predominantly labeled; silver grains are observed over the cytoplasm, frequently associated with the lamellar bodies. Alveolar macrophages are first observed in the explants on gestation day 19. Prenatal alveolar macrophages have an immature appearance with very few intracellular organelles and phagocytic inclusions. Postnatally, large amounts of cell debris and phospholipid-rich materials are found in these cells which

contain more organelles such as lysosomes and rough endoplasmic reticulum. Fewer macrophages are found in the halos around the prednisolone-treated explants compared to the control, suggesting that prednisolone has an inhibitory effect on the production of alveolar macrophages.

From the present study, it is clearly demonstrated that after day 18 prednisolone enhances the differentiation of the epithelial cells of fetal rats considerably. Maximal uptake of tritiated prednisolone before birth is observed around day 20 and coincides with the initiation of cytodifferentation. Glucocorticoid receptors are present in the cuboidal epithelial cells on gestation day 18; and subsequently present in the type II cells which are actively involved in the synthesis, storage and secretion of pulmonary surfactant. A few alveolar macrophages are first observed on gestation day 19. They are probably serving as scavengers of the excess pulmonary surfactant secreted during the perinatal period. Prednisolone may influence the production of alveolar macrophages by inhibiting the mitotic division of the interstitial cells which are believed to be the direct precursors. This inhibitory effect may be of clinical importance as the steroid-treated newborns may be more susceptible to infection.

INTRODUCTION

REVIEW OF RELATED LITERATURE

DEVELOPMENT OF NORMAL LUNG

In the early embryonic stage, the lungs, trachea and extrapulmonary bronchi develop from an endodermal outgrowth from the foregut. The tubular lung bud later undergoes extensive branching to form the bronchial tree. The cells that eventually make up the pulmonary epithelial lining (ciliated, goblet, alveolar epithelial cells, and epithelial derivatives, such as secretory cells of bronchial glands) are derived from endoderm whereas other structures, such as pleura, muscle, cartilage, blood vessels, etc. form from lung-associated mesoderm and mesenchyme.

Although lung development proceeds in a smooth and continuous fashion rather than abrupt steps, the intrauterine and perinatal growth and development of rat lung has been divided into three stages. The glandular period is the most extensive period in utero for the rat. During this time the lungs consists of a loose mass of mesenchymal connective tissue with branching airways which are lined by columnar or cuboidal epithelial cells. Blood vessels are generally not in direct contact with the epithelial lined tubules at this time but are widely distributed in the mesenchyme. In fetal rat lung, glycogen accumulates in large quantities in the cuboidal

epithelial cells from day 17 to 19. As the type II cell differentiates in late gestation (days 19 to 21 in rat), the size of the cell decreases together with a rapid depletion of the intracytoplasmic glycogen content. This is accompanied by the appearance of the lamellar bodies in the type II cells. The first appearance of surfactant in the fetal lung coincides with this morphologic change which usually occurs at approximately day 20 of gestation in the rat (Buckingham and Avery, 1962). Subsequently, the complete cuboidal epithelial lining of the tubules transforms into functional type II cells, marking the transition from the glandular to the canalicular phase of development (O'Hare and Sheridan, 1970).

Physiological changes within the fetal lung marking the change from glandular to canalicular are accompanied by several morphological changes. Blood capillaries became apposed to the cuboidal epithelium and appear to bulge into the airways. At this point, as cytodifferentiation occurs, attentuated epithelium is observed to give a primitive air-blood barrier. The rat is born in this phase of the lung development (Thurlbeck, 1975). It has no alveoli but rather a lung built up of primitive air sacs which have very thick septa and contain two capillary networks, each one facing an air sac (Burri,1974). This pattern remains practically unchanged for the first 5 days of life. The development of lung from the canalicular to the alveolar stage involves a change in degree rather than kind and takes place postnatally.

The transition from canalicular to alveolar stage is marked by the appearance of secondary crests which subdivide the primary terminal air sacs into alveoli. During this process the septa becomes thinner and the interstitium is reduced to a single capillary network between adjacent alveoli. Three postnatal pulmonary developmental stages in the rat are described by Burri (1974). During the first four postnatal days, the lung enlarges by expansion of the existing air saccules, followed by a period of rapid tissue proliferation. In this period, up to 2 weeks after birth in the rat, there is a vigorous increase in tissue mass followed by increased capillarization. This process of septation, which produces actual alveoli, greatly increases the surface area of the lung. The equilibrated growth phase begins some time after 2 weeks and continues until the lung reaches maturity. Newly formed alveoli are slowly enlarged and some alveolarization still takes place at a reduced rate. Although approximately 40 cell types can be identified in the adult lung (Sorokin, 1961), the alveolar epithelial cells are of particular concern to this study.

