The Role of Hyperoxia in Abnormal Development of Fetal Lung

A Thesis Submitted For The Degree Of MD In The University Of Leicester

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Contents

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I dedicate this thesis to my wife and children

Abbreviations

CLD	Chronic Lung Disease of Prematurity
TGF-beta	Transforming growth factor beta
PCNA	proliferating cell nuclear antigen
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nickend labelling
ARDS	Acute Respiratory Distress Syndrome
RDS	Infant respiratory distress syndrome
TNF	Tumour necrosis factor
IL	Interleukin
NFκB	nuclear factor-kappa-B
ROS	Reactive oxygen species
ROP	Retinopathy of Prematurity
N48/N72	Cultured in normoxia (air) for 48/72 hours
H48/H72	Cultured in hyperoxia (95% oxygen) for 48/72 hours
IQR	Interquartile range
VEGF	Vascular endothelial growth factor

Chapter One:

Introduction

Prematurity and Chronic lung disease

Chronic Lung Disease of Prematurity (CLD) affects almost half of all infants ventilated below 32 weeks gestation and contributes significantly to the long-term morbidity of preterm infants[1 2]. The disease is a structural and functional disturbance of respiration in the infant who has been exposed to factors that result in abnormal lung development, at a time when the lungs are meant to be in an intrauterine environment, rather than an extra-uterine environment.

There are many risk factors for the development of CLD, including hyperoxia. The exact pathophysiology is still not fully elucidated in the role of oxygen to the contribution to this condition. In attempting to understand the processes involved in the development of this debilitating and sometimes fatal disease, this thesis is directed to the development and study of models of lung development to help determine the role of oxygen in CLD.

Normal lung structure

The mature lung of a human adult consists of specialized tissue types, each with a distinct role to play to enable the host organism to absorb environmental oxygen into the circulation, and to excrete gaseous waste products- i.e. carbon dioxide, back into the atmosphere.

A number of properties must be fulfilled for this to be possible. The lungs must have a large surface area for gaseous exchange to occur over. Air must be efficiently conducted to these surfaces via a series of airways of minimum resistance to limit the amount of energy expenditure. The supporting structure must be resilient enough to

withstand shear stress from repeated expansions/contractions of the airway system. The work involved in expanding the airway system must not be excessive i.e. the airways must be compliant, yet possess a degree of elasticity to allow the lungs to return to normal size after inspiration. Finally, the circulatory system must be closely apposed to the respiratory airways to enable efficient exchange of gases.

All of the above properties are provided for admirably in the mature human lung.

Conducting airways comprise the trachea, the bronchi and the bronchioles. These progressively increase in number resulting in each airway, giving rise to two subsequent smaller airways. The final airways of the conducting system are the terminal bronchioles. The epithelium of these airways becomes progressively thinner as they progress down the tree, with the terminal bronchioles lined by cuboidal epithelium. Each airway is surrounded by smooth muscle arranged in tight spiral sheaths that are able to provide some structural tone maintaining the patency of the enclosed airways. By contraction or relaxation of these muscle bundles, constriction or dilation of the airways is respectively enabled. These airways are unable to provide any functional gaseous exchange across the epithelium primarily due to the relative thickness of the epithelial cells, and the intervening smooth muscle bundles surrounding the airways. There are approximately 16 generations of conducting airway in the adult lung.

Respiratory airways are continuations of the conducting system maintaining the pattern of dichotomous branching previously described. The epithelium lining however is much thinner than preceding generations, and comprises two specialized cell types. Type I epithelial cells are extremely thin with a large surface area. They

provide little resistance to the diffusion of gases across their depth, with a thickness of only 0.2μm. This contrasts with an epithelial thickness of nearly 10μm for cuboidal epithelium. These Type I cells do not possess the ability to proliferate. The Type II epithelial cells are scarcer, and are responsible for the production of surfactant[3]. This fluid mixture consists predominantly of glycerophospholipids - substances that have detergent-like properties and thus act to reduce surface tension in the easily collapsible respiratory airways. Also contained within surfactant are apoproteins, which stabilize the functions and structural integrity of the glycerol components of surfactant. Type II cells also possess stem-cell like properties, and act as precursors of Type I cells when these latter cells are injured.

The respiratory bronchioles are essentially conduits for air, but which have the ability to exchange gas along their length. There are three divisions of respiratory bronchioles, which themselves divide into two atria. These are funnel shaped air sacs lined in thin respiratory epithelium. The epithelium is arranged in minute pouches divided by crests to maximize potential surface areas. The pouches of air sacs are termed alveoli, and these form the final and most important sites of gaseous exchange. In all there are 7 generations of respiratory exchange structures beyond the conducting airways.

Vascular compartment- the lungs possess their own circulatory system that runs in parallel to the systemic circulation. Thus pulmonary arterioles run through the lung mesenchyme alongside the proximal airways, progressively dividing with the accompanying airway tree until reaching the respiratory exchange structures. Here, the vessels form a mesh-like network of capillaries that lie between the alveoli, closely apposed to the epithelium such that there is a gap of less than 1μm between

the red blood cells and the air within the alveoli. The alveolar crests found between alveoli, each have a capillary passing through within their substance where the majority of gaseous exchange can take place. The flow to these vessels is regulated by changes in tone in the pulmonary arterioles. These vessels are extremely sensitive to changes in oxygen tension; increasing resistance to flow to areas of under-oxygenated lung, and thus promoting perfusion within more adequately oxygenated areas. This physiological system prevents the occurrence of ventilation: perfusion mismatch when functioning adequately.

Mesenchyme- The interstitium of the lung comprises the vascular compartment, the muscular components of the airways and blood vessels, and the supporting structures of the lung. The tensile properties of the lung are mainly provided by a scaffolding of collagen that runs through the interstitium. This is present within the extracellular matrix between the cellular components, but is adherent to these cells by a complex proteoglycan binding system. Elasticity is provided by a fine mesh of elastin, existing as a spiral protein, also found in the extracellular matrix. Within the literature, the terms mesenchyme and interstitium have often been used interchangeably. Where mesenchyme tends to refer to undifferentiated cell mass found outside the epithelial compartment, interstitium tends to refer to the more mature correlate when it has become more scarce and differentiated into a highly organised structure. Throughout this thesis I will use these terms within these contexts.

Normal lung development

The gestation of the human fetus is 40 completed weeks. A baby is considered premature if delivery has occurred before 37 weeks. Survival of human infants has

been reported from as little as 22 weeks gestation. During gestation, the respiratory system develops in a well-defined manner, both structurally and functionally.

The intra-uterine environment is patently different from the extra-uterine. Thus, there is an expectation that respiratory functional requirements must be suited for one or the other. In these circumstances, lungs develop with the primary aim of supporting extra-uterine life, and thus have no functional role to play *in-utero*. Before delivery, the placental system provides for all the fetus' respiratory exchange needs.

The placental system however is limited by the amount of oxygen that can be provided to the fetus. As such the oxygen tension of the fetal vascular supply is only 3-4kPa, compared to 10-14kPa in the newborn infant. Thus the fetus develops in a relatively hypoxic environment.

I shall hence discuss the development of the fetal lungs in the confines of the relatively hypoxic intra-uterine environment, before considering the deviations in development that early delivery produce. More detailed examination of particular aspects of lung development will be undertaken within the discussion of this thesis.

Anatomists have categorised the structural development of the lung into defined 'stages'. There is discrepancy between investigators regarding the exact timing of these stages and their definitions, however they have been generally accepted as follows[3-6]:

<u>Embryonic stage: 0-6 weeks.</u>

This stage represents the formation of the main lobes of the respiratory system. The lung mass at the commencement of this stage constitutes undifferentiated

mesenchyme, derived from the embryonic mesoderm. The trachea and bronchi originate from an endodermal diverticulum of the upper gastrointestinal tract. This endodermal branch invaginates into the lung mesodermal mass at its cephalic end. Subsequent dichotomous branching of this primitive airway into the mesenchyme occurs until each prospective lobe has its own airway supply.

Pseudoglandular stage: 6-16 weeks.

The pseudoglandular stage represents the initial phase of lung development within the fetus, as opposed to the embryo. This stage represents the development of the conducting airway system. These constitute the small bronchi and bronchioles up to the terminal bronchioles and are destined to carry gas to and from the more peripheral airway structures responsible for gaseous exchange[7]. The term pseudoglandular is given due to the histological appearance of the lung at this gestation resembling a collection of glands within the stroma (Figure 1.1a).

There is a continuation of the pattern of dichotomous branching of airways into the surrounding mesenchyme. The mesenchyme undergoes concurrent developmental changes with differentiation of peri-airway mesenchyme into a continuous smooth muscle layer. These are present throughout the conducting airways, and are only deficient around the most peripheral 'terminal buds'. These buds are the 'newest' of the formed airways and the site of maximal growth/differentiation. Vascular proliferation is noted with new blood vessels developing around terminal buds as a capillary plexus.

Canalicular stage: 16-26 weeks.

The term 'canaliculisation' refers to the massive increase in vascularisation of the

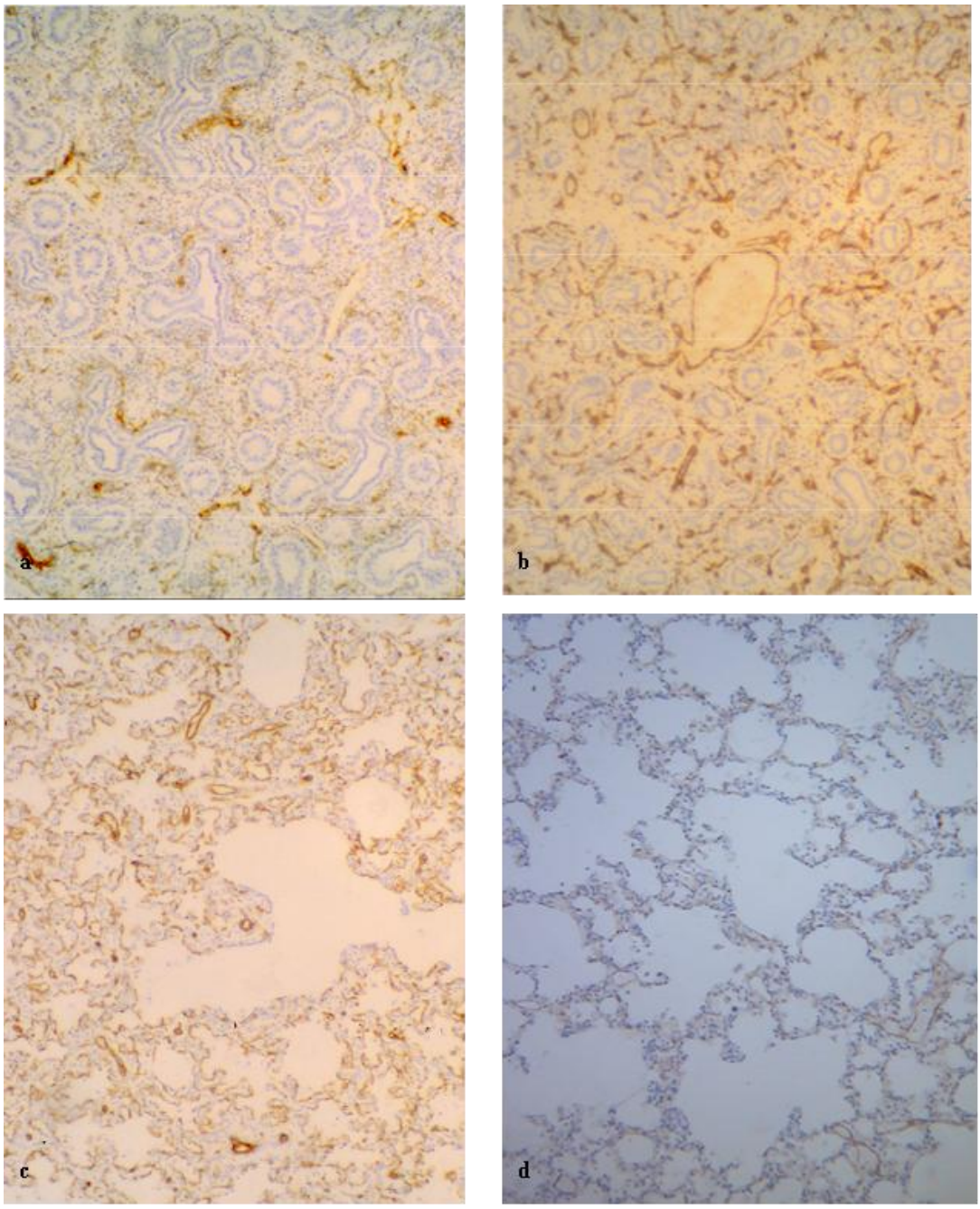


Figure 1.1: Histological appearances of lung from pseudoglandular (a), canalicular (b), saccular (c) and alveolar (d) stages of lung development. Sections have been stained for CD31 (brown) revealing vasculature, with haematoxylin counterstaining (blue).

lung, and it is this process that dominates histological analysis (Figure 1.1b). Concurrent with the vascular changes, is the development of the primitive acinus. The canalicular stage sees the development of the respiratory bronchioles of which there are three generations. The epithelium of the acinar structures contains cuboidal (Type II) pneumocytes, interspersed with flattened (Type I) cells through which gaseous exchange occurs. The Type II cells are first noted at 24 weeks of gestation[4 8] and are responsible for the formation of surfactant, which decreases airway surface tension. It is with the formation of these distal airways and cells that survival in the extrauterine environment first becomes feasible.

Saccular stage: 26-36 weeks

This stage is characterised by the dilatation of the acini structures, with the formation of early alveolar precursors. Three more airway generations are formed during this stage, corresponding to the alveolar ducts and alveolar sacs. Prior to the formation of mature alveoli however these simple dilated structures are referred to as saccules and are separated from one another by the formation of primary septae (Figure 1.1c). The formation of the saccular structures is promoted by the rapid involution of the mesenchyme compartment (as the interstitium is known at this stage). Although the interstitium contains a primitive capillary network in close apposition to the airway epithelium, the collagen network is very delicate. Elastin fibres are actively deposited *de novo* within the mesenchyme, and correspond to the sites of future secondary septae formation, and thus the sources of alveoli.

Alveolar stage: 36 weeks onwards.

The formation of alveoli characterises the alveolar stage of lung development. This is

deemed as the mature lung of the newborn infant, capable of independent respiratory exchange (Figure 1.1d). This ignores that there is still structural maturation involved in this stage. In particular there is formation of vascular fusion within the alveolar septae, allowing optimal oxygen transfer. Continued maturation and development of alveolar structures occurs until 18 months of age postnatally.

Control of airway branching in the fetus

From the onset of airway branching when the outpouching of the ventral part of the endodermal tube into the splanchnic mesoderm occurs giving rise to the trachea, and subsequently to the main bronchi, there exists an interaction between the mesenchyme and epithelium. That the mesenchyme exhibits control over the characteristics of the adjacent epithelium has been demonstrated by the transplantation of distal mesenchyme onto primitive trachea. The effect of this grafting is to induce the differentiation of tracheal epithelium into the mature surfactant-producing phenotype[9]. The nature of this interaction is influenced by the site of mesenchyme source, and thus implies varying properties within the spatial distribution of what appears to be an amorphous mesenchymal mass. Conversely, if mesenchyme is stripped from the airway tube, it will continue to elongate but not divide[10].

The control of airway branching has been investigated using both normal and genetically altered animals. Immunohistochemistry of mice at this stage of lung development reveal that at sites destined to form branch points, there is altered expression of a number of growth factors:

Transforming growth factor beta (TGF-beta) is a multifunctional cytokine that evokes a number of potential responses dependant on the target tissue. Both mesenchyme and

epithelium are sources for this protein in its various isoforms. The effect of TGF-beta on epithelium is to suppress proliferation[11]. Increased expression of TGF-beta has been demonstrated in murine pseudoglandular lung at the sites on terminal buds that are destined to become clefts[12]. It is believed that the up-regulation of TGF-beta at these points inhibits proliferation at these focal sites whilst those epithelial cells on either side continue to proliferate resulting in formation of a branch-points.

Alternatively, epithelial-mesenchymal interactions may be to promote proliferation rather than suppress it. Fibroblast Growth Factor-7, also known as Keratinocyte Growth Factor (KGF), is expressed in mesenchyme within the developing lung. The receptor for this factor is located at opposing epithelial cells stimulating cell division and thus promoting branching at these sites[13].

The control of various factors including Platelet Derived Growth Factors (PDGF) and Epidermal Growth Factor (EGF) [14] has a complex relationship when considered in their combined actions on the developing lung. However, it is by their various influences on mesenchyme and epithelium that the development of a respiratory tree in the pseudoglandular lung can occur.

Pulmonary vascular development in the fetus

It has been previously assumed that the small vessels of the lung develop by a process of angiogenesis i.e. by sprouting of vessels from more proximal arterioles. Recently, the consensus is that these vessels arise *de novo* rather than sprout from preformed vessels, and therefore are formed by vasculogenesis, not angiogenesis[15]. As the airway tree is being formed in the pseudoglandular lung, condensations of mesenchyme surrounding terminal airway buds differentiate into endothelium and

form capillary plexi. These are at first discontinuous from more proximal vessels, but gradually form interconnections with more proximal arterioles formed earlier. In turn, many of these vessels develop smooth muscle layers which are believed to be derived from the nearby airways, and thus are themselves transformed into arterioles. Pulmonary endothelial plexi that do not develop into arterioles, mature into a network of interconnecting capillaries. Initially, these are organised around the terminal airways, but remain at a distance from epithelium. During the canalicular stage, these vessels become more closely apposed to the nearby epithelium, providing the potential for gaseous exchange to occur. Subsequent development of the capillaries increases the efficiency of contact of blood with the maximal respiratory epithelial surface area. The most important alteration occurs during alveologenesis. At sites where capillaries and epithelium are in direct contact, an invagination of epithelium is formed into the saccule, within which interstitium protrudes. This mesenchyme carries with it a loop formed by the blood vessel, thus forming a secondary septae within the saccule possessed of a double capillary layer. This is the process by which an alveolus is formed during the perinatal period. Subsequent maturation of the alveolar capillary network occurs during postnatal life during which the double layers of capillaries within alveolar septae fuse to form single capillary layers, the hallmark of the adult alveolus. Further discussion regarding pulmonary vascular development will be undertaken in Chapter 3, page 114

The regulation of cell numbers during lung development

Evidence for an important role for both proliferation and apoptosis in the normal development of the lung exists which propose these two processes occur in tandem within the developing human. It is believed that apoptosis provides a regulated

manner in which excessive cells laid down in tissue through proliferation can be removed to provide the desired final organ structure[16]. Investigations have determined that these two processes can be affected directly or indirectly by the amount of oxygen in the environment[17-20].

It is also becoming apparent that alterations in the normal rates of apoptosis and proliferation might lead to abnormal lung development in preterm infants delivered into a hyperoxic environment.

Cell Proliferation

Cells undergoing proliferation in somatic tissue do so by undergoing mitosis. This is a tightly controlled process that proceeds through a series of well defined steps. The dividing cell can be found in one of two major phases, mitosis and interphase. Mitosis is the active division of cells into two daughter cells whereas interphase is the period of time wherein the cell prepares itself for mitosis by undergoing DNA replication, protein synthesis and cell growth. Interphase is itself divided into a series of specific steps; G1 during which the cell grows and is metabolically active, S phase where DNA replication occurs, and finally G2 when proteins are synthesised in preparation for mitosis[21].

Not all cells continuously proliferate, but instead exist in a state of rest until they receive a signal inducing an entry to the cell cycle (Figure 1.2). This is characteristic of some cells in the adult organism where cell division might only occur in response to injury. Thus hair follicles have a turnover measurable in hours, whereas cardiac myocytes in adults may have resting phases lasting many years. The resting state is denoted as G0 using standard nomenclature and may last for hours through to years

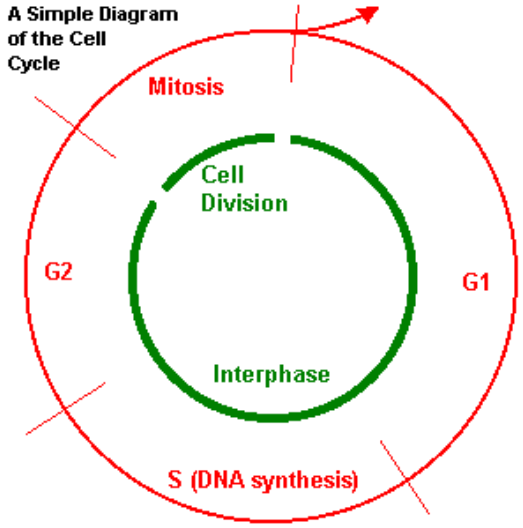


Figure 1.2: Simplified schemata of cell cycle. Two main stages of mitosis and interphase are further divided according to the metabolic and synthetic functions of the cell into G1, S and G2 phases. G0 represents movement to a quiescent state outside the cycle.

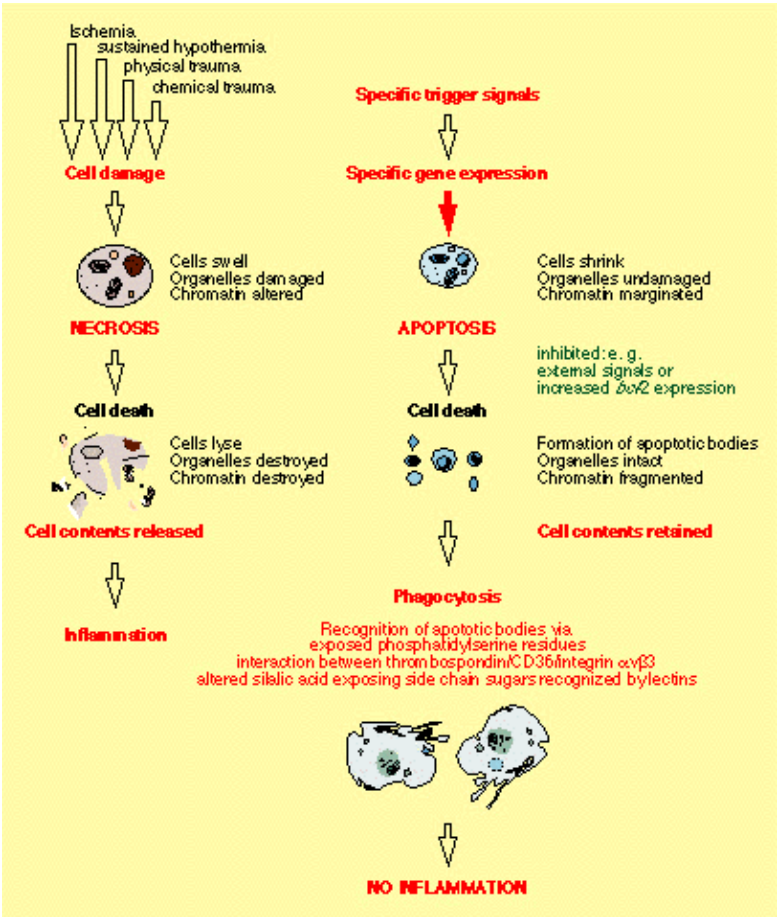


Figure 1.3: Representation of main differences between apoptosis and necrosis. From Molecular Biology of the Cell, Albert B et al[20].

dependant partly on the nature of the tissue. Cells enter G0 after exiting the cycle at G1 whereupon they become quiescent although they are still metabolically active.

It is essential that cells do not enter a stage until they have completed the previous stage or unless conditions are appropriate for the new stage to be completed successfully. For this reason, a system of regulatory pathways exists that are known as cell cycle checkpoints. The cyclin dependant kinases (CDK's) [16] are the major determinant of entry into G1. By the activation/inactivation of these complexes, it is possible to alter the rate of proliferation in tissues, and these have been found to be dysregulated in various tumours. Once passage through the 'restriction point' has occurred by virtue of activation of the CDK's, the cell enters an inevitable program leading towards S phase and mitosis.

Methods of assessing proliferation

Methods may be divided broadly into two categories. In the first, markers are added to tissues whilst still in culture, that are taken up by proliferating cells. These markers are retained within cells for the duration of their lifespan, thus providing a measure of all new cells produced since the addition of the marker to the tissues. Examples of these are radiolabelled thymidine- a DNA nucleotide taken up during mitosis, and bromodeoxyuridine. The second method is to detect- usually by immunostaining, proteins that are expressed specifically by a cell during proliferation. Examples of these are Ki67 and PCNA (proliferating cell nuclear antigen).

Role of proliferation in lung development

Proliferation and apoptosis both contribute to the normal development of the fetal

lung. It is fairly self evident that since the lung increases in cell mass manifold during fetal gestation and this is not accompanied by cell hyperplasia, then the growth observed must be predominantly contributed to through cell proliferation. Epithelial proliferation is vital during the development of airway branching[22], and is particularly active during the formation of the airway system. Stiles's[23] assessment of proliferation in rats between 15 and 21 days of age show that proliferation rates are extremely high when using bromodeoxyuridine uptake with values of 450 and 370 cells per 1000 cells in the epithelial and mesenchyme compartments respectively. May[24] used stillborn fetuses as controls for the assessment of proliferation in ventilated preterm infants. Approximately 2/1000 cells stained positive for Ki67 between 22 and 36 weeks gestation, although no comment was made regarding the location of these cells. This represents a particularly high rate during the pseudoglandular and canalicular stages of lung development, with marked reduction during the saccular stage.

Apoptosis

Loss of cellular tissue by cell death is expected to occur by apoptosis or necrosis. Necrosis was recognised as a form of cell death as long back as the discipline of histology was established, and in the established pathology textbooks of yesteryear, this was the only form of cell death described. Necrosis was defined as 'the morphological changes caused by the progressive degradative action of enzymes on the lethally injured cell'[25]. Thus, cell injury was assumed to be a natural prerequisite to cell death, whether it is due to an acute overwhelming insult, or sustained chronic damage. Cells did not die due to 'old age', but as a result of chronic low grade injury.

Apoptosis was postulated as an alternative form of cell death as recently as the

1970's[26]. Derived from the Greek phrase for the 'dropping-off' of leaves, this form of cell death was put forward as distinct from necrosis. Whereas necrosis is the result of injury to a cell culminating in death associated with an inflammatory response, with apoptosis there is an energy consuming process that the cell undergoes resulting in its removal without such a response (Figure 1.3).

Apoptotic signalling pathways

It is important to be able to closely regulate the number of cells that enter and complete apoptosis in the developing and diseased organism. The steps involved in apoptosis and the regulators of this process have become more clearly defined over the past decade and will be outlined here[27 28].

Essentially, apoptosis occurs in response to one of two pathways: the intrinsic and extrinsic. The intrinsic pathway occurs in response to intracellular damage whereas the extrinsic pathway occurs when receptors on the cell surface are activated by specific ligands that trigger an intracellular response that leads eventually to apoptosis of the cell (Figure 1.4). Both mechanisms finally meet at a common pathway that leads irreversibly to apoptosis of the cell.

The final aspects of the pathways that directly lead to cell autolysis involve the caspase family of proteins. These aspartate-specific proteases possess a cysteine at their active end and exist in cells as synthesised inactive precursors known as procaspases. Once activated, an amplification cascade occurs where procaspases are

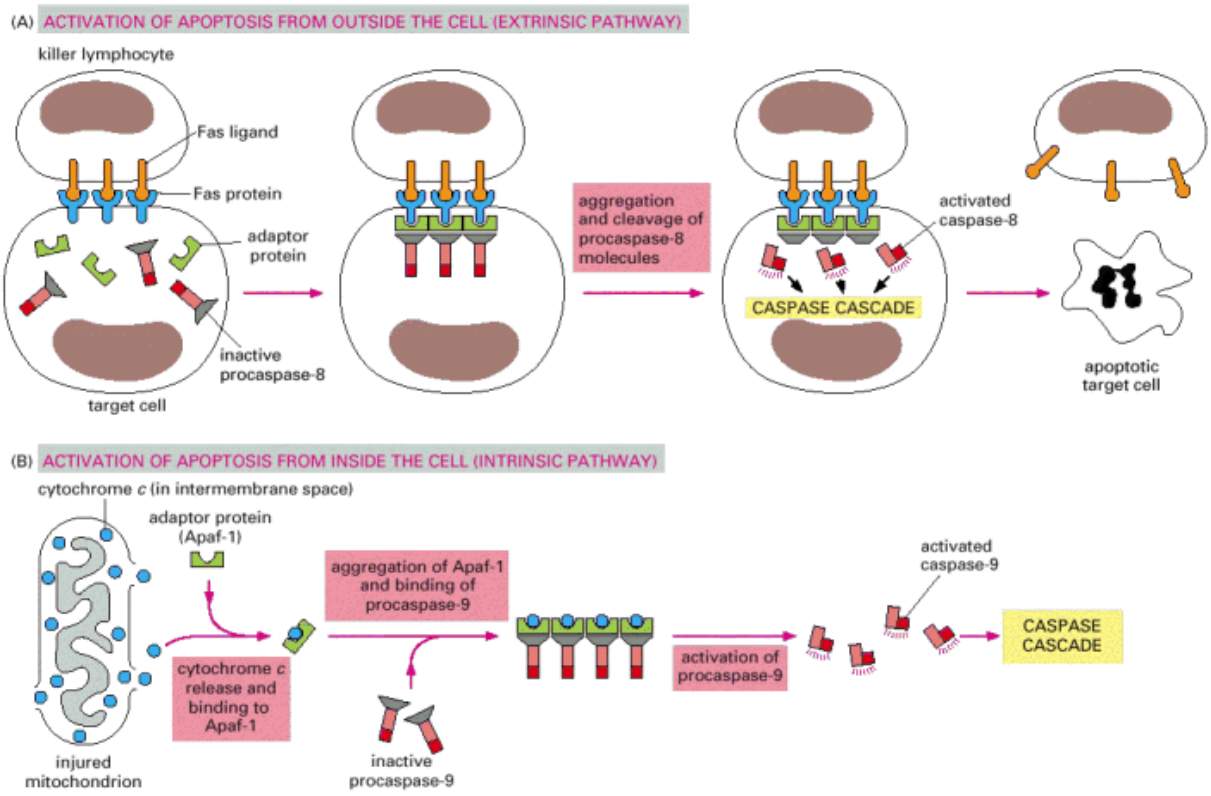


Figure 1.4: Schemata representing main differences in signalling between the extrinsic (A) and intrinsic (B) pathways[20].

activated, and then cleave other procaspases and so on until finally there is production of large quantities of caspases, which are capable of cleaving key cell proteins such as the nuclear lamina. A key protein that is cleaved is the inactive form of DNAse, releasing the active form, which in turn dismantles the cell's nuclear DNA. Importantly, proteins on the cell surface are specifically altered allowing these to be recognised by other cells responsible for the removal of apoptotic cells once the target cell has neatly dismantled itself in preparation for ingestion by neighbouring cells or macrophages.

A number of different caspases exist, but these are broadly divided into two groups, the initiator caspases which act upstream to enable the amplification process to commence, and the effector caspases which occur downstream and which are responsible for the 'digestion' of the cell's internal structure.

As procaspases lie dormant in all cells, the mechanism for their activation is tightly regulated. Activation tends to occur by the action of adaptor proteins which act to bring together the initiator procaspases into an aggregate enabling the proteins to cleave each other and thus trigger the amplification process[28].

In internal apoptosis, a number of different mechanisms can lead to procaspases aggregation[27 28]. The adaptor protein Apaf-1 can be activated by cytochrome c. This electron carrier protein is released by mitochondria into the cytosol in most forms of internal apoptosis providing the alternative name for this process as mitochondrial apoptosis. The release of cytochrome c from mitochondria is regulated by a host of competing agonists and antagonists for the process. Examples of this include bcl-2 which inhibits cytochrome c release, and *bax* and *bad* both of which stimulate

cytochrome c release.

Extracellular signalling again can act via a number of mediators, however the best known and characterised pathway involves the TNF family of ligands and receptors, specifically the *fas* ligand and *fas* 'death' receptor protein. The *fas* receptor is widely expressed in cells but particularly prevalent in inflammatory cells and mesenchyme in the lung[27 29 30]. Thus, killer cells of the inflammatory system can induce cell death by producing the *fas* ligand which then binds receptors in susceptible cells. Clustering of *fas* proteins are able to recruit intracellular adaptor proteins and hence initiate the caspase pathways that ultimately result in the death of the involved cell.

Role of apoptosis in lung development

Apoptosis is increasingly being identified as a major factor in lung development. Despite this, very little work has been published demonstrating the contribution of apoptosis to human fetal lung development using in vivo specimens. Del Riccio[31] summarised the contribution of apoptosis to lung development at the various developmental stages as follows:

Embryonic stage: Apoptosis in mesenchyme around branch points and regions of new lung bud formation.

Pseudoglandular stage: Apoptosis of interstitium contributes to mesenchymal involution.

Canalicular stage: Mesenchymal involution continues secondary to interstitial apoptosis, but in addition, epithelial apoptosis occurs for the first time.

Saccular and alveolar stages: Epithelial and mesenchymal apoptosis continues.

During the vascular maturational stage in the postnatal period, there is formation of secondary alveolar septae with transformation of the bilayered capillary network into a single layer capillary network where the intervening connective tissue is removed. The overall reduction in mass of interstitial tissue is accompanied by a reduction in fibroblast numbers by 10-20% and epithelial cells by approximately 10%, both by a process of apoptosis[32 33].

These observations were however extrapolated from the use of animal models, and very little work has been carried out in a range of pathological human lung specimens to delineate the rates of apoptosis at the various stages of human lung development. Stiles et al[23] who quantified the rates of apoptosis in rats sacrificed at 15 to 21 days of gestation. They determined that apoptosis was most active at D18 to 21, approximating to the canalicular and saccular phases of lung development in the rat.

My own work (unpublished) has quantitatively analysed apoptotic rates in archived human lung tissue over gestation ages representing late pseudoglandular lung, right through to alveolar lung. I concluded from my results that there are obvious gestation related differences, with significantly increased apoptosis occurring during the phases of interstitial regression, namely the late canalicular and the early saccular stages of lung development.

The delivery of the fetus into the postnatal environment has been studied in animals and in human tissue with the use of explant culture methodology and is believed to be associated with a rapid increase in the rate of apoptosis in lung tissue.

Kresch[34] analysed the progression and distribution of apoptosis in the lungs of rats

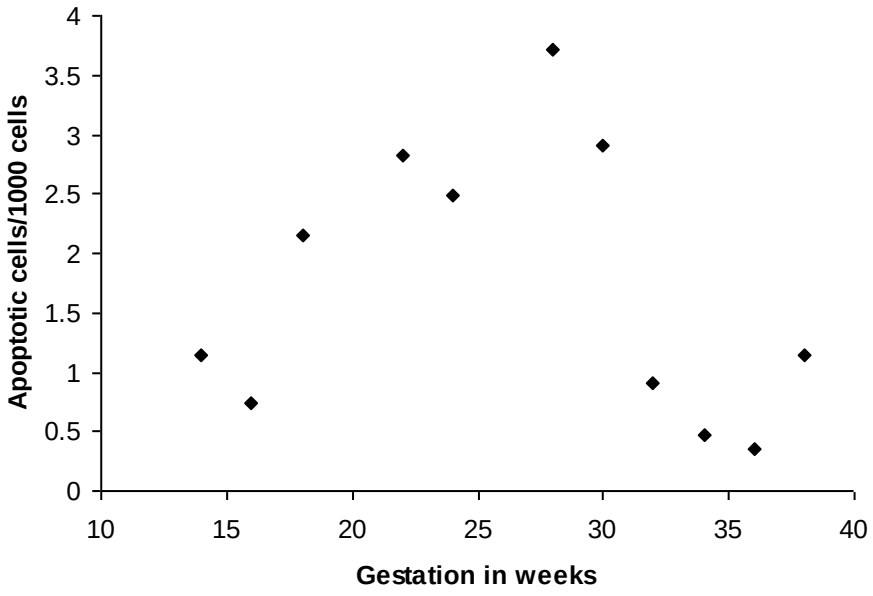


Figure 1.5: Apoptotic indices plotted against gestation show an increase in apoptosis with gestation until 30 weeks, followed by a sudden decrease in apoptosis.

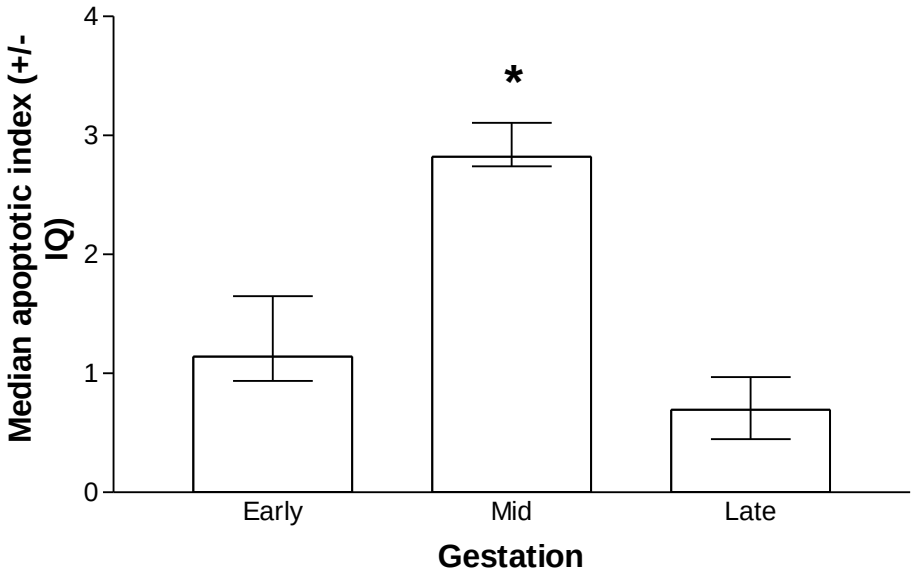


Figure 1.6: Apoptotic activity is significantly raised during the mid-gestation period when compared to early and late gestation (* $p<0.05$) n=11.

at a range of gestations commencing in the pseudoglandular stage. Using both electron microscopy and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining, their group was able to demonstrate apoptosis limited to the mesenchyme during the earlier stages of development, then progressive involvement of the epithelial compartment as the saccular and alveolar stages were underway. Delivery of rats results in a 14-fold increase in apoptotic rates for 48 hours, before returning to baseline rates. The cells that underwent apoptosis immediately after birth were not identified in this work however, and it is therefore not clear whether epithelium or interstitium is primarily involved during this accelerative phase. What was particularly of interest was the demonstration that the increased rate of apoptosis was present in lungs immediately after delivery. The implication therefore is that this is a process carried out in anticipation of imminent delivery, rather than exclusively a response to delivery into a postnatal environment.