Alveolar Epithelium--Structure

In the normal adult mammalian lung, the alveolar surface is lined by continuous epithelium, formed by two types of cells, both derived from endoderm. The continuity of the alveolar epithelial lining was first observed by Low in 1952 with an electron microscope.

The greater part of the alveolar surface is lined by flattened cells which have a diameter of about 50-60 μm; they have very thin attenuated cytoplasm that is not clearly visible by light microscopy. These cells are commonly known as type I pneumocytes, but are also referred to as membranous pneumocytes, type A cells, small alveolar cells, or pulmonary epithelial cells. Type I cells characteristically have scanty perinuclear cytoplasm containing very few intracellular organelles. They cover most of the alveolar surface, and they sometimes partly cover the other alveolar cell--the type II pneumonocyte. This cell type is also known as granular pneumonocyte, great alveolar cell, large alveolar cell, alveolar cell, septal cell, type B cell, Niche cell or corner cell. The type II cells are usually found in the angles of alveoli and are cuboidal with a diameter of about 10 μm. Many intracellular organelles are well developed in the cytoplasm; in particular are the characteristic osmiophilic lamellar bodies. Type I and type II cells interdigitate by tight junctions and by desmosomes.

Most recent studies suggest that the type II cell is the stem cell of the alveolar epithelium. It has been shown that in normal lung, it is the type II, and not type I, cells that label within 2 hours after a single injection of ^{3}H thymidine (Kauffman et al., 1974). If animals are allowed to live some days after administration of tritiated thymidine, labelling is found in both

type I and type II cells (O'Hare and Townes, 1970). This suggests that type II cells act as progenitor cells of the alveolar lining. Further evidence that type II cells may differentiate into type I cells was obtained in a serial cytokinetic and morphological study of epithelial injury and repair by Adamson and Bowden (1974). They have shown that during the alveolar epithelial regeneration following oxygen poisoning in mice, the recovery phase was characterized by proliferation of type II cells. Only type II cells were arrested in metaphase by colchicine. In a pulse labeling experiment, an increase in the labeling of type I cells was coincident with the halving of grain counts in type II cells. It is suggested that replacement of type I cells selectively damaged by oxygen is accomplished by proliferation of type II cells and their transformation to the squamous type of epithelium. It is also clear from the experiment that the type II cells are comparatively injury-resistant and they serve as a reservoir of dividing cells for the replacement of the injury-susceptible type I cells.

Adamson and Bowden (1975) also demonstrated that in the developing rat lung the normal evolution process of the alveolar epithelium was similar to that after injury. Formation of squamous alveolar epithelium occurred by division of type II cells with subsequent transformation to type I epithelium. From gestation days 18-20, maximal DNA synthesis and cell division was noted when the

the alveoli were lined exclusively by type II cells. Intermediate cell forms between type II and type I cells were observed after day 21. Very few type I cells were labeled by ^{3}H-thymidine and mitotic figures were not seen in this cell type. Postnatally, the alveoli were lined by the normal mixed population of epithelial cells. Thus, this study provides additional evidence that the type II cell is the progenitor of type I epithelium. Formation of type I epithelium is probably influenced by the development of the capillary system. From gestation day 21 onward, type I cells are frequently observed in the rat lungs, a finding that coincides with an increase in labeling of capillary endothelium. Since type I cells overlie the capillaries, it may be postulated that the bulging action of new capillaries pushing into the alveoli may be a factor in the transformation of type II cells to the attenuated form. The importance of the pulmonary vasculature in influencing the structural development of the lung was stressed by Noack and Schwarz (1971).

Alveolar Epithelium--Function

The type I cell lining the capillaries has an extensive surface area of about 4000 μm^2 and is structurally well adapted to efficient gas exchange. The type II cell appears to have two major functions: (a) as already discussed, it acts as a stem cell for the alveolar epithelium and is particularly active in proliferating

to repair any injury of the type I cells; (b) type II cell has been implicated in the synthesis, storage and secretion of pulmonary surfactant, the substance that is necessary for proper airway inflation.