It can be hypothesised that either exposure to an oxygen-rich extra-uterine environment or humoral factors associated with onset of delivery, result in apoptosis enabling rapid adaptation to gaseous exchange.

Scavo et al[35] studied human explant cultures of fetal lung from the pseudoglandular stage, analysing the change in rates of apoptosis using DNA laddering techniques and TUNEL staining. They demonstrated a massive increase in apoptotic rates commencing shortly after culture was established when compared to the preculture state, with rates approximately increased by a factor of x50. In comparison to my work, they cultured their tissue in 21% oxygen conditions only and in doing so showed a similar response to my tissue, which had been cultured in 95% oxygen. In contrast, the medium they utilised was serum-free. Thus, the implication of the papers

of Scavo and Kresch is that humoral factors must influence apoptosis in the developing fetus and in explant culture.

May and Speer's group studied histopathological slides from two groups of human infants[24]. The first group were stillborn fetuses, whereas the second had died after a period of time ventilated in oxygen. Using only TUNEL staining, they demonstrated a significantly greater rate of cell apoptosis in the ventilated infants, which was predominantly restricted to the epithelial compartment. Their inference from these observations was to regard oxygen and ventilation as being the factors responsible for the increased rates of apoptosis. It was not alluded to in the discussion of this paper that the differences were partly explained by the fact that the stillborn fetuses had not prepared for delivery by undergoing labour, or had not undergone the stress responses of delivery entailing as it does, massive changes in circulating catecholamines and hormones. It is these factors which may well have contributed to the apoptotic rate increase. They were however, able to demonstrate a relationship between number of days ventilated and the degree of apoptosis seen. Mean apoptotic indices for stillborn infants were 0.8% compared to 2.8% for infants ventilated for <1 day, 3.3% for infants ventilated for 1 to 3 days, and 13.3% for infants ventilated for more than 3 days. This gives strength to oxygen exposure as being a likely candidate for apoptosis stimulus.

In contrast, Hargitai et al[36] examined 24 autopsy cases of preterm infants born between 24 and 36 weeks gestation and assessed the degree of apoptosis rates in the epithelial cells of the airway tubules using TUNEL together with Haematoxylin and Eosin staining. No attempt was made to quantify mesenchymal apoptosis. They demonstrated very little apoptosis in lungs of infants who died in the first few days of

life with relatively early stage CLD. For those who survived to develop more advanced stages of CLD before succumbing, there were higher rates of apoptosis noted. The implications of these findings are that apoptosis is not so much a feature of acute lung injury, but of sustained injury and remodelling, thus contrasting with my traditional acceptance of apoptosis being an intrinsic part of acute lung injury or adaptation in the immediate postpartum period.

Thus evidence for the role of oxygen in the regulation of apoptosis is only indirectly provided by such human pathological studies, which are unable to clearly differentiate the effect of preterm delivery and oxygen exposure on this process. A clearer understanding of the pathogenesis can only be provided by in vitro models and in vivo controlled animal studies.

Detection of apoptotic cells in experimental work

The optimal method of detection of apoptosis in an experimental sample draws as much controversy as the interpretation of the results obtained. In reviewing the literature, it becomes evident that an array of methodologies exists in interpreting the degree and type of cell death[28 37 38]. A number of choices exist when contemplating assessment of apoptosis:

First is the nature of the sample. Histological identification of cells undergoing apoptosis requires a test that is amenable to immunohistochemical detection. Alternatively, in homogenous tissue as used in Western Blot analysis, individual cells cannot be identified, so markers of apoptotic activity are sought.

Second is the degree of commitment to apoptosis as a process. One may be satisfied

with identifying cells that are being made more likely to undergo apoptosis, or cells that are undergoing apoptosis irreversibly, or finally identifying cells that have undergone the final stages of apoptosis.

Morphological identification of cells

The original works describing apoptosis[26] utilised electron microscopy to define the hallmark changes and are still used by many as the gold standard for identifying apoptotic cells. Apoptosis was noted to have appearances dependant on the stage of apoptosis being undergone. Thus at the onset of apoptosis, the cell and in particular the nucleus shrinks, nuclear chromatin becomes condensed with margination of sharply defined masses within the nucleus against the nuclear membrane.

Subsequently, the cell becomes detached from neighbouring cells, and fragments into well defined 'buds' including the breaking up of the nucleus (karyorrhexis). Each of these buds is enclosed within a sealed plasma membrane and is referred to as apoptotic bodies. These are then phagocytosed by neighbouring cells which may have these bodies identified within them for some time. This method is considered precise, however its disadvantages include a requirement of high level of expertise, the ability to screen only limited amounts of tissue and difficulty in providing a quantitative assessment of apoptosis other than being able to comment that apoptotic cells were rare or frequent.

Light microscopy using tissue or cytospins stained for haematoxylin and eosin can provide a less precise morphological identification process for apoptotic cells. Again, cells are described with nuclear condensation, cytoplasmic shrinkage and detachment from neighbouring tissue. It is however very time consuming to identify these cells

over a large tissue section when attempting to quantify rates of apoptosis. The process of selecting out these cells is made easier by the use of ethidium bromide nuclear stains that can help differentiate nuclei that are healthy, condensed or display secondary necrosis.

In general, these methods are rarely used for studies where quantitative analysis of apoptosis is being utilised, and tend to be used to provide confirmatory evidence for apoptosis where other less precise methodologies are employed.

Biochemical markers of apoptosis

In describing the differing markers of apoptotic activity, I shall proceed in a chronological order of the processes involved in apoptosis.

Of the cell receptor mechanisms of initiating apoptosis, the presence of the *fas* ligand and/or receptor is most used for defining proapoptotic activity, rather than as a marker of apoptosis itself[29 30]. It is particularly useful in defining if extrinsic signalling is responsible for induction of the apoptotic process, which must then be detected with other means.

In defining the role of intrinsic apoptotic pathways, a number of differing markers are used and have become synonymous with apoptotic activity *per se* in a number of studies. Of these, *bax* and *bad* as apoptotic agonists are most commonly used, often in comparison with activity levels of the antiapoptotic factor *bcl*-2[20]. Also used are the other markers of intrinsic activity, cytochrome c and Apaf-1[39 40].

Although these markers give clues as to why apoptosis is occurring and can provide evidence for proapoptotic effect of study interventions, it is with the caspases that

more formal identification of the apoptotic process occurs[41 42]. The most upstream of the caspases are 2,8,9 and 10 which act as initiators of the apoptotic process. However these do not all belong to a common pathway, and whilst caspase 8 belongs to the death receptor initiation pathway (extrinsic), caspase 9 belongs to the mitochondrial pathway (intrinsic). Thus, the detection of one may exclude detection of cells or activity in the other pathway.

The more accepted method for using caspases to detect apoptosis is in detection of the activated (cleaved) forms of the downstream (effector) caspases, in particular 3, 6 and 7. When detected, they signify cells that are committed to undergoing apoptosis and are demonstrated regardless of the pathway undergone- intrinsic and extrinsic. Activated caspase 3 has become progressively popular for this purpose and is readily available for immunostaining of tissue sections[20 43 44].

Finally, one can search for the biochemical products of apoptosis. These involve both cytosolic and nuclear constituents, of which DNA itself is a prime candidate for detection. The most commonly used test for apoptosis in histological sections and cytological specimens has traditionally been TUNEL This test relies on labelling cells with agents with affinity for the terminal portions of fragmented DNA and provides and easy method for identifying apoptotic cells and producing reliable cell counts. The weakness of the test has been that it is possible for necrotic cells to produce false positive results with this test.

In a similar vein to TUNEL, a non-histological technique similar to Northern Blotting uses electrophoresis of DNA from samples and again, labels the free end of the DNA. In necrosis, DNA is broken down into fragments of random size and thus produces a

smear on labelling. In contrast, with apoptosis, the nature of the DNA fragmentation is that fragments are of a particular size and multiples thereof. Resultantly a characteristic laddering pattern is produced which indicates the degree of apoptosis in the given sample[35 45]. This DNA laddering has been used to provide evidence for apoptosis but without the strengths of good quantitative assessment, or ability to identify where in the tissue the apoptosis is occurring.

Immunohistochemical staining of end products of apoptosis is becoming available for progressively more targets. PARP (poly(ADP-ribose) polymerase is a nuclear enzyme which is cleaved during apoptosis. Antibodies to the cleaved form produce nuclear staining of apoptotic cells. A novel recent target is M30, [19] fragments of cytokeratin 18 that result after digestion by caspase 6. Although this is a specific test for apoptosis when present, its main limitation is that cytokeratin is a cytoskeletal protein found exclusively in epithelial cells, thus resulting in apoptotic changes within the parenchyma being missed.

It can thus be seen that in the assessment of proliferation and apoptosis, there exists a plethora of tests, each with their own strengths and weaknesses. These can be summarised as in Table xxx.

Oxygen delivery in the intrauterine environment

The delivery of oxygen to the developing fetus is an essential process that is enabled by the complex circulatory interactions between the fetus and the mother via the placental unit.

Group	Specific	Pro's	Con's
Morphological identification	EM. Light microscopy.	Precise	Laborious Needs expertise Poor for quantitative analysis
Markers of proapoptotic activity	Fas.	Specific to extrinsic signalling	Not markers of committed apoptosis. No markers for common signalling (instrinsic and extrinsic)
	bax, bad, cytochrome c.	Specific to intrinsic signalling	
Initiator caspases	2,8,9,10.	Sensitive for early apoptotic activity	Cells not committed. 8 may miss intrinsic. 9 may miss extrinsic.
Activated caspases	Activated 3,6 or 7 caspases.	Sensitive and specific to committed apoptotic cells	
DNA lysis products	TUNEL	Easy to do	Detects necrosis
	DNA laddering	Very sensitive Easy to use with homogenised tissue	Difficult to quantitate Unable to localise to specific cells. May be increased in necrosis
Cell apoptosis products	PARP, M30	Very specific	May be confined to certain cell groups

Table 1.1: Summary of major strengths and weaknesses of various apoptosis detection methodologies.

The delivery of oxygen is dependant on a number of factors. These comprise 1) the content of oxygen in the transporting medium (plasma and red blood cells), 2) the rate of delivery of the medium to the tissue, and 3) the ability of the medium to give up oxygen to the destination tissue.

The content of oxygen in solution follows a linear relationship that comprises the

solubility of oxygen in the given solution, and the partial pressure of the oxygen itself. This is calculated by the formula:-

Oxygen content in solution	=	PO2 (Oxygen tension in kPa)	X	0.022 mlO_2/dl/kPa (Oxygen solubility in plasma)

The quantity of oxygen that can be carried in this form is small and insufficient for the needs of a complex organism. For an oxygen tension of 13kPa, found in arterial plasma, this is equivalent to only 0.3ml of oxygen in 100mls of plasma. The development of specialised oxygen carrying components in the circulatory system has therefore developed, which in humans comprises haemoglobin carrying red blood cells (erythrocytes).

Haemoglobin consists of globin (a protein) and haem, which comprises a combination of ferrous iron and protoporphyrin. Globin is derived from 4 polypeptide chains that each binds one haem group, to form one haemoglobin molecule. Each molecule has four reversible binding sites to oxygen, one on each haem group. As oxygen binds to the haem rings, occupying all four binding sites, the configuration of the complex structure alters to provide increased binding of the carried oxygen. The opposite holds for when the complex loses oxygen. Loss of a single oxygen molecule results in a change in structure, making oxygen affinity less effective for the remaining sites, and thus promoting oxygen dissociation. The physiological role provided by this effect is stable oxygen carriage (high affinity) under high oxygen tensions. In contrast, within

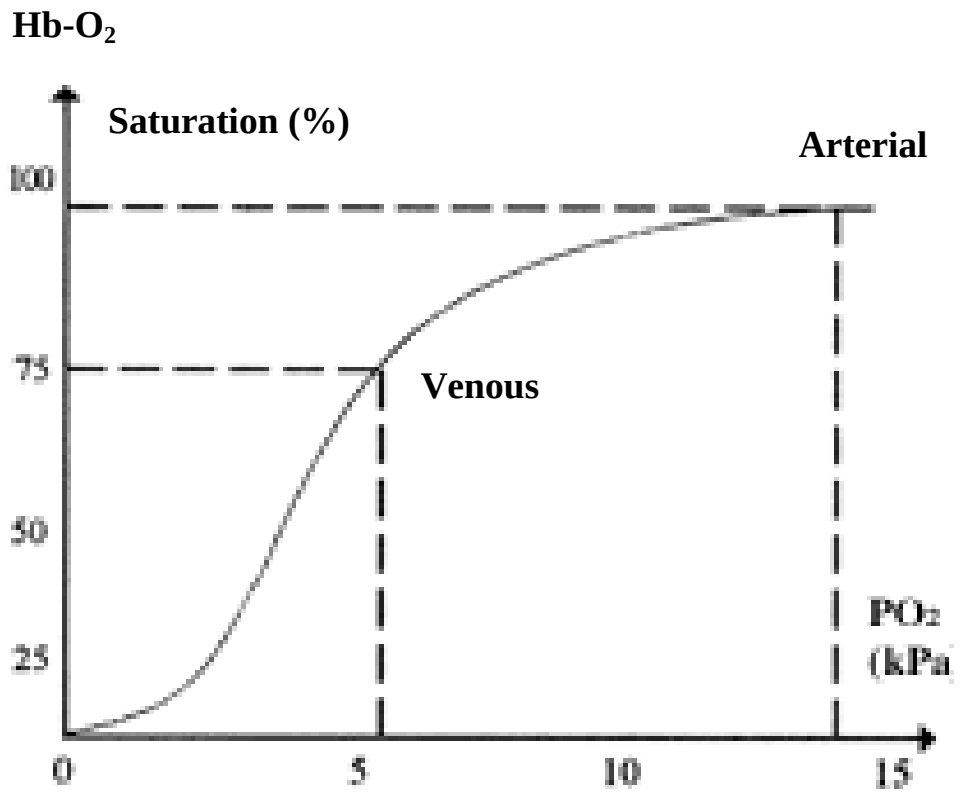


Figure 1.7: Oxygen dissociates from haemoglobin with relative ease at lower oxygen tensions. Thus offloading of oxygen occurs where there is relative tissue hypoxia. Fetal oxygen tensions are far lower than the arterial and venous tensions shown above in the postnatal human. Taken from Respiratory Physiology. Widdicombe and Davies. 1983

areas of low oxygen tension, oxygen is efficiently given up. This may be represented by the oxygen dissociation curve that is shown (Figure 1.7). The curve is sigmoid in shape, and demonstrates that as oxygen tension rises, there is a concomitant rise in oxygen saturation. The term oxygen saturation denotes the proportion of haemoglobin within a given blood volume that exists in the oxyhaemoglobin form. Beyond approximately 13kPa, adult haemoglobin is almost 100% saturated, thus further increases in oxygen tension are unable to increase oxygen binding beyond this maximal value.

With the additional oxygen load carried by erythrocytes, the oxygen content of blood can be represented by the following formula:

Oxygen content (ml/dl)	=	Oxygen bound to haemoglobin (Haemoglobin g/dl x %saturation)	+	Dissolved plasma oxygen (PO_2 x 0.022 mlO_2/dl/kPa)

Oxygen is deposited in hypoxic regions by virtue of these areas having a lower oxygen tension than within the carrier fluid, thus oxygen dissociates from blood and diffuses to the tissues, following a tension gradient.

For fetal blood to take oxygen from maternal blood via the placenta, there needs to exist a difference in oxygen tension between the two circulations. The maternal blood is arterial, and therefore has an oxygen tension of approximately 12kPa under normal conditions. Fetal blood at the exchange site is essentially fetal deoxygenated blood

arising from the umbilical artery, and thus at a lower tension, approximately 2kPa. To assist the uptake of oxygen, fetal haemoglobin has a different globin structure, providing a higher affinity for oxygen than adult haemoglobin. This provides fetal haemoglobin with an oxygen dissociation curve, which is to the left of the adult haemoglobin curve. Factors that shift the curve to the right reflect a decrease in affinity of haemoglobin to oxygen, whereas a shift of the curve to the left represents an increased oxygen binding affinity.

The final factor influencing oxygen delivery is the rate of fluid delivery to the organ or organism. Thus the placental blood flow and fetal blood flow are both linearly related to oxygen delivery to the fetus.

These rules apply to the delivery of oxygen within an *in vivo* environment; however, a number of principles may be applied to the *in vitro* scenario. These shall be considered in Chapter 2, page 98.

Causes of prematurity

A variety of pathways may lead to the premature delivery of a human infant. In some cases, the cause is iatrogenic, whereby a medical decision is made to delivery the fetus for maternal or fetal reasons. In a significant proportion of cases spontaneous preterm labour occurs due to unknown causes. In some cases, subclinical infection with chorioamnionitis may initiate the labour by mechanisms as yet incompletely understood[46]. Structural anomalies of the uterus or cervix may result in prolapse of amniotic membranes, rupture and resultant premature labour or inflammation.

Functional limitations of the preterm lung

Delivery of preterm infants may require variable degrees of respiratory support dependant on both structural and functional maturity of the respiratory system. Babies born at 36 weeks gestation have achieved sufficient development of alveolar structures and have an almost full complement of surfactant rendering the need for respiratory support redundant other than in only the most unwell infants, forming fewer than 10% of the patient group.

Infants born at 23 weeks gestation represent the threshold of viability in neonatal intensive care. Survival rates in these patients are as little as 10% dependant on the survey data available[47]. For infants born at this gestation, there are a number of deficiencies. Surfactant is present in negligible amounts; alveoli are not present with respiratory bronchioles existing as the most terminal organ of gas exchange. In addition, the lack of musculature in the extremely preterm infant with overly compliant thoracic wall provides insufficient strength for the infant to overcome the inherently high elastance of the described anatomy. These infants invariably require mechanical ventilation to inflate and deflate the lungs in rapid cycles in an attempt to excrete carbon dioxide, and to promote the delivery of oxygen to the most terminal airways.

The delivery of room air containing 21% oxygen may prove to be insufficient for the adequate provision of transferred oxygen into the pulmonary circulation. In these situations increased oxygen concentrations may be required.

Acute lung injury to the preterm lung

Oxygen has long been recognized as being toxic to the lung. The mechanisms by which oxygen exerts its toxicity on the lung is discussed more fully in this chapter (page 57). The extents of its effects are determined by its concentration, and the susceptibility of the host to its effects. The latter is greatly influenced by the stage of development of the subject. In adults, the administration of 100% oxygen to the lung for prolonged periods leads to the development of Acute Respiratory Distress Syndrome (ARDS) [48]. An initial destruction of endothelial cells is followed by type 1 epithelial cell denudation. This results in an alveolar leak, resulting in diffuse exudation of plasma into alveolar spaces. It has long been recognized that neonates of most species are more resistant to the toxic effect of hyperoxia than adults. This observation has been demonstrated in animal models with the increased survival of newborn rats exposed to 100% oxygen when compared to adult counterparts who all die within 1 week of exposure[49].