In 1929, Von Neergard first described the effects of surface tension on the lung air-liquid interface and emphasized its importance in the newborn. He found that lungs were more difficult to inflate with air than with fluid and postulated that the high surface tension of the air-water interface was lowered by a monolayer of unknown composition. Pattle (1955) proposed for the first time that the bubble film was composed of surface active material (SAM) from the alveolar surface. Clements et al. (1957, 1958) demonstrated that surface tension of the lung fell to very low levels upon compression in a modified Wilhelmy surface balance, while upon re-expansion it rose rapidly. The surface active material came to be called pulmonary surfactant.

Pulmonary surfactant may be defined as a unique lipo-protein, particularly rich in dipalmitoyl phosphatidyl-choline and containing lesser amounts of cholesterol, neutral lipid and other phospholipids (Goekke, 1974). Disaturated phosphatidyl choline and phosphatidyl glycerol are the major phospholipid constituents responsible for the surface tension-reducing properties of pulmonary

surfactant and are probably secreted into the air sacs attached to a protein molecule (Clements and King, 1973). Gluck et al. (1973) has shown that two chemical pathways in the human lung synthesize surface active lecithin (phosphatidylcholine). In pathway I choline is phosphorylated and then converted to cytidine diphosphate choline (CDP) and the phosphorylcholine moiety is transferred to diglyceride molecule to form phosphatidylcholine. In pathway II (methylation pathway) ethanolamine is phosphorylated, activated and transferred to a diglyceride molecule to form phosphatidyl-ethanolamine which then reacts three times successively with S-adenosyl methionine to form lecithin. In rat and rabbit, the choline pathway is the predominant route of lecithin synthesis. In 1954 Macklin was the first to suggest that the large, type II cell manufactured and secreted an alveolar lining material. Within the type II cell, multilamellar bodies seem to act as storehouses of surfactant. Woodside and Dalton used the electron microscope in 1958 to study mouse lungs and noted that lamellar inclusions were absent until the 17th or 18th day of gestation (full term in mice is 19 - 21 days). Buckingham and Avery (1962) found that they could not detect surfactant in the lungs of fetal mice until the 17th or 18th day of gestation; the appearance of lamellar bodies and surfactant at about the same time led them to propose that the lamellar bodies secrete surfactant. This view is supported by many investigators who have shown that pulmonary surfactant is stored within the type II cells

as lamellar bodies, and is secreted into the alveoli when required. Askin and Kuhn (1971) using tritiated palmitate in vivo, showed that labelled precursors of surfactant were preferentially incorporated in type II cells. The number of lamellae increases as the forming lamellar body enlarges, but the mechanism by which phospholipid is secreted is not understood. Once in the air spaces, the membranes of a lamellar body unwind and become transformed into tubular myelin. The enzyme phosphatidic acid phosphatase which is involved in lecithin synthesis is also associated with lamellar bodies (Meban, 1972). The widely accepted view concerning the relationship between surfactant and the lamellar bodies of the type II cells is largely based on: (1) the lipid nature of both, (2) the coincidental appearance of lamellar bodies and surface activity in the fetal lung (Kikkawa et al., 1968), (3) morphologic evidence that lamellar bodies are extruded into the air sacs and, (4) the fact that the surface-active lung washings contain whorls of osmiophilic material (Frosolono et al., 1970). Recently, surface activity was demonstrated in preparations of isolated lamellar bodies (Rooney et al., 1975). Adamson and Bowden (1973) used an organ culture system to show that the perilamellar membranes of the type II cells as the site of surfactant synthesis. The internal lamellae, which show the characteristic osmophilia of unsaturated lipids or lipoproteins, appear to be accumulations of whorled membranes with little enzymatic activity. They may be by-products resulting from rapid membrane turnover at the active perilamellar site. Because of the close association between

lamellar bodies formation in type II cells and the appearance of surfactant activity in the fetal lung, visualizing lamellar bodies is a useful index of type II cell maturation.