The preterm infant does not appear to have the same resilience to oxygen toxicity, and very quickly develops changes similar to ARDS. These acute changes result in widespread loss of the endothelial and epithelial compartments with accumulation of protein rich 'hyaline membranes' into the primitive alveoli. The pathological correlate of this 'Infant Respiratory Distress Syndrome' is termed 'Hyaline Membrane Disease' (Figure 1.8). As will be discussed later, this is soon followed by an influx of inflammatory cells and release of proteolytic enzymes, all of which contribute to further damage to the fragile preterm lung. With the influx of these materials into the airways and acini, there is a resultant impairment to gaseous exchange. Unfortunately, this leads to a vicious cycle whereby more concentrated oxygen must be delivered to the lung artificially and at high ventilator generated inspiratory pressures. Both of

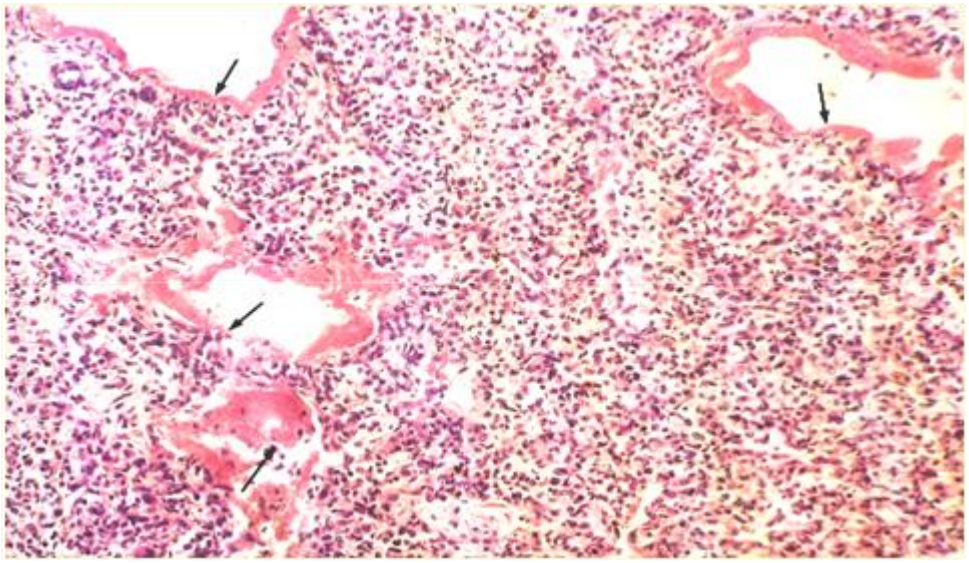


Figure 1.8: Lung from an infant who died shortly after delivery in the early saccular stage of lung development. There is complete collapse (atalectasis) of the small airways and saccules making identification of epithelium difficult in the absence of immunohistochemical markers. Distended bronchioloar structures are lined by amorphous ‘hyaline membrane’ debris making gaseous exchange difficult.

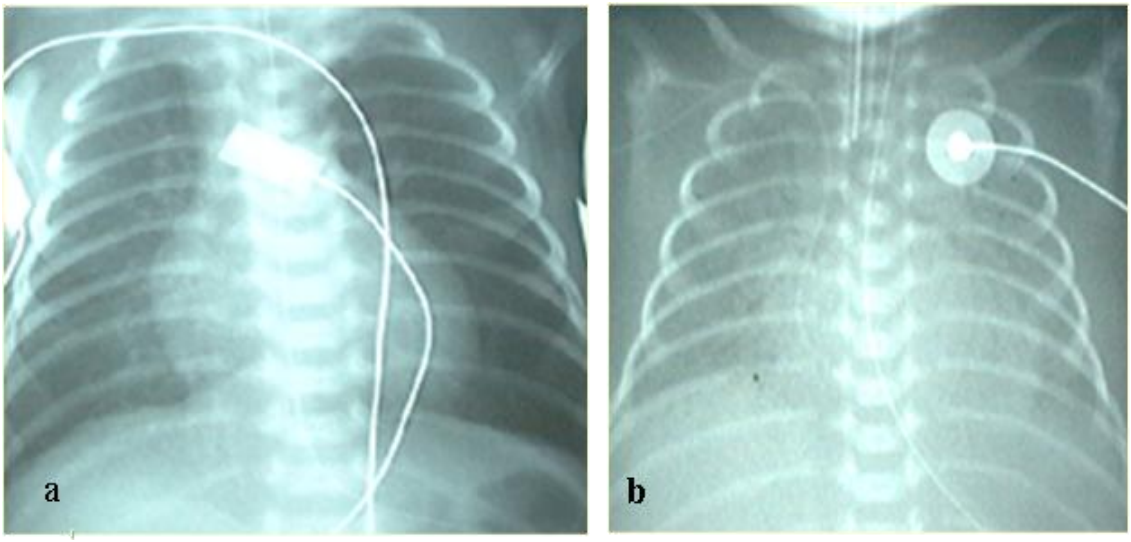


Figure 1.9: Chest X-ray films demonstrate the radiological features of RDS. A normal x-ray (a) has clearly visible heart and diaphragmatic borders with translucent lungs. In RDS (b), the lung is opaque with a characteristic ‘ground-glass’ appearance

these result in further injury by oxygen toxicity and shear stress respectively. Some of this vulnerability of the preterm infant appears to be secondary to an immature antioxidant system. Studies investigating the full term infants' well-developed defences against oxygen induced lung injury[50] [51] show a high concentration of antioxidant enzymes both intracellularly and systemically. These include Vitamin A, superoxide dismutase, glutathione and catalase, all of which possess the ability to scavenge reactive oxygen free radicals and thus avoid the effects of direct toxicity of oxygen or ROS.

Investigation of the preterm infant reveals this antioxidant activity is markedly reduced in comparison to more mature term controls with reduced measurable enzyme activity across the board[50]. In recognition of this antioxidant deficiency, clinical research has attempted to redress this balance by the administration of exogenous antioxidants. Overall, results have been generally disappointing and is discussed later in this chapter. It is recognised however that endogenous production of antioxidants can be increased in the at risk infant in a number of ways. The administration of antenatal corticosteroids is known to upregulate the production of a number of antioxidant enzymes[52] and is again discussed in the therapeutics section in this chapter. Exposure to high tensions of oxygen itself induces an antioxidant response. This has been elegantly demonstrated by experiments where by first pre-exposing adult rats to supplemental oxygen, ARDS-type pathology can be prevented on subsequent exposure to 100% oxygen[53]. Once again unfortunately, it is the preterm infant that possesses an impaired upregulation of antioxidant production in response to hyperoxia when compared to their term counterparts[54]. It is for this reason that most evidence for acute lung injury occurs in the first few days of life in the preterm infant,

where even relatively low concentrations of supplemental oxygen may potentially result in lung injury.

With acute lung injury or chronic exposure to high levels of oxygen, the consequences are subsequently seen as Chronic Lung Injury of the newborn, which has also been historically defined as the pathological correlate of Bronchopulmonary Dysplasia. I now discuss this disease, its pathogenesis, prevention and treatment.

Historical context of Chronic Lung Disease

Before the advent of Neonatal Intensive Care in the 1960's, the survival of premature infants of less than 2500 grams was extremely poor. The underlying pathophysiology for these infants was Respiratory Distress Syndrome (RDS), where the immature lungs produced insufficient quantities of surfactant at birth. The poor respiratory compliance that resulted from this deficiency produced a clinical picture characterised by respiratory distress, hypoxaemia and respiratory acidosis with a characteristic radiological picture (Figure 1.9).

The definition 'CLD of Prematurity' as a disease has itself developed from early descriptions initially by Northway in 1967[55]. At this time, the care of preterm infants had become revolutionised by the introduction of intensive care therapies, predominant amongst which, was the use of ventilators, introduced by Reynolds[56] in the 1960's to the field of neonatology. The use of these technologies, in combination with oxygen therapies, which had previously been the only form of respiratory support, provided a new found means for infants to survive at 30 weeks gestation or less.

At these early stages, infants who previously had succumbed were surviving. Some of these infants unfortunately were left with a degree of respiratory insufficiency, resulting in chronic requirements for supplementary oxygen therapy, often for the first few years of life.

Northway first defined the disease as 'Bronchopulmonary Dysplasia'. This was a pathological term given to a disease in which, for the surviving patients, a histological diagnosis would be unattainable. For this reason, a clinical definition for the disease was proposed:

"Bronchopulmonary Dysplasia- the requirement of supplementary oxygen in a premature infant who has reached 28 days of age, accompanied by characteristic radiological findings"

Northway's original publication provided radiological, pathological and clinical correlates for the disease[55].

Despite improvements in respiratory care over the last 10 years and the widespread use of exogenous surfactant and antenatal steroids, there has been little alteration in the incidence of this disease. In part, this is thought to be due to improvements in survival of extremely preterm infants who now go on to develop CLD. It has also been noted that the nature of CLD has altered. Previously, CLD affected larger infants with severe RDS who were exposed to high ventilatory pressures and oxygen concentrations, and was characterised by an inflammatory response to this treatment. Now, CLD affects extremely preterm infants who may not have significant RDS at birth but have immature pulmonary development. In contrast to the older babies with significant early lung injury, these infants with alveolar maldevelopment have become

regarded as those with the 'new BPD' pathology. It is this new BPD that has tended to be reclassified as CLD in recognition of its very different pathological picture. Exposure to the extra-uterine environment and to treatment modalities results in this abnormal lung development[57]. It is likely that there exists a spectrum between these two pathologies, and that common underlying processes are occurring. I shall aim to refer to the disease as CLD for the rest of this thesis.

Histological evidence of inflammation in CLD

Evidence has accumulated from a number of studies linking inflammation with CLD. These initially relied on interpretation of histological samples of lung obtained from infants dying from CLD. These, however, represented the most severely affected babies. They do nevertheless demonstrate inflammatory cellular infiltrates in the lungs of ventilated babies when compared to those who succumbed to non-respiratory causes[58]. These infants were characteristically preterm babies born at gestations of 28-34 weeks who had been exposed to high pressure ventilation and to high concentrations of oxygen. Histology confirmed the presence of interstitial fibrosis, alveolar wall thickening and smooth muscle hypertrophy. Recent studies looking at infants who were delivered at more immature gestations (23-28 weeks) have revealed different features. A simplification of the alveolar unit predominates with less striking inflammatory changes. In particular, there appears to be a failure to develop secondary alveolar septation and an associated pruning of the microvasculature. It is hypothesised that this failure of vascular development leads to this malseptation[59]. These findings are less likely to be due to hyperoxic lung damage, and more likely to be related to both ante- and postnatal inflammation resulting in dysregulated lung development. These pathologies are each represented in Figure 1.10.

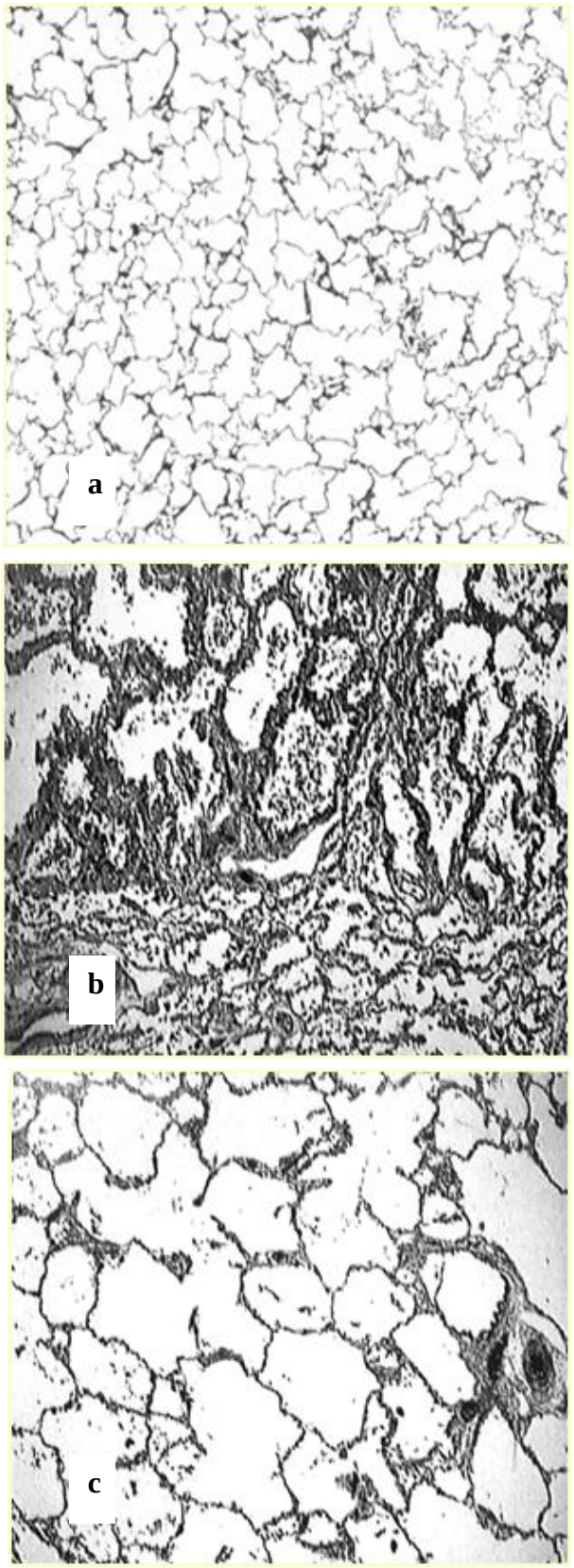


Figure 1.10: Comparative histological appearances of normal alveolar stage lung (a), lung from an infant with ‘old BPD’ (b) characterised by septal fibrotic hypertrophy, areas of overexpansion with neighbouring atalectasis. Finally (c), new BPD or CLD characterised by impaired alveolarisation. Reproduced with permission

The role of cytokines in the initiation of inflammation

Inflammation plays a role in host defence. It has evolved to protect against a multitude of insults particularly microbial pathogens and abnormal host cells. However it has become apparent that the inflammatory response can at times, be debilitating whilst being mobilised excessively against self-limiting insults, or chronically against continued stimuli such as ventilation and thus proves counterproductive[60].

The response of any tissue to injury can be classified according to the stages of an inflammatory response. After initial recognition of injury, a coordinated response must be initiated both locally and remotely, to allow the recruitment of effector cells. These have a role of containing the injury. The predominant effector cell during early stages of inflammation is the neutrophil, which once recruited and activated, either phagocytoses bacteria, or releases granule contents into the local environment. Subsequent cellular influx include macrophages which at times play an initial role in coordinating effector response, but with time also have a role in controlling and finally downregulating inflammation. Once removal of the offending agent has been accomplished, a reparative phase follows, re-establishing normal structure and tissue function, or alternatively, resulting in fibrous scar tissue.

Initial injury can be due to a number of agents including hyperoxia, or infection. The classical response to endotoxin injury commences with the recognition of lipopolysaccharide (LPS). This is a component of the bacterial cell wall, and specifically binds CD14 sites located on macrophages. These cells act as the initiators of the inflammatory response and when appropriately stimulated, respond with the production of signalling molecules termed cytokines[61], which share the ability to alter the functional characteristics of target cells. Cytokines are a diverse collection of

peptides which interact as part of a complex network to initiate and coordinate the inflammatory and reparative processes.

Cytokines have been categorized into various hierarchical categories. Traditionally these have been based upon the source of the cytokines hence interleukins are derived from leukocytes, lymphokines from lymphocytes, and monokines from monocytes. These categories have become more arbitrary as investigations have progressively revealed that all cells are capable of producing a range of cytokines. It is this that enables differentiation from hormones which are produced by specialized cells and tissue, and act upon specific end-organs. The early response cytokines Tumour Necrosis Factor-alpha (TNF-alpha) [62] and Interleukin-1 (IL-1) [63] are produced by the response cells including macrophages, endothelial cells, epithelial cells, fibroblasts and smooth muscle cells with the lung. IL-1 is a 17kDa protein produced predominantly by monocytes and is induced by other cytokines, particularly TNF-alpha. TNF-alpha[62 64] is a 17kDA protein which derived its name from the observation that the injection of this protein into tumour tissue leads to rapid destruction of small supply blood vessels within the tumour leading to involution. These together are classed as proinflammatory cytokines and act on macrophages to induce their own production by positive feedback. Their ability to recruit other macrophages locally enables a rapid amplification step, priming the region with numerous, activated macrophages.

Chemoattractants

Recruitment of neutrophils is mediated predominantly via the CXC chemokines (*chemo*tactic cyto*kines*) [61]. These polypeptides range in size from 7-10kDa and are

structurally related by the presence of four cysteine residues. Grouping into families is determined by minor differences in the cysteine arrangement. CC chemokines, which possess adjacent cysteine residues, include Monocyte chemotactic protein-1 (MCP-1), Macrophage Inflammatory Protein (MIP) and RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted). These are chemoattractant for monocytes, lymphocytes, mast cells and eosinophils.

The CXC family possess an aminoacid residue between the first two cysteines. They are further subdivided into ELR(+) or ELR(-) groups depending on the presence of the Glu-Leu-Arg motif. ELR(+) chemokines are attractant for neutrophils and act as angiogenic factors[65]. They include Growth Regulated Protein-alpha (GRO-alpha), but Interleukin-8 (IL-8) is the most potent chemokines for neutrophils. ELR(-) chemokines include Platelet Factor 4 and are attractant to monocytes and inhibit angiogenesis.

The migration of neutrophils to the area of injury is mediated by a series of processes. The adherence of neutrophils to the endothelial wall by attachment of the ligand L-selectin, to P- and E-selectins expressed on endothelial surfaces, produces slowed movement of neutrophils at these vessel sites, termed 'rolling'. The expression of L-selectin by endothelial cells is upregulated at inflamed areas due to the effect of TNF–alpha and IL-1. Subsequent firm adherence of neutrophils and migration across the endothelium is partly mediated by the $beta_2$-integrins, e.g. CD11b/CD18 which are upregulated on the neutrophils surface after activation, usually in response to IL-8. The $beta_2$-integrins attach firmly to the receptor, ICAM-1 (intercellular adhesion molecule) expressed on endothelium[66]. In addition, the lipid mediators Platelet Activating Factor (PAF) and Leukotriene B4 (LTB4), together with complement, all

of which are produced as part of an inflammatory response, act to increase vascular permeability.

The expression of these facilitators of neutrophil migration are increased by the action of interleukin-6 (IL-6). This 16-21kDa glycoprotein has many sources including macrophages, endothelial cells, smooth muscle cells (SMC's) and fibroblasts, in response to proinflammatory cytokines. Although IL-6 acts systemically to produce an acute-phase response, it also has been shown to have a negative feedback response, resulting in decreased production of TNF-alpha and IL-1 *in vitro*.

The inflammatory cascade in the lung as described is complex and mediated by both humoral and cellular elements. These are summarised diagrammatically in Figure 1.11.

The role of hyperoxia in inflammation

As discussed earlier, exposure to all species to hyperoxic conditions result in lung injury. This has been best characterised in adult species exposed to 100% oxygen. Accompanying the widespread respiratory cell injury is the release of pro-inflammatory cytokines and subsequent recruitment into the lung of inflammatory cells. Release of cytokines such as IL-6 results in a systemic acute phase response. These pathophysiological changes result in the clinical correlate of ARDS, characterised by severe respiratory failure with diffuse pulmonary infiltrates in the absence of congestive heart failure[67].