Attention has been directed to the development of alveolar epithelial cells because of the possible relationships of pulmonary immaturity, surfactant production, and hyaline membrane disease in the newborn. Hyaline membrane disease, also known as respiratory distress syndrome (RDS), is the leading cause of neonatal death in developed countries. RDS is characterized by increased alveolar surface tension, which produces progressive atelectasis, and at death hyaline membranes are usually found lining the alveoli. In 1959, Avery and Mead first showed that pulmonary surfactant could not be found in extracts from the lungs of infants with the disease. The deficiency is mainly due to the immaturity of the surfactant synthesis and/or secretion system in the lung (Gluck and Kulovich, 1973). Attempts have been made by many investigators to accelerate the maturity of the lung with various chemicals such as aminophylline, thyroxine, and glucocorticoid.

Effects of Glucocorticoids on Fetal Lung Maturation

Glucocorticoids are known to affect the metabolism of many organs in the body. Buckingham et al. (1968) suggested that glucocorticoids may accelerate differentiation of the lung cells

capable of synthesizing pulmonary surfactant. This suggestion was later strengthened by the observations of Liggins in 1969 that lambs delivered after prenatal glucocorticoid administration were viable at an earlier gestation day than untreated controls. Delemos et al. (1970) infused cortisol into one of twin lambs and found that the lung of the treated lamb was more mature than that of the twin control. Further studies by Kotas and Avery (1971) on rabbits established a similar finding. Wang et al. (1971) conducted a semiquantitative study of the morphologic changes following glucocorticoid injection to fetal rabbits. As compared to control, the treated animals demonstrated earlier appearance and greater numbers of osmiophilic inclusion bodies in the type II cells, less glycogen and more flattened epithelial cells. These findings were confirmed by Kikkawa et al. (1971) who, in addition, carefully looked for and failed to find morphologic evidence of glucocorticoid toxicity. Taeusch et al. (1973) observed that premature rabbit pups who had been treated 2 days earlier with hydrocortisone survived with no distress, while untreated littermates exhibited respiratory distress and died with pulmonary atelectasis. These studies confirmed the earlier findings of Liggins with premature lambs which led to the discovery that corticosteroid accelerates lung development.

Corticosteroids are secreted by the adrenal glands. Evidence

for a natural increase in fetal and maternal cortisol levels near term has been presented (Mulay et al., 1973). The role of the fetal adrenal in the timing of lung maturation has also been studied by interrupting the pituitary-adrenal axis. In rabbits and rats, intrauterine fetal decapitation was associated with a delay in appearance of the pulmonary surfactant. Blackburn et al. (1972, 1973) observed that surgical decapitation of fetal rats on day 16 of gestation retarded lung differentiation. The pneumocytes failed to differentiate and retained large volumes of glycogen in their cytoplasm. Very few lamellar bodies were formed and subsequently surfactant production was reduced. Blackburn concluded that retarded development of fetal lung was related to reduced levels of circulating hormones secreted by the pituitary-adrenal-thyroid axis.

Effects of glucocorticoids on lung cell maturation have been observed in in vitro experiments. Adamson and Bowden (1975) incubated rat lung explants from day 18 fetuses in medium containing prednisolone and showed that after three days incubation there was a marked increase in the incorporation of palmitic acid, a known precursor of phospholipid surfactant. This enhanced uptake of lipid precursors coincided with the appearance of lamellar bodies within the cuboidal epithelial cells of both adult and fetal explants cultured with prednisolone. Ekelund et al. (1975) performed a similar experiment with organ culture of human fetal lung. They found that the fetal lung explants incubated with cortisol accumulated

a significantly higher amount of phospholipids. Thus the organ culture system appears to be suitable for the study of steroid effects in the lung.

From these various experiments, it is concluded that glucocorticoid can accelerate lung maturity in late fetal life. Glucocorticoid (glucocorticosteroid) is a 21-carbon steroid that is secreted by the adrenal cortex and that acts primarily on carbohydrate, lipid and protein metabolism. The common, naturally-occurring adrenal glucocorticoids are corticosterone, 11-deoxycorticosterone, cortisone and cortisol (also known as hydrocortisone). In addition, several synthetic steroids have been produced with potent glucocorticoid activity and compounds such as dexamethasone, prednisolone and triamcinalone are widely used therapeutic agents. Dehydrogenation at the 1 and 2 positions of the hydrocortisone and cortisone molecules yields prednisolone and prednisone, which possess more potent anti-inflammatory properties.

Mechanism of Steroid Action for the Acceleration of Lung Maturation

(a) Receptor sites--Specific cytoplasmic receptors for glucocorticoids and their nuclear binding sites have been shown to exist in fetal rabbit and lamb lungs (Ballard and Ballard, 1972; Giannopoulos et al, 1972). In the fetus, the lung has the highest

concentration of glucocorticoid binding sites (Ballard and Ballard, 1972). Using fetal rabbit lung, it has been shown that there is good correlation between the affinity of steroids for receptor and their biological potency (Giannopoulos, 1973). The presence of specific glucocorticoid receptors in fetal lung suggests that glucocorticoids influence the lung directly rather than as a consequence of their effects on other tissues. The hormonal responsiveness of a tissue is dependent on the stage of its development. The evidence to date indicates that the critical period during which the fetal lung is capable of responding to glucocorticoid is limited to a time shortly before that in which differentiation normally takes place (Farrell and Avery, 1975). Receptors with high affinity for glucocorticoids are most numerous at this period of development (Ballard and Ballard, 1974; Ballard et al, 1974). In rat, receptor concentration is 0.41 $\pm$ 0.03 pmoles/mg. protein in the fetal animal at day 19 of gestation (Ballard et al., 1974). During this time, maximal DNA synthesis and cell division is observed in the type II epithelium (Adamson and Bowden, 1975). The receptor concentration decreases to 0.02 pmole/mg. protein by seven weeks of age which coincides with a lower mitotic rate in the lung. In fetal rabbit lung, the nuclei have been shown to contain macromolecules which have the properties of physiological receptors for cortisol. The nuclear receptors increase in density with gestational age and reach a peak concentration in the rabbit at 28 days when surfactant

production is demonstrable (Giannopoulos et al., 1972). Ballard and Ballard (1972) also produced evidence that there are receptors for cortisol in the cytosol of cells. Thus, changes in cytoplasmic receptors level during development may regulate hormonal responsiveness of the lung. In all these studies of whole fetal lung, the identity of the cell type binding the steroid was not directly demonstrated.

(b) Intracellular mechanisms--The binding of glucocorticoids is quite similar in different types of cells: the steroid enters the cell, and in each and every cell that exhibits a response to glucocorticoids, these steroids are initially bound to a cytoplasmic protein receptor with a high affinity (Giannopoulos, 1973). Subsequent transfer of the steroid-receptor complex to the nucleus is a temperature-sensitive process and the complex undergoes a change in shape before combination with the nuclear chromatin (Giannopoulos et al., 1972; Giannopoulos, Mulay and Solomon, 1973). These receptor sites may be specific for certain steroids and thus determine which steroids are transported into the nucleus. In the nucleus, the complex depresses specific genes, leading to transcription and translation. In the lung, the proteins formed may be enzymes involved in the biosynthesis of surface-active phospholipids or enzymes involved in the breakdown of glycogen. In addition to choline phosphotransferase, other pulmonary enzymes

demonstrated to exhibit greater activity after glucocorticoid treatment include lipoprotein lipase and glycerol-phosphate phosphatidyltransferase (Hamosh et al., 1976; Rooney et al., 1975). This process of enzyme induction and protein synthesis is expressed phenotypically by differentiation of the immature alveolar cells into type II and type I cells. Enhancement of enzymes involved in lipid synthesis is circumstantial evidence in favor of a steroid effect on type II cells. Glycogen may also provide the energy or material for cell proliferation, accounting for its presence in the areas of rapid cell multiplication. Sorokin (1961) observed that cyanide which destroys terminal oxidase activity, or malonate which interferes with the Krebs tricarboxylic acid cycle activity, did not alter lung growth in culture. Fluoride, which prevents glycolysis, stops lung growth. Therefore, it appears that steroids may direct glycogen-derived biochemical activity from cell division to cell differentiation, which in type II cells is indexed by surfactant synthesis and secretion.

Adverse Effects of Glucocorticoids on Lung Cells

Since glucocorticoids are used in treatment of respiratory distress syndrome, possible adverse effects of glucocorticoids have been studied. Experimentally it is noted that cortisol injection inhibits lung cell division. Carson et al. (1973) found an approximately 12% reduction in cell number in steroid-treated rabbits. The decreased cell number leads to lighter lungs but is not accompanied