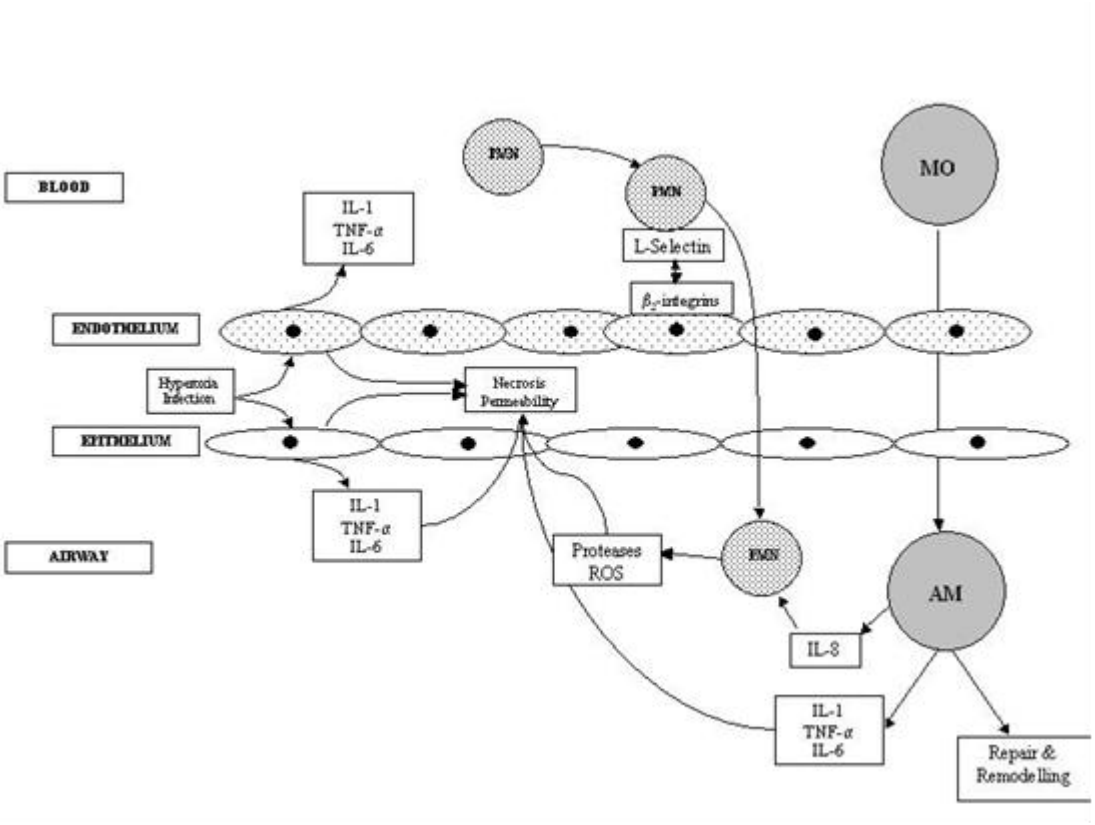


Figure 1.11: The figure shows a simplified version of the events that are likely occur in infants at risk of developing CLD. Risk factors such as oxygen therapy, ventilator-induced injury and infection are likely to lead to the recruitment of neutrophils (NEUTROPHIL) to the lungs where the production of proteases and reactive oxygen species (ROS) results in lung tissue damage. The cells of resolution and repair namely alveolar macrophages (AM) are derived from peripheral blood mononuclear cells (MO) are potently producers of cytokines both pro-inflammatory, chemoattractants and growth factors which are important in repairing the acute lung injury. Many of the growth factors, which are important in normal repair and remodeling, are also important in normal lung growth. Abbreviations: IL-1 – interleukin-1beta, IL-6 – interleukin-6, IL-8 – interleukin-8.

More recent studies have demonstrated the link between hyperoxia and inflammation in the developing animal lung. Warner et al[68], utilised the newborn mouse as a model of hyperoxic lung injury as alveolar development progresses in the early postnatal period. Exposure of these animals to 85% oxygen for 1 month resulted in 40% mortality. Survivors demonstrated decreased alveolar septation with increased terminal airspaces and evidence of lung fibrosis- findings consistent with CLD in human infants. Lung lavage showed that hyperoxia resulted in a neutrophilic infiltrate, peaking 2 weeks after exposure to hyperoxia. Analysis of neutrophil mRNA revealed an upregulation in expression of proinflammatory cytokines IL-1 and MIP.

The modulatory effect of oxygen on cytokines and growth factors

The association between hyperoxia and CLD is believed to stem from oxidant stress. The increased availability of oxygen results in leaking of reactive oxygen species (ROS) from the mitochondrial electron chain. This oxygen load cannot be dealt with by the immature antioxidant enzyme system present in preterm infants when compared to their term counterparts[54]. The formation of these oxygen radicals also results from the process of hypoxia/reoxygenation, whereby the conversion of xanthine dehydrogenase to oxidase results in the generation of superoxide anions[69]. Another source of ROS, once inflammation has been initiated, is from activated neutrophils recruited to the lungs in RDS. Via the NAPDH oxidase pathway, these cells produce and release of hydrogen peroxide, superoxide and hydroxyl radicals.

A number of pathways exist by which these agents exert their effect. A major determinant is the direct toxicity they possess, inherent from their ability to react with

cellular constituents. In particular, lipid peroxidation leads to cellular membrane disruption, followed by cellular necrosis. The release of a variety of intracellular constituents has chemotactic properties, resulting in the recruitment of neutrophil's to the lung[70]. Evidence for increased lipid peroxidation in early CLD infants has been demonstrated by Pitkanen[71], by the measurement of the expired lipid by-products, ethane and pentane.

ROS have been shown to assist in recruiting inflammatory cells by inducing the prolonged expression of specific neutrophil binding proteins on endothelial cell surfaces in particular Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1) [72] and Granule Membrane Protein-140 (GMP-140) [73]. This upregulation of specific protein expression provides an example of the role ROS have in signal transduction[74], in particular the activation of nuclear factor-kappa-B (NFκB) [75]. This transcription regulatory protein is located within cytoplasm, bound to an inhibitory protein. This is cleaved to its active form by ROS, resulting in attachment of the activated NFκB to its promoter sites on specific genes in the cell nucleus, enabling their transcription. Many immunomodulatory targets exist for NFκB including transcription of TNF-alpha, IL-1, IL-6, IL-8, MIP-1alpha and the cell adhesion molecules. A secondary effect of NFκB activation is the inhibition of neutrophil apoptosis via transduction of cell death regulators. Apoptosis is considered an important contributor to neutrophil removal[76], thus inhibition of this process prolongs inflammation.

The mechanisms by which oxygen has a regulatory effect on cytokine expression may give insights to potential future therapies specifically targeted to these mediators of inflammation.

Therapeutic interventions for hyperoxia mediated inflammation in CLD

The role of cytokines in coordinating inflammation have made them a target for potential therapies for CLD. Drug therapies have focused on removing stimuli for cytokine expression, suppression of production, or blocking their terminal effects. In addition, the use of cytokines themselves have become a therapeutic option.

Antioxidants

Supplementing the immature antioxidant system of the preterm infant has been attempted using a number of pharmacological agents including Vitamin E, Vitamin A and superoxide dismutase (SOD) [77] [78]. Vitamin A supplementation has been associated with a modest reduction in CLD amongst survivors at 36 weeks. However administration is via repeated intramuscular injections[79]. No clinical improvement has been demonstrated after the use of Vitamin E in infants less than 1500g[77].

Animal studies have shown a reduction in lung injury after intratracheal administration of antioxidants including Cu-Zn-Superoxide Dismutase (Cu-Zn-SOD), alpha–tocopherol, and N-acetylcysteine[80] [81]. Human studies have demonstrated a reduction in inflammatory mediators with the use intratracheal Cu-Zn-SOD, but as yet no improved clinical outcomes have been demonstrated conclusively other than reduced hospital admissions for respiratory illnesses in the first year of life[82-84]. This limited effect may be secondary to limited abilities to deliver these agents to the lung. Newer methods of delivery may show promise, in particular, recombinant human CuZn SOD used in combination with a surfactant vector[85] [86].

Infection

The use of antenatal antibiotics to prevent the inflammatory cascade associated with subclinical infection during preterm labour was believed to be a suitable and simple means for preventing CLD. This hypothesis was recently tested during the ORACLE trial[87]. The use of antenatal antibiotics in preterm labour was not shown to significantly reduce the incidence of CLD, although the use of erythromycin when rupture of membranes had occurred led to a small reduction in CLD on subgroup analysis[87 88]. An increased incidence of necrotising enterocolitis associated with the use of Augmentin during this trial reminds us of the potential hazards of any therapy.

The role of postnatal antibiotics for preterm infants has been poorly investigated. *Ureaplasma urealyticum* has been associated both ante- and postnatally with the development of CLD. Treatment with erythromycin has not altered the development of this disease, nor has it altered cytokine profiles in broncheoalveolar lavage (BAL) from treated infants[89 90].

Corticosteroids

These currently provide the mainstay of pharmacological treatment of CLD-associated inflammation. Steroids interact via glucocorticoid receptors and subsequent regulation of several genes which code for proteins with anti-inflammatory actions. This may be upregulation of production e.g. lipocortin-1 synthesis, resulting in inhibition of prostaglandin and PAF production. More commonly, inhibition of the expression of pro-inflammatory genes occurs e.g. IL-1, IL-6, IL-8 TNF-alphą RANTES, MIP-1alphą and adhesion molecules[91].

The use of antenatal steroids in mothers has been proven an effective therapy for preterm infants[92]. Although outcomes for the incidence of CLD have not been altered by its use, it is likely that this may be related to increased survival of the more immature infants who are more likely to develop CLD. There is no evidence for a direct antenatal anti-inflammatory effect of antenatal steroids. However, an induction of antioxidant enzymes and surfactant proteins is thought to be promoted by antenatal corticosteroids[93].

The use of systemic steroids postnatally in preterm infants has been associated with reduction in inflammatory markers and neutrophils in BAL, and has been shown to result in hastened extubation[94]. Optimal timing of steroid administration remains controversial with a failure to demonstrate increased survival in infants given postnatal steroids and from the association of steroid use with later neuromotor disability[95-97]. Concerns have also arisen regarding the detrimental effect steroids have on normal postnatal alveolar development[93]. Animal studies have demonstrated that steroids during the postnatal period impair the normal septation process, reducing overall alveolar numbers[98]. The concerns related to the systemic side effects of corticosteroids, have led to attempts to utilise the inhaled route of administration. In one study, the use of inhaled steroids in preterm infants was found to reduce concentrations of inflammatory cytokines in BAL fluid and reduce need for systemic steroids subsequently[99]. A reduction in the incidence of CLD has not been demonstrated in larger studies addressing this question, and at present, their use is not widespread[97 100].

Non-steroidal anti-inflammatory drugs

A number of non-steroidal anti-inflammatory agents have been assessed for the prevention and treatment of CLD. Ibuprofen has theoretical potential to block inflammatory prostaglandin pathways. However, in vitro studies utilizing infant monocytes incubated with ibuprofen, show an increase in pro-inflammatory cytokine production and decrease in anti-inflammatory cytokines[101]. A clinical study comparing the use of ibuprofen with placebo in preterm infants showed only a trend towards reduced ventilator days in the treatment group[102]. The effect of anti-inflammatory drugs such as ibuprofen and indomethacin on closure of the ductus arteriosus are more likely to contribute to preventing the development of CLD.

Cromolyn acts to stabilize mast cells, reduce neutrophil chemotaxis and activity[103 104]. An early study looking at the use of nebulised cromolyn from day 1 in babies under 1000g [105], in the pre-steroid/surfactant era, showed no difference in survival or incidence of CLD. A later study where cromolyn was administered from day 3 [106], again showed no reduction in the development of CLD. However, lavage samples taken during this study showed significant reduction in TNF-alpha and IL-8 concentrations, implying an effective anti-inflammatory effect. This illustrates the difference between clinical and cellular outcome measures that can exist, and reinforces the need for large, well-designed clinical studies.

Anti-inflammatory cytokines

The culture of tracheal cells from preterm infants with RDS, demonstrates a dose dependent suppression of IL-1, TNF-alpha, IL-6 and IL-8 protein production by recombinant human IL-10 [107 108]. The systemic administration of this cytokine to mice

exposed to bacterial pneumonia, results in decreased lung injury and mortality[109]. The upregulation or administration of IL-6 has become another potential target. Transgenic mice, overexpressing IL-6 and IL-13 appear to be protected from hyperoxic lung injury and resultant death[110]. This however is at the expense of remodelled airways with emphysematous lungs[111]. At present, these treatment modalities remain experimental. A future role for the treatment of respiratory disease in preterm infants remains to be seen.

Chemoreceptor antagonists

Antagonism of cytokines can be produced directly using antibodies either to the cytokine molecule, or to their receptors. CXCR2 antagonists target the CXC chemokine receptors (type 2) present on neutrophil's. These have been administered to newborn rats exposed to hyperoxia to block IL-8 chemoattractant effects. Together with reducing inflammatory infiltrates, there was prevention of alveolar septal wall thickening[112 113]. Similarly, in rats with immune-complex mediated lung disease, the administration of antibody to IL-8 reduces inflammatory responses in lung injury via blockage of E-selectin mediated recruitment[114]. Using a hyperoxia-ventilation injury model in rabbits[115], the administration of IL-1 receptor antagonists reduced lung albumin and elastase concentrations together with neutrophil influx. There was however, no improvement in respiratory dynamics in this model.

The role of oxygen therapy in established Chronic Lung Disease of Prematurity

The requirement of oxygen as a drug in the acute phase of care of the newborn

preterm infant is to facilitate aerobic metabolism. An absence of oxygen delivery relative to the metabolic needs of the peripheral tissues results in anaerobic metabolism with the consequences of energy failure and build up of toxic by-products of anaerobic metabolism i.e. lactic acid. Acutely this results in metabolic acidosis, the inhibition of surfactant production and hypoglycaemia. The supply of oxygen sufficient for the needs of the infant have to be balanced not only with the toxic effects of high concentrations of oxygen within the premature lung as discussed previously, but also with the potential effects of relative hyperoxaemia.

The newborn term infant possesses an arterial oxygen tension of approximately 13kPa when nursed in room air. This may equate to a measured saturation of 98% i.e. proportion of haemoglobin that is in the form of oxyhaemoglobin. With the introduction of supplemental oxygen as a therapeutic agent in the 1950's, preterm infants with cyanosis were given generous concentrations of oxygen either to provide a saturation of 98% or more likely with an absence of any effective form of oxaemic monitoring. The effect of this attempt to reproduce conditions for full term infants was disastrous with an association becoming obvious between oxygen therapy and blindness due to Retinopathy of Prematurity (ROP) [116]. This complication was subsequently determined to be related to relative hyperoxia in the early neonatal course, but that hypoxaemia in the chronic state might lead to progression of the disease in the older preterm infant with established CLD. Recognition of the association resulted in a swing in the therapeutic pendulum to another extreme with maintenance of preterm infants in a relatively hypoxaemic state. The effect was as dramatic with a significant rise in the incidence of cerebral palsy and infant death[117] [118]. Attempts since then have been made repeatedly to determine the optimal

tension/saturation of oxygen within the arterial blood.

The recent practice of maintaining saturations above 95% in infants with CLD was challenged with the STOP-ROP [119] study carried out in the US. It was gaining support that the pathogenesis of ROP included a late hypoxic state in the retina after an initial perinatal vascular insult. Thus, it was hypothesised that administration of oxygen to maintain a relatively hyperoxaemic state would prevent the progression of retinal disease in preterm infants with this prethreshold ROP. To the surprise of many, the maintenance of saturations of 96-99% when compared with 88-94% controls was not associated with an alteration in progression to severe ROP. More importantly, the group that was maintained at the higher saturation range were found to have a higher rate of pulmonary complications and were more likely to be discharged home in oxygen. The publication of STOP-ROP encouraged the reporting of practices in centres who had always professed a preference for maintaining relatively low oxygen saturations whilst managing preterm infants. In 2000, Win Tin *et al* from the Northern Neonatal Network in the UK [120] published an observational study comparing respiratory outcomes, ROP rates and developmental outcomes in 4 neonatal units with differing target ranges for saturations as part of their unit guidelines (80-90%, 88-94%, 90-95% and 93-98%). These targets were employed from the outset of neonatal care for approximately 8 weeks of life, as opposed to the practice in STOP-ROP where these targets commenced from an average corrected gestational age of 35 weeks. The rates of ROP were found to be highest in the units where a high oximetry target was employed (28% rate) and lowest in the unit with the least generous oxygen provision (6% incidence). This bore out the assumption that hyperoxia in the initial phase of the postnatal course was the impetus for a vascular insult to affect the

preterm retina. The pulmonary outcomes again dissimilar with infants remaining ventilated for much longer (31 days median) in the 'high-sats' unit compared to the 'lowest-sats' unit (14 days median). Encouragingly, there appeared to be no differences in rates of disability between the units providing a degree of reassurance for those keen to adopt the relatively hypoxic practices described.

The more recent BOOST study[121] provides support to the maintenance of lower tensions of oxygen in the blood comparing the use of 'low' saturation targets with 'high' saturation targets. This study was carried out in a double-blinded randomised manner. Monitors were developed that either under- or over-read true measured saturations by 3%. Thus in obtaining assumed target saturation readings of 93-96%, babies were actually being subjected to saturation ranges of 91-94% or 95-98%. Like the STOP-ROP study, these involved very preterm infants (mean gestational age 26 weeks) who were a few weeks of age (corrected gestational age 32 weeks) and thus had established lung disease. The intervention was continued through to discharge home. Growth and neurodevelopmental outcome were similar in each group, but the infants maintained at lower saturations were less likely to be discharged home in oxygen (17% vs. 30%).

These studies have challenged the view that maintaining oxygen saturation levels at lower target ranges are associated with poor outcomes and appear to support the use of these lower targets to prevent worsening of lung disease.

It is important however to distinguish these relatively short term studies with other observations that the maintenance of saturations in infants with CLD below 94% is associated with poor outcomes[122]. A comparison of two studies looking at Sudden

Infant Death Syndrome (SIDS) [123], [124] in two era's show a reduction in rate of SIDS from 11% in infants with CLD to 0% where the latter group were maintained at saturations above 93%. This has been supported by other observational studies and anecdotal evidence. Weight gain improved by maintaining SpO2 above 93% in high-risk infants, with discontinuation of oxygen therapy associated with slowdown in weight gain[125]. Additionally, poor weight gain has been reported when sleep hypoxia (<91% SpO2) was discovered on prolonged monitoring of high risk infants during long term follow up[126].

Pulmonary hypertension in adults is an expected complication of longstanding hypoxia in adults. This phenomenon is replicated in ex-preterm infants with CLD. Physiological studies confirm increased pulmonary vascular reactivity in infants with CLD. In these patients, an increase in SpO2 from 82% to 93% with low flow oxygen resulted in 50% decrease in measured pulmonary arterial pressure[127]. Additionally, a reduction in Fio2 resulting in a drop in tension from 62 to 54mmHg (equivalent approx to SpO2 reduction from 90 to 85%) causes an increase in pulmonary arterial pressure[128].

From these observations, it can be concluded that although high inspired oxygen tensions are associated with acute lung toxicity and increased clinical respiratory complications, the long term management of infants with established lung disease favours the maintenance of relatively 'normal' oxygen tensions to promote growth, and avoid pulmonary vascular remodelling. Thus, the dual role of oxygen as a toxin and as a therapy coexists in preterm infants, and continues to provide a fascinating challenge to neonatologists in obtaining the optimal balance between the two.

Methods of study

Although there are many excellent animal models of CLD, their relevance to human disease is unclear[68],[129], [130]. Cell culture has provided data regarding the toxic effects of hyperoxia and ROS on human cell lines[18], [43] ,[131]. ROS production is partly dependant on oxygen tension, and has been recognized as being important in intracellular signal transduction[17]. However, the use of single cell type culture methodology provides limited scope for examining the interaction of separate cell types on each other. Epithelium and mesenchyme are known to interact during lung development, thus models enabling culture of both cell types have established a role in lung development studies. In this respect, the bilayer cell culture technique has enabled certain interactions between cell types to be observed[132], [133].

Fetal lung organ culture[134] provides a means of investigating the effects of oxygen within an architectural framework encompassing several cell types. The use of an extracellular matrix substitute, such as collagen[135] provides an architectural framework to support growth of explanted tissue. Studies carried out with this methodology have been able to demonstrate maintained functional characteristics of lung. These include epithelial fluid secretion[136], airway smooth muscle contraction[137] and accelerated epithelial maturation with production of surfactant proteins[8] and Type II pneumocyte morphology.

Although the use of in vitro models provides a method to study a number of physiological processes in the absence of inflammatory cells, there is still a place for the study of human postmortem tissue. The chronic remodeling of lung in CLD occurs in an environment where the lung is not isolated, but influenced by systemic factors such as blood borne chemokines. Smooth muscle has been recognized as being

abnormally remodeled in CLD, however this remodeling has not been fully characterized.

Aims

The mechanisms underlying the effect of hyperoxia on developing lung has not been demonstrated to my knowledge within the fetal human explant culture model.

I propose to utilise the human fetal lung explant model to examine the effect of hyperoxia on acute structural and physiological processes in the mesenchyme and epithelium.

My specific aims within this thesis are:

1) To determine the effects of hyperoxia on the morphology and morphometry of the cultured explants.

2) To assess the specific effects of hyperoxia on epithelium, mesenchyme and vasculature in this model.

3) To examine the relative roles of apoptosis and proliferation in the hyperoxia mediated changes.

I hypothesise that hyperoxia causes altered rates of proliferation and apoptosis in cultured human fetal lung, resulting in accelerated lung development, and that this provides a model of preterm lung adaptation to an extra-uterine environment after delivery.

Chapter Two:

Development of a hyperoxic human fetal lung explant model.

Introduction

The analysis of lung development under various oxygen tensions requires a stable culture environment for fetal lungs. A number of methodologies have been utilized to carry out this process, the majority of which have been in animal species. The use of lung explants to analyse the processes involved in normal lung development have most commonly used in mice and rats[23 34 138 139]. Murine lung is relatively small, allowing ease of diffusion of nutrients into the central parts of the lung, without the benefit of a functional circulatory system.

Maintenance of tissue in culture requires a framework to maintain the tissue structure in a three dimensional environment, the provision of nutrients, hormones and survival factors to prevent death of the tissue. Finally, the factor under investigation needs to be able to be tightly controlled by the researcher

Human lungs at the pseudoglandular stage of lung development are many times larger than the murine lung at a comparable stage of development. This precludes the use of entire lungs in culture methodology, as only the peripheries of the tissue would receive sufficient oxygen and diffusible nutrition. Thus, these tissue specimens are first cut into smaller fragments before placing into culture, to allow satisfactory diffusion of survival factors into the centre of each explant.

The use of a sealed flask is a feasible option to allow controlled oxygen environments to be produced in which the explants can be cultured. This can be combined with standard methodologies developed to maintain viable human fetal lung explants in a semi-solid gel culture medium[135].

The human lung develops in utero at oxygen tensions of 3-4kPa. This is far lower than the normal ex utero environment where lung is then exposed to approximately 21kPa oxygen. Preterm infants who are delivered while the lung is still in its early stage of development may be exposed to extremely high concentrations of oxygen as part of their therapeutic management, with oxygen tensions as high as 100kPa. This may result in acute toxic changes in the lung and ultimately results in an abnormal development of the lung characterised by the histological appearances of Chronic Lung Disease of Prematurity. We are therefore intending to examine the effects of these two postnatal environmental influences on the development of lung explants.

Analysis of this tissue could be carried out by qualitatively describing the alterations in lung appearance on phase contrast microscopy. By fixing these tissues by freezing or by paraffin embedding, thin sections can be obtained. This allows staining of tissue using specialized histological techniques and thus determine the influence of culture differences on individual cell types and the expression of functional proteins.

Hypothesis

Some workers have utilised modular chambers to generate a hyperoxic environment during culture work[17]. A number of investigators have utilised fetal human lung explants in a semisolid culture medium. I proposed that combining both methodologies would provide a viable means to determine the effect of hyperoxic (100kPa) oxygen tension on short-term lung remodelling responses when compared to normoxia (21kPa). I proposed these conditions would alter developmental characteristics within a short period of time that could be quantified on microscopy utilising morphometric techniques.

Methods and Materials

Explant culture methodology

Culture of fetal lung specimens was carried out using a methodology modified from the work of McCray et al[137]. Tissue was obtained from the Medical Research Council tissue bank at the Hammersmith Hospital, London. Ethics had previously been obtained from the Leicestershire Local Research Ethics Committee. Lung tissue was obtained from therapeutic terminations of pregnancy where there was no suspicion of fetal abnormality. Gestations of fetuses varied but were consistently from the pseudoglandular stage of lung development. Information on the exact gestational age of the tissue was not consistently available for tissues and was therefore not applied to our data collection. The tissue bank provided notice of tissue availability on weekdays, during the afternoon. This tissue had been harvested on the same day. Due to logistical considerations, transportation would be arranged for the next morning, to arrive that same afternoon or at latest, the following morning. Based upon these factors, arrival of tissue at my laboratory occurred within 48 hours of harvest from the deceased subject. Samples were transported in RPMI (Roswell Park Memorial Institute) 1640 medium without glutamine (Gibco PRL), under sterile conditions and on ice to preserve tissue viability.

The specimens were transferred to a tissue culture grade flow cabinet (Ultimat flow cabinet, MAT, UK) (Figure 2.1). All materials described hereon used for culture methods are sterile. Tissue was dissected into individual pieces using stainless steel microdissection scissors. Each piece was 1-2mm^3 in size approximately and was obtained from peripheral lung tissue. This was ensured by removal and discarding of

tissue hila structures including pulmonary arteries, main bronchi and immediately surrounding tissue.

Each piece was placed in a 35mm petri dish within 300μl of Vitrogen based embedding medium. The medium was pre-prepared as follows- 3.5ml distilled water, 1ml fetal bovine serum (FBS), 0.33ml of 7.5% NaHCO3, 2ml 5X Ham's F12 (Sigma N6760), 0.1ml 0.1M $CaCl_2$, 0.1ml of 1mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3ml Vitrogen (Cohesion inc, Palo Alto), 0.1ml 1M HCl to produce a final volume of 10mls of 10% FBS semisolid gel. Before this solution was able to solidify, the 300μl aliquot was carefully placed in the centre of the petri dish, and the piece of tissue then gently submerged into the pool without disrupting its circumferential boundaries.

After approximately 45min at 37ºC, the medium had solidified and was immersed in 2ml of feeding medium. To make 100mls of medium I added to 88mls of Ham's F12 with HEPES (Sigma N8641), 1ml of Penicillin/Streptomycin, 1ml of glutamate, 160μl 1M NaOH and 10mls of fetal bovine serum. The organ explants were then incubated at 37ºC in 5% CO_2/95% air. The explants were inspected at 24hrs after culture (Day 1) for evidence of cell migration into surrounding gel matrix to signify viability. Non-viable tissues were discarded[124].

Hyperoxia

A number of explants were removed at Day1 and placed in hyperoxic chambers (Figure 2.2) which were flushed with a gas mixture of 95%O_2/5%CO_2 for 10minutes at 10L/min then sealed and maintained at 37ºC for either 48 or 72 hours. Chambers had been tested to ensure FiO_2 remained at 95% O_2 for 72hrs. For each explant under

Figure 2.1: Flow cabinet used for culture tissue and preparation. Laminar flow of air prevents contamination of tissue specimens by bacteria and fungi. When not in use, ultraviolet light maintains sterility. All instruments introduced into the cabinet are sprayed with alcohol to sterilise

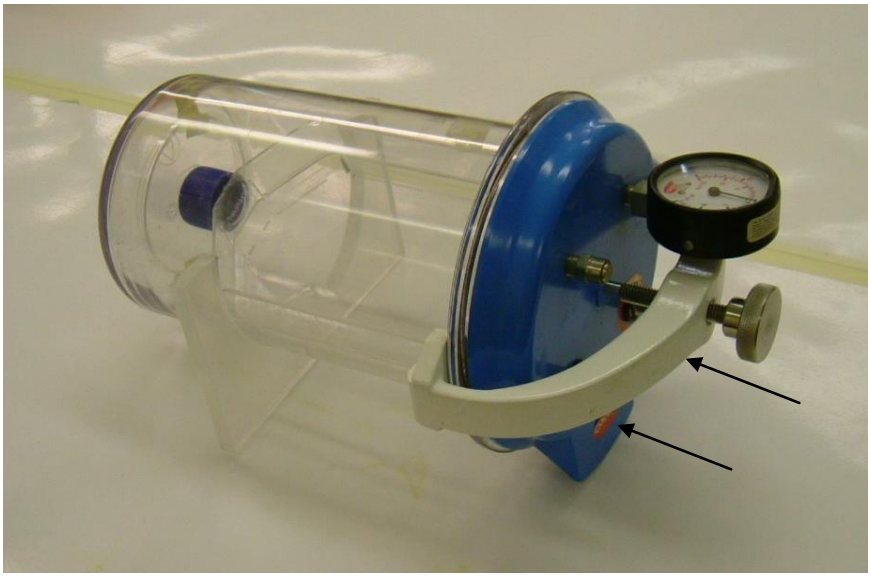


Figure 2.2: Modular gas chamber. Items for culture can be placed within, after which the lid is clamped. Two vents are available (arrows), one to flush gas into the chamber, with the other allowing gas to escape. Pressure gauge provides direct correlation with rate of gas flow

these conditions, a control was maintained in the standard incubator in 5%CO_2/95%air. Explants were harvested at Day 1, 3 and 4. For Day 3 and 4 samples, paired hyperoxia and normoxia exposed explants were harvested. Maintained cultures were fed at 1 and 3 days. The culture, harvesting and feeding schedule is set out in Figure 2.3.

Analysis of the validity of the hyperoxic environment was carried out with multiple methodologies. Initially, an atmospheric oxygen sensor was placed within a flask. This was in routine use on the local neonatal unit and was 2 point calibrated in air and 100% oxygen. The flask was then flushed with 95% oxygen/5% CO2 for 10 minutes at 10 litres/minute. The reading on the sensor was recorded at the time of sealing, and subsequently again 24 hours later.

Confirmation of oxygen tension equibrilation of the culture system utilized the Neotrend Oxygen sensor (Diametrics, UK), developed for measurement of oxygen tensions within a liquid phase. A petri dish was set up for culture with semisolid gel, and feeding medium as performed for explant culture. Prior to solidification, the probe was placed with the tip at the centre of the gel. Measurement of oxygen was carried out after medium was added in atmospheric conditions. The apparatus was then placed within the hyperoxic chambers and flushed as described previously with 95% oxygen/ 5%CO2. Repeat measurements of oxygen tension were then carried out.

Harvesting of samples

To assist maintenance of sample integrity during harvesting, I developed an agar mould technique. Briefly, 1.5% agar was heated in a water bath to 90ºC until melted. Feeding medium was carefully aspirated off, from the culture petri dish. Agar liquid

	Day 0	Day 1	Day 2	Day 3	Day 4
Feeding schedule	Feed	Feed		Feed	
Control tissue	Plate all explants	Remove x2 (Day 1 tissue)		Remove N48	Remove N72
Hyperoxia Experiment		Place hyperoxic samples		Remove H48	Remove H72

Figure 2.3: Timeline demonstrating interventions according to day of culture. Day 0 represents the plating out and feeding of all explants. On Day 1, 2 random samples are harvested. The remaining have media replaced and divided into those placed into normoxic and hyperoxic conditions. These are maintained until Day 3 where 2 samples from each condition are harvested, and those remaining for another 24hours again have their feeding medium changed.

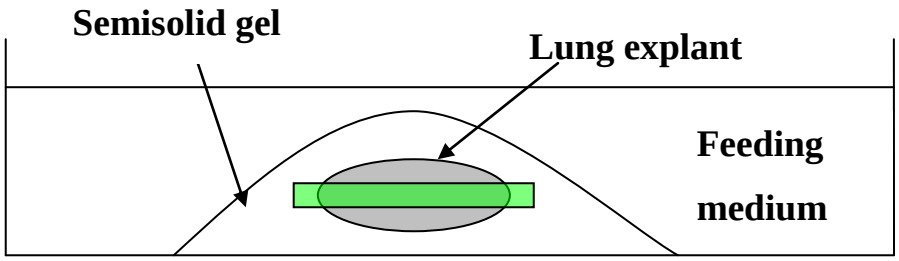


Figure 2.4: Cross section demonstrating explant tissue submerged within semisolid collagen based feeding gel. Sections for staining and morphometric analysis were obtained from the central section as demonstrated by the green shading.

was then allowed to cool to 50ºC, at which point 1ml was aspirated using warmed pipette tips and poured over the collagen gel. The dish was placed in a fridge for 5-10 minutes until the complex had suitably solidified. Once solid, the dish was upturned, allowing the collagen gel with explant to be peeled out intact. Samples were dissected from the collagen gel, and then dropped into 10% formaldehyde at 4ºC for 24hrs. Subsequent to this, tissue was kept in 70% industrial methylated spirits (IMS) until processing was possible. Paraffin embedding was carried out on my behalf by the Pathology Department at the Leicester Royal Infirmary.

Section preparation

Paraffin embedded specimens were cut into 4um sections using a microtome. Sections were mounted onto glass microscope slides. Slides had previously been coated with Vectabond (Vector labs, Burlingame, USA) to provide additional adhesion. Cut specimens were allowed to dry at 37ºC for 3 days to ensure optimal drying and bonding.

Immunostaining

Paraffin tissue sections were immunostained for monoclonal mouse anti-human pancytokeratin to denote epithelial cells. Sections were deparaffinised by placing in xylene for 5 minutes, then moving to fresh xylene a further 5 minutes. They were then rinsed twice in 100% IMS for 3 minutes each, then rehydrated through 95% IMS for 3 minutes, 70% IMS for 3 minutes, and finally placed in distilled water. To retrieve antigens, I transferred slides to boiling citric acid (100mM, buffered to pH 6.0). This was sealed within a porcelain pressure cooker, which was replaced into a microwave

oven and reheated at full power. Heating was discontinued four minutes after the container had reached optimal pressure. After slides were placed into distilled water, endogenous peroxidase was inhibited with 0.3% hydrogen peroxide in methanol for 30 minutes. I replaced the solution for Phosphate Buffered Saline pH 7.2 (PBS) to terminate this step. To contain subsequent solutions to specific regions of the slide and to reduce volumes of reagent, wax rings were 'drawn' around tissue sections. Non-specific binding sites were blocked with non-serum protein block (Dako, California, USA) for 30 minutes. Slides were incubated overnight at 4ºC with the primary antibody diluted in 0.05% bovine serum albumin/PBS at optimal dilution (pancytokeratin 1:200 dilution). Thereafter the slides were washed in PBS and incubated for 30min in goat anti-mouse biotinylated secondary antibody (Sigma-Aldrich, Poole, UK B9904) diluted 1:250. Note that in between steps the sections were rinsed twice with phosphate buffered saline (PBS) for minutes. After further rinsing, slides were incubated with avidin-peroxidase reagent (Vector laboratories) followed by 3,3-diaminobenzidine for 3 minutes. Sections were lightly counterstained with Ehlich's haematoxylin, with brief dipping in acid alcohol to differentiate stain.

Materials

Vitrogen was purchased from Collagen Corporation, Palo Alto. Monoclonal mouse anti-human pancytokeratin (C-2931) was purchased from Sigma-Aldrich, Poole, UK. All other reagents were purchased from Sigma-Aldrich, Poole, UK.

Image analysis

<u>Airway size</u>

Cells stained for pancytokeratin were used to indicate epithelium. To ensure that the sections of lung explant were comparable as possible, only sections corresponding to centre of the explant (determined by the number of sections cut from each explant) were used for architectural analysis (Figure 2.4). Tissues were analysed using digitally captured images at x40 magnification and image analysis software (Leica Qwin, Leica Imaging Systems, Cambridge, UK).

Images were obtained which provided views of the entire tissue section. If this were not possible, composite pictures were created from overlapping photographs. Using a graphics tablet, a digital overlay was created of the entire tissue section using facilities within Qwin. Manual drawing of every airway were then carried out using pancytokeratin as an airway marker. These images were then coded and digitally stored to be analysed subsequently. Quantification of the number of airways, the cross-sectional areas of each airway (a) and the area of the entire cut tissue (A) were carried out for Day 1, normoxic and hyperoxic tissue samples.

Calculations were carried out for each tissue sample as follows:

$$\text{mean airway size } (\mu m^2) = \frac{\text{sum of airway areas } \sum a_{1\ldots n}}{\text{number of airways } n}$$

$$\text{percent of tissue coverage} = 100 \times \frac{\text{sum of airway areas } \sum a_{1\ldots n}}{\text{area of entire tissue } A}$$

In addition, the results of airway diameters were pooled together for all specimens at

Day1, N48, N72, H48 and H72 respectively. These were combined to formulate cumulative airway size profiles.

Epithelium thickness

Epithelial cell thickness was determined for each tissue over three random high power (x400) fields for each section. For each field, epithelial thickness of each airway was measured at three separate equidistant points to minimise biased selection. Mean epithelial thickness for each tissue was calculated.

Statistical analysis

Comparisons between Day1, normoxia and hyperoxia exposed matched lung sections were made with the SPSS software package using paired-t-test analysis for normally distributed data, and Wilcoxon signed rank paired analysis for non-parametric data. Significance for two-tailed tests were set at $p<0.05$.

Results

Tissue culture

Specimens came from anonymous pregnancies at gestations ranging from 8-16 weeks. Frequency of tissue supply was variable with occasional gaps of upto 2 months. Attrition rate was low (below 10%), with the majority of specimens demonstrating migration of cells into surrounding collagen gel. Reasons for failure of culture included detachment of gel from the underlying petri dish, tearing of the gel spontaneously and fungal infection of feeding medium.

Gas distribution

Oxygen composition of flask gas contents immediately after flushing with hyperoxic gas mixture was 95% using the gas oxygen sensor. Oxygen was maintained at 95% of the gas mixture for at least 48 hours after the sealing of contents, reflecting maintenance of intra-flask environment.

Fluid oxygen tension in room air was 20kPa when measured in the medium in gel. When the flask achieved 95% oxygen content after flushing and was allowed to equilibrate for 30 minutes, oxygen tension in the middle of the solidified collagen gel was 100kPa. Withdrawal of the probe into the medium, at the same oxygen partial pressure, maintained oxygen tension at approximately 95kPa. Thus oxygen tension was maintained throughout the culture media and semisolid culture gel.

Macroscopic tissue appearance

Culture under normoxic conditions enabled tissues to be maintained for up to 6 days. By 5 days in culture, a number of specimens tore away from their gel precluding their use. I did not utilize any tissues beyond 96 hours in culture for any further experiments.

Microscopic tissue appearance

For each human fetal lung studied, I compared hyperoxic exposure with air exposure. All samples after the initial 24 hours in air showed that the airways at the edge of the cultured lung had become relatively dilated compared to those more central within the tissue. On low power microscopy, it could be seen that there was little change in structure between 24 hours in culture and 3 subsequent days in normoxic culture

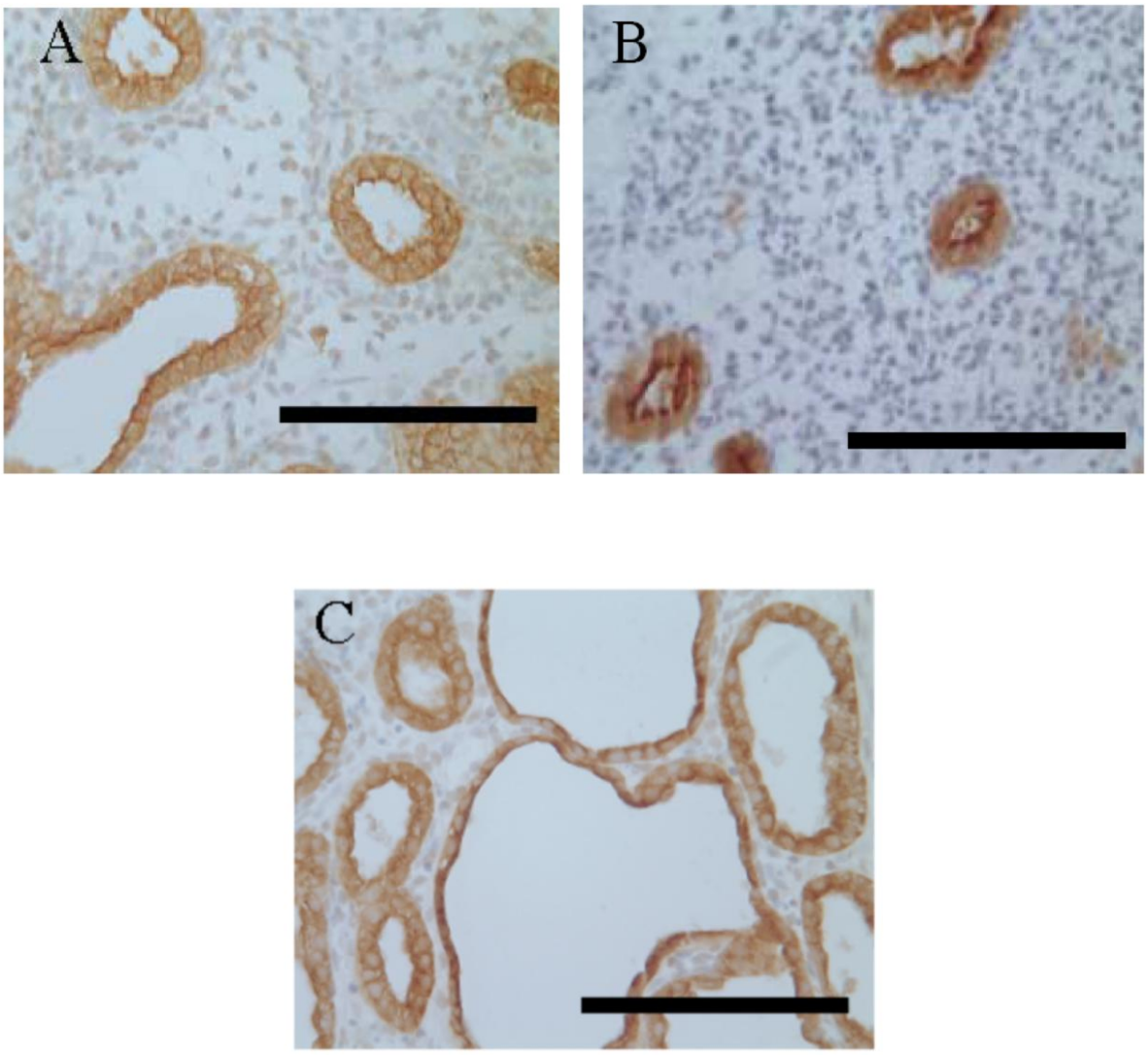


Figure 2.5: Day 1 cultured lung (A) predominantly consists of mesenchyme with interspersed airways lined with columnar epithelium. Culture for a subsequent 72hrs in normoxic conditions (B) maintains this appearance although airways appear marginally smaller. However, alternative culture in hyperoxic conditions for 72hrs results in a thinned epithelium lining the dilated airways and a generalised paucity of mesenchyme. Tissue sections imunstained for pancytokeratin with brown DAB representing epithelium. Light haematoxylin counterstaining. Bar=100μm (n=6)

(Figure 2.5a and b). The architecture of fetal lungs cultured in hyperoxia for up to 72 hours was markedly different to explants cultured in normoxia for the same period (Figure 2.5b and c). The airways in lung sections from hyperoxia-exposed explants were much larger than in normoxia-exposed tissue. In contrast to the normoxic exposed tissue, the epithelial cells appeared flattened within the dilated airways.

The total airway sizes for six lungs at 24 hours of culture were pooled and compared with six corresponding lungs cultured for a further 72 hours. Medians and cumulative curves were generated. During this time there was little change in the median airway size, the median airway at 24 hours had a cross-sectional area of 2900μm^2 compared to 2600μm^2 after a further 72 hours in culture. The overall profile for airway sizes after 1 and 4 days in culture were similar (Figure 2.6).

Effect of hyperoxia on airway sizes

Morphometric analysis was performed on the lung explants exposed to the various conditions. The cross-sectional area of the airways was largely similar between the explants cultured for up to 72 hours in normoxia when compared to day 1 tissues (Figure 2.7, day 1 median 4400μm^2 (IQR: 3900-6500), 48 hours of normoxia 5900μm^2 (IQR: 3700-9300) and 72 hours of normoxia 5800μm^2 (IQR: 3300-8800) respectively). This is more clearly shown in Figure 2.6 which shows the cumulative airway size pooled for all lungs studied. The median cumulative cross-sectional surface area was 2600μm^2 in the day 1 samples and did not change significantly after a further 72 hours in normoxic culture. In contrast, the median cross-sectional area of the explants exposed to hyperoxia was 17600μm^2 (IQR: 10200-26600) at 48 hours and 14000μm^2 (IQR: 10500-22000) at 72 hours which was significantly increased

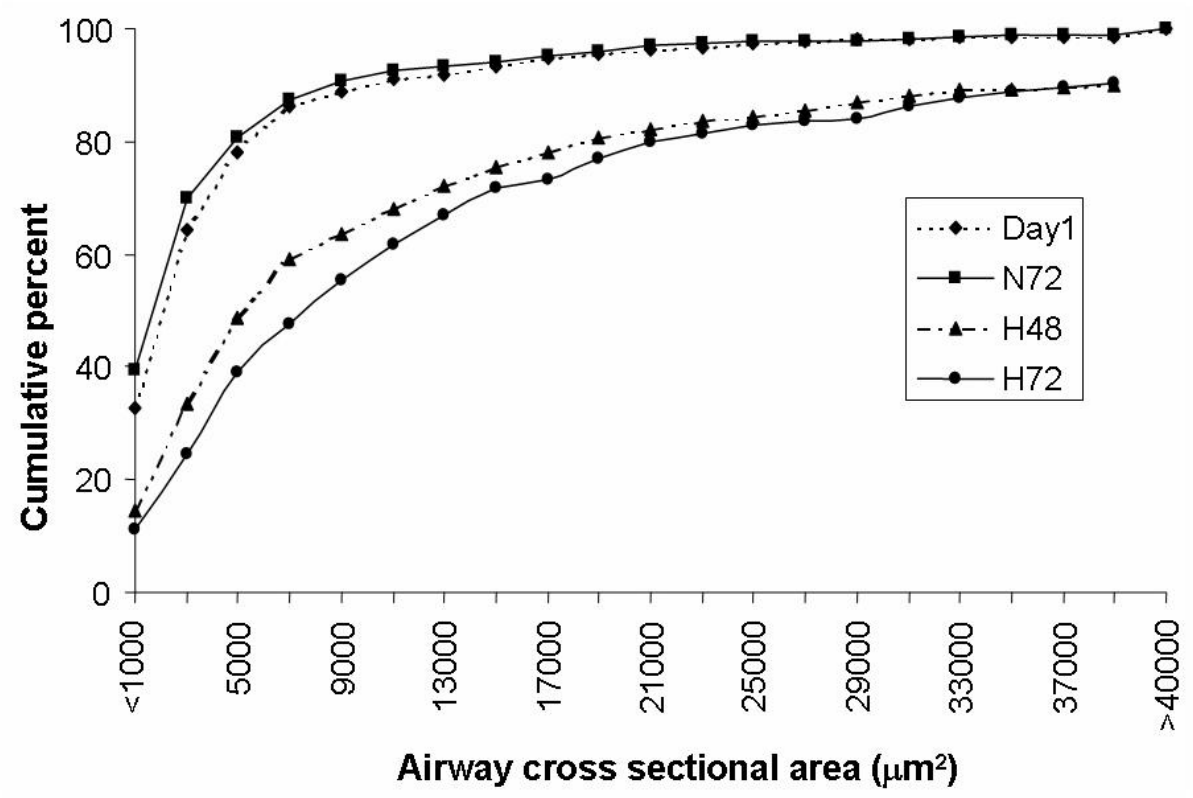


Figure 2.6: Cumulative airway size profiles from pooled lungs explants (n=6) show that the majority of airways in Day 1 lung and normoxic lung (N72) have a cross sectional area below 5000um2. Hyperoxic lung however has airways with a much greater cross sectional area and culture for 48 and 72 hours in hyperoxia produces very similar airway profiles

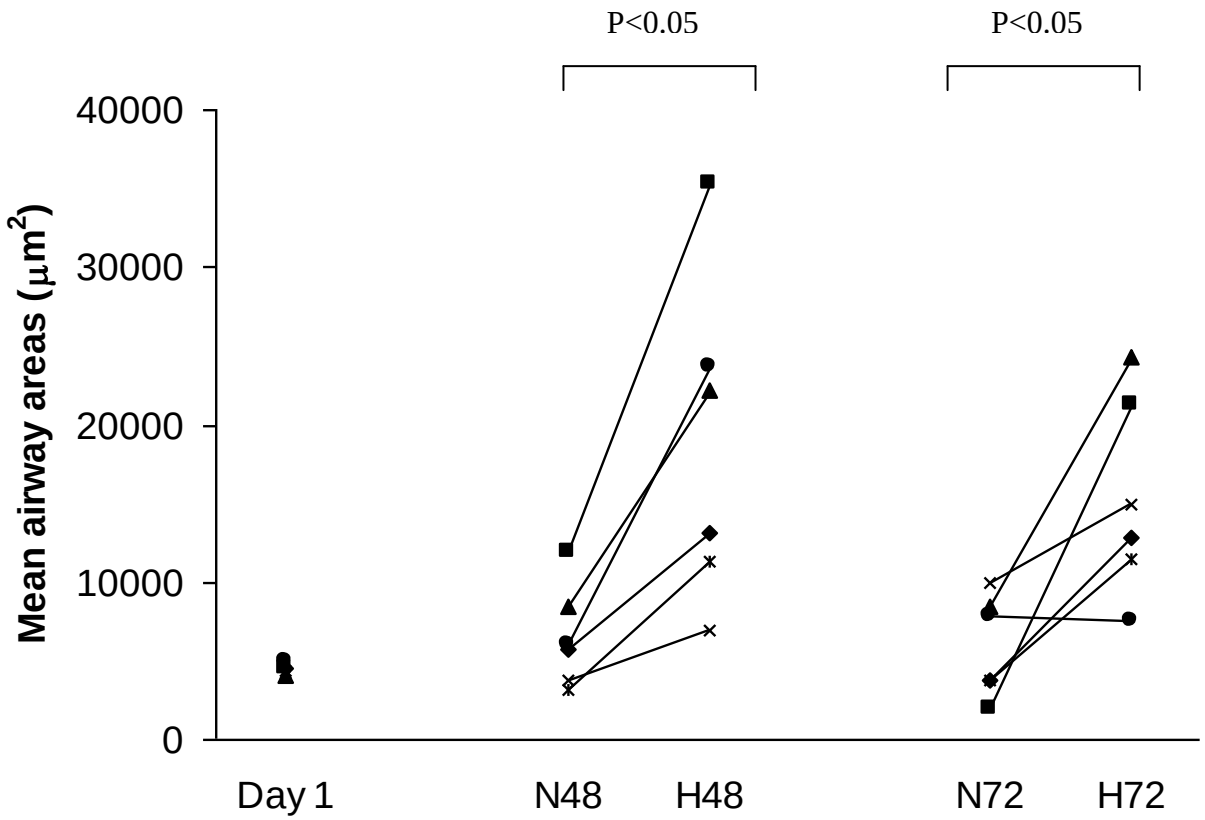


Figure 2.7: Mean airway cross sectional areas for each explant section. Normoxic cultured tissue (N48 and N72) have similar airway sizes as lung shortly after culture is established (Day 1). Culture in hyperoxic conditions for 48 hours and 72 hours (H48 and H72 respectively) results in dilated airways compared to normoxic controls(n=6).

when compared to day 1 and normoxic tissues (all $p<0.05$ when hyperoxic tissue compared to corresponding normoxic tissue or day 1 samples, Figure 2.7). This was further demonstrated by the median cumulative surface area of the pooled tissues which was increased in both the hyperoxic conditions (median 6100μm^2 at 48 hours and 8600μm^2 at 72 hours, Fig 2.6).

Effect of hyperoxia on airway contribution to total tissue

The surface of the sections occupied by airway was similar at day 1 (median 15%, IQR:13-16%) and normoxic tissue at 48 hours (15%, IQR: 14-21%) and 72 hours (15%, IQR: 12-23%). However, this area was significantly increased in the hyperoxic tissue (Figure 2.8) [37% (IQR: 25-52% at 48 hours) and 43% (IQR: 24-48%) at 72 hours, $p<0.05$ when compared to day 1 and corresponding normoxic tissue]. I counted the number of epithelial and mesenchymal cells in 8 high power fields on each section. The relative contribution by epithelial cells was similar in the day 1 [median 30% (IQR: 25-31%)] and normoxic tissues at 72 hours [29% (IQR: 26-34%)]. In contrast, the hyperoxic-exposed tissues were comprised of a higher percentage of epithelial cells [38% (IQR: 33-46%); $p<0.05$ when compared to day 1 and N72 conditions].

Effect of hyperoxia on airway density

This increase in surface area occupied by the airways was not due to increased numbers of airways as the number of airways per surface area were similar in the tissues exposed to normoxia or hyperoxia at 72 hours of culture [N72 33/mm^2 (IQR: 22-40) vs. H72 29/mm^2 (IQR: 14-38), p=NS].

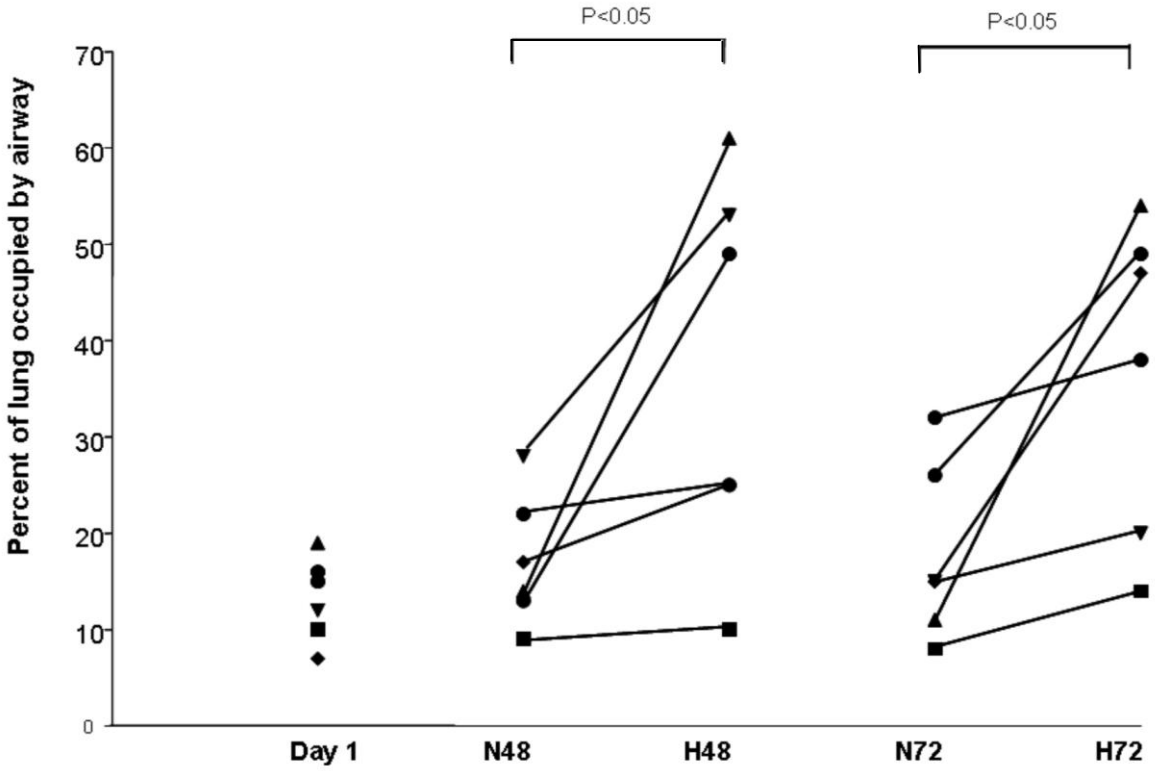


Figure 2.8: Culture of fetal explants maintains the proportion of lung surface area that is occupied by airway. The use of hyperoxic culture conditions leads to proportionally greater contribution of airway to the lung, either by increased airway size, loss of mesenchyme or both (n=6).

Effect of hyperoxia on epithelial thickness

Inspection of tissue under high power revealed that epithelium was significantly thinner in hyperoxic tissue. Airways were initially lined with columnar epithelium, but by 72hrs in culture were cuboidal in appearance. Hyperoxic exposure produced cells that had a flattened appearance (Figure 2.5c). Mean epithelial thickness for hyperoxic lungs was thinner than all normoxic pairs (6.4μm and 8.7 μm respectively, $p<0.05$) (Figure 2.9).

Discussion

I determined that the use of modular gas flasks enabled sterile tissue culture to be carried out at predetermined oxygen concentrations. Culture in a 21% oxygen environment enabled tissue structure to be maintained over a 96 hour period. Airway size was maintained, as was the proportion of tissue volume occupied by airways. Culture in hyperoxic conditions (95% oxygen) after an initial stabilisation of culture produced an enlargement of the airways found in the tissue. This change was most dramatic in the periphery of the explant tissue. These changes in airway size and relative contribution to tissue were statistically significant when compared to tissue at the initiation of culture, and compared to tissue subsequently maintained in normoxic conditions.

The epithelial changes included a thinning of epithelial layer cells in cultured lung. High-powered microscopic sections demonstrated a change from columnar and pseudostratified epithelium to a cuboidal appearance. I did not carry out electron

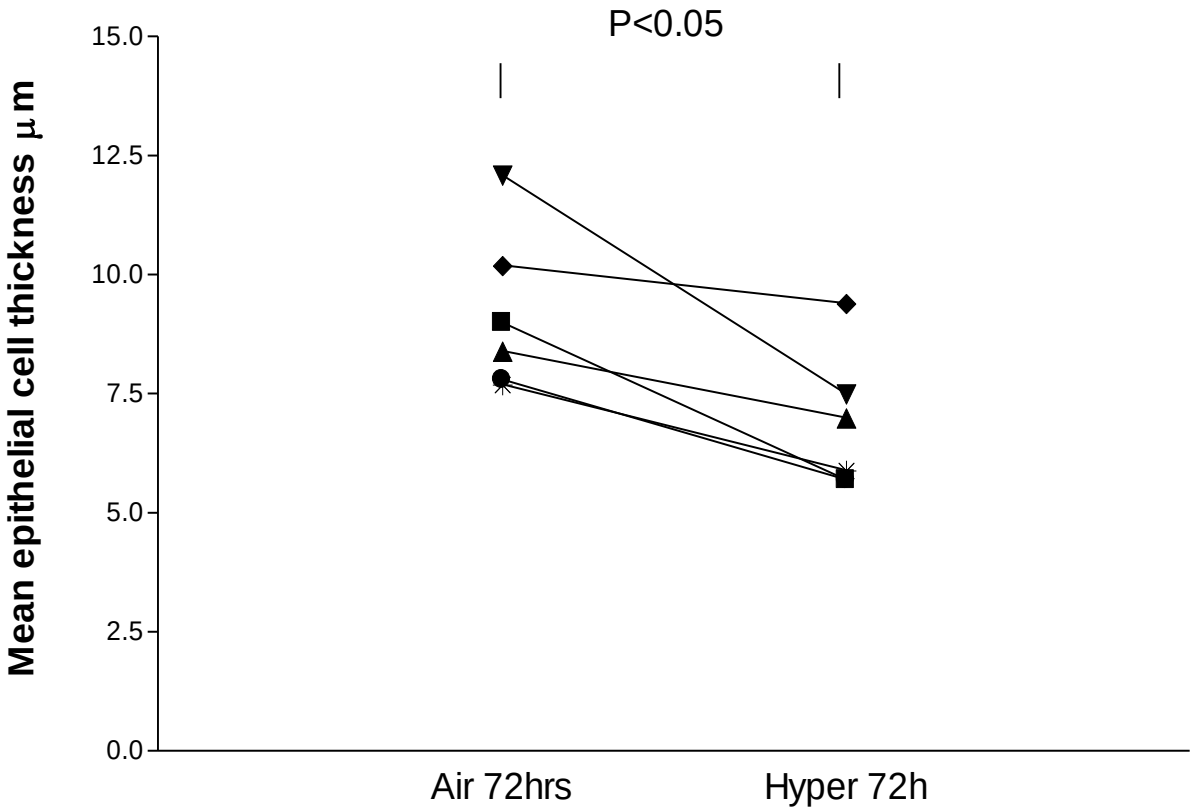


Figure 2.9: After the first day in culture, exposure to 95% oxygen for a subsequent 72hrs resulted in airways lined by significantly thinner epithelium than air cultured pairs. $P<0.05$, (n=6)

microscopic analysis to confirm this at an ultrastructural level, however this has previously been described as an effect of culture[135]. In those studies, epithelial morphological changes are accompanied by functional maturation. There is shown to be activation of Surfactant Protein A transcription with resultant production of SpA protein. These changes are expected at 24 weeks of gestation during normal development, but are observed within 4 days of culture in lungs at 12 weeks gestation[8]. I found that hyperoxia produced a further change in morphology with a flattening of epithelial cells, reminiscent of Type I pneumocytes. The properties of these flattened cells have not been characterised. It must be noted however, that flattening of an epithelial cell does not necessarily indicate maturation to Type I morphology. It is possible that this may represent a dysplastic response or flattening due to increased intraluminal pressure. Culture of Type II cells on plastic base is known to induce a number of possible changes in cell morphology. Included in these is a flattening of cells unaccompanied by differentiation and may thus represent metaplasia[140].

The dilatation of airways seen predominantly in the periphery of tissue can be ascribed to a number of possible causes. This could be due to an increase in the number of epithelial cells due to hyperoxia, either due to increased proliferation, or reduced cell death. Alternatively, there might have been a reduction in the mesenchyme compartment. Mesenchyme cell depletion similarly could be due an alteration in proliferation/cell death ratios. A final proposition is that there was an increase in fluid within the airways.

The presence of fluid in the developing lung was historically ascribed to the continuity of the fetal airway tree with the amniotic fluid collection within the uterus. Previously, most clinicians were of the belief that amniotic fluid, produced partly by amniotic membranes and added to by fetal urinary output, effectively filled the fetal airway tree thus providing support. Recent work has placed the fetal lung as an effective fluid generator, by virtue of a Chloride anion (Cl-) secretory ability by epithelium throughout gestation[141 142]. There is a net efflux of fluid from the lungs via the trachea into the amniotic space during fetal development. At the time of delivery, a rise in circulating catecholamines results in a conversion of the lung to fluid absorption[143]. Thus the airways are prepared for their ensuing air respiration function.

Oxygen is also able to exert a modulatory effect on the production of a number of developmental proteins by gene regulation, including Vascular Endothelial Growth Factor(VEGF) [144], Surfactant protein A (SpA) [145] and Tissue Inhibitor of MetalloProteinases (TIMP) [146]. The mechanisms for oxygen control over protein synthesis are not fully elucidated but are believed to be multiple. In some circumstances the presence of oxygen generates ROS which act as inhibitors of normal cellular processes. Many of these are in an attempt to protect replicating cells from the effect of oxygen toxicity on DNA at a vulnerable stage by inhibiting proliferation. Aconitase is an example of an enzyme required for mitochondrial respiration. By inactivation of this enzyme by the superoxide radical, the ‘closing down’ of mitochondrial function prevents the subsequent mitochondrial generation of ROS which can lead to cell injury and death as explored in Chapter 4, page 154. The activation of p21 protein formation by oxygen is another example. p21 epxression is upregulated by hyperoxia and acts to halt cell proliferation during mitosis[147 148].

Alternatively, oxygen may act to encourage cellular activity and physiological processes. These include activation of the ROS-sensitive transcription factor, nuclear factor (NF)-κB[149 150]. It is via NFκB that induction of cytokine production may proceed enabling an inflammatory response as a means to recruit the immune system to repair the site of oxidant injury.

The best known oxygen dependant expression of a physiological protein is the production of VEGF[151 152], which has a role to play in both normal vascular growth and in a variety of disease processes. This protein will be further explored in Chapter 3 but again it should be noted that the effect of oxygen is to suppress production of VEGF.

A possible role for oxygen regulation of fluid production by the explant epithelium can be proposed by analysis of the role of Keratinocyte Growth Factor (KGF) [14] in the developing lung. KGF belongs to the Fibroblast Growth Factor (FGF) family of growth factors. Within this, family, KGF is designated FGF-7, as an alternative nomenclature. The FGF family, possess key roles in embryonic growth and development. Transcripts for KGF are located in lung mesenchyme at sites where there is active airway branching[13]. Receptors for KGF are expressed in epithelial cells, and when stimulated by exogenous KGF, have been found to promote proliferation and maturation of Type II cells in cell cultures[153], and when administered to rats intratracheally[154]. Work carried out with human fetal lung explant culture has demonstrated that the administration of KGF to growth medium, results in an increase in liquid secretion by the lung epithelium, resulting in distended airways[136], as seen in my experiments. A role for KGF in the distension seen with my experiments can be hypothesised based upon experiments that demonstrate that hyperoxic conditions

applied to neonatal rabbits resulted in upregulation in KGF mRNA expression[155]. I have not investigated this particular phenomenon within my experiments.

The culture methodology set out, was adapted from work carried out by McCray et al[137]. The particulars of this method are that it aims to maintain viability of tissue pieces in culture whilst maintaining structural integrity. Variations occur in lung culture methodology, with major differences being grouped into tissue source, use of serum supplementation in culture medium, and the use of supporting structures.

As mentioned in the results section, we estimated an approximate loss of 10% of specimens plated out into culture conditions. Although we used lungs from 6 fetuses for the experiments described in this chapter, many more specimens were originally utilised in the developmental stage of our work. A few of these were utilised to develop optimal culture conditions e.g by altering the pH of the culture media, and varying the methods of harvesting tissue specimens without tearing the fragile explants, and two were lost to fungal infection during culture. In particular, 8-10 specimens were cultured and frozen at harvesting. These specimens were maintained in frozen state after sectioning and then immunostained. After varying degrees of success with methodology, I changed to formaldehyde preservation of cultures after harvesting with far more consistent staining results which provided the data for these and following chapters. Only one lung failed to culture with any appropriate maintenance of morphology in the final epoch of experimentation requiring discarding.

The use of human lung for explant culture has a number of advantages when compared to animal equivalents. Primarily, the research involved is ultimately linked

to human disease processes and thus, human tissue has greater relevance than tissue from other species. Alternative lung explant work has been carried out predominantly with mice[156 157] and rats[35 158 159]. The practical advantages of using these species are that the small size of the lung buds enables oxygen and nutrient diffusion throughout the tissue. Hence, entire lung buds may be used in culture, as opposed to pieces of tissue with human explant culture.

Exposure of human fetal lung to varying concentrations of oxygen in serum-free conditions, cultured on artificial membranes has been studied. In 21% oxygen, Cossar et al [160] described expansion of the intraluminal compartment, similar to the changes I demonstrated with 95% oxygen. Similar experiments by Accaregui et al[161] using stepped oxygen concentrations between 1% and 21% oxygen showed that explants maintained at 1% oxygen maintained a lumen volume density of 15% as compared with 50% for those maintained in air. In their later experiments, no morphological changes occurred when oxygen concentration was increased from 21% to 95% [162]. Others who used similar methodology found similar dilation of airway spaces in 21% oxygen[35 163].

As our work involved the use of tissues that had been maintained in the extrauterine environment for unto 48 hours before we commenced culturing under controlled conditions, we remained mindful that these conditions may have influenced the subsequent development in culture, both under normoxic and hyperoxic conditions. We were reasonably reassured that time from harvesting these tissues and commencement of culture were fairly consistent between tissues and unlikely to vary by more than 24 hours. More importantly, our main objective was the comparison in changes in tissue morphology that occurred from the time culture began, rather than

any particular effect of harvesting or initiation of culture on the tissue.

The presence of serum within explant culture is not universally utilised. The hormones in serum-supplemented media have been shown to promote Type II cell maturation during culture[164]. This may mask the ability to study the effects of individual growth factors on lung development.

Variation in the use of supporting structures varies from the culture of explants on inert grids or semi permeable membranes, which are then immersed partially in culture media. These may be associated by the spreading out of tissue over the membrane, with a potential loss of 3D structure. It is assumed that this effect would be amplified in human culture methodology, as the tissue is not maintained within the connective tissue sheath that envelops entire lung lobes. The use of collagen as an adherent has been utilized whereby the membranes upon which the explants are cultured, is pre-coated with a liquid collagen mixture which is then allowed to gel.

The culture of tissue in a semi-solid gel enriched with collagen has been previously used[137], and provides a matrix into which the lung can migrate into, whilst limiting the spreading out of tissue sometimes seen in fetal lung cultured on membranes. This collagen may itself alter the growth characteristics of the lung tissue[165], as it does in developing fetal lung in vivo.

As there is no oxygen consumption by this gel, oxygen tensions are maintained between the closed gaseous environment and the tissue surface. To my knowledge, the gradient of oxygen tension within the tissue has not been elucidated, and is dependant on the density of the tissue and its oxygen consumption[166]. It was for this reason that I elected to analyse the periphery of the lung explants, thus enabling

comparisons of oxygen tension. It is likely that the centre of explants may be relatively hypoxic in both sets of conditions. The greater the oxygen consumption by the cells, the more steep the drop in oxygen gradient within the tissue. Gassman's analysis of PO2 gradient within embryonic blastocysts did however demonstrate a surprisingly well maintained oxygen tension as the core of the blastocyst was approached as measured by advancing an oxygen microelectrode in 50μm steps from the periphery of bodies that measured approximately 70μm in diameter[166] . Figure 2.10 demonstrates that even at the centre of the embryonic body, PO2 is relatively well maintained when compared to the periphery. Oxygen consumption studies on bodies of varying sizes also indicate that the larger the body, the lower the oxygen consumption of each individual cell thus maintaining a reasonable oxygen tension towards the centre of even relatively large solid structures dependant purely on diffusion of oxygen for metabolic requirements.

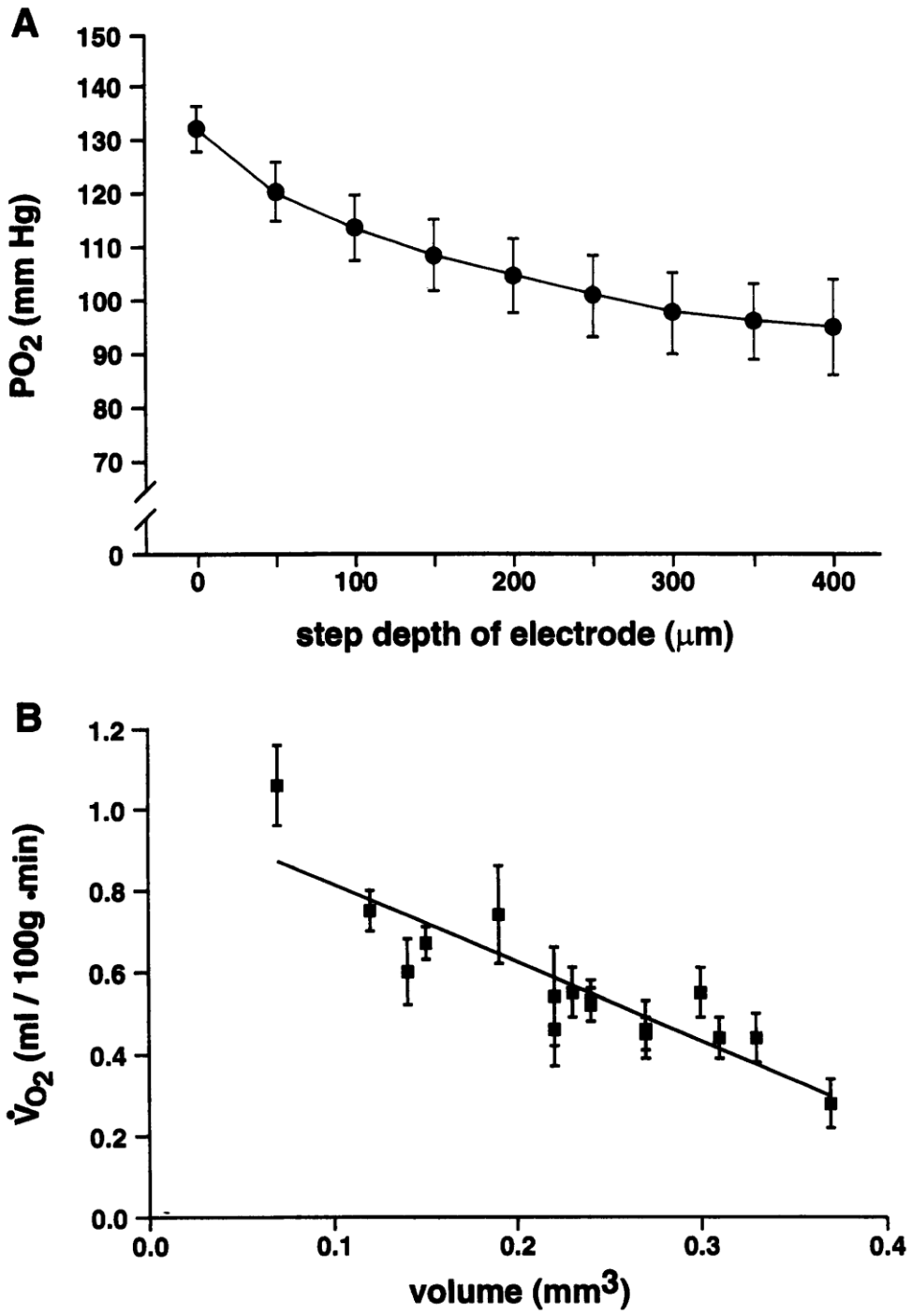


Figure 2.10: Measurement of oxygen tensions at varying depths within a solid embryonic sphere displays relatively well maintained tensions as far as the centre of the structure (A). In analysis of oxygen consumption of embryonic bodies of various diameters, it is apparent that as the size of the body increases, the relative oxygen consumption per unit mass decreases preventing excessive tissue hypoxia. Diagrams reproduced from Gassman M et al[166].

Summary

I have demonstrated that the fetal human lung explants can be placed into culture using a collagen based semi-solid gel medium to maintain structural characteristics and aid harvesting of tissue. The additional use of serum supplemented feeding medium enabled tissue to be maintained in culture for up to 96 hours.

The use of modular gas chambers allowed explants to be grown under varying oxygen tensions reliably, once cultures had been established.

I found that culture in hyperoxic conditions (95% oxygen content) produced visible structural differences when compared to explants maintained in normoxic conditions (21% oxygen). Hyperoxic tissue demonstrated relative dilatation of airways, an increase in the proportion of tissue occupied by airway lumen, and thinning of epithelium in dilated airways.

These findings could be due to a number of mechanisms which include an increase in fluid secretion in the lung, loss of mesenchyme by increased cell death and/or reduced proliferation, of relative expansion of epithelium by opposite alterations in cell death/proliferation.

Applying the observations of increased airway dilation, loss of mesenchyme and thinning of epithelium to the developing human could have a number of possible parallels in vivo.

As suggested by many of the explant model research thus far carried out, this could

represent an acceleration of lung development as a physiological response to prepare the lung for the extrauterine oxygen rich environment. The changes thus represent movement of pseudoglandular lung into the saccular phase and has previously been supported by demonstration of flattening of epithelial cells and expression of surfactant proteins at a precocious stage of lung development.

Alternatively, these findings could be related to the acute effects of oxygen toxicity whereby it is via tissue damage and increased vascular permeability that I noted my observations, making this a model of lung injury in the preterm infant.

Finally, I may be witnessing dysregulated lung development in response to hyperoxia that has its closest equivalence in CLD. This may rely in part on the accelerated lung development mentioned above, but in a disordered non-physiological manner.

I shall investigate these alternate hypotheses in the following sections by examining the changes in the vascular components of the explant model, and investigating the processes involved in the remodelling of the lung I have reported.

Chapter Three:

Effect of hyperoxia on the vasculature in human fetal lung explant culture

Introduction

The exposure of the preterm infant to hyperoxic conditions results in an acute lung injury, the earliest component of which includes the destruction of the pulmonary vascular endothelium. Direct toxicity by the generation of ROS is believed to contribute to this process. It appears however, that secondary to hyperoxia there is loss of blood vessels from tissue as part of a physiological response. This is exemplified in preterm infants by the pruning of blood vessels in the retina by prolonged high blood oxygen tensions. This obliteration of small blood vessels in response to hyperoxia is believed to be mediated in part by suppression of the angiogenic factor Vascular Endothelial Growth Factor (VEGF) [167].

In contrast, regional tissue hypoxia is known to promote the proliferation of blood vessels within the tissue- again in response to VEGF[168]. In the developing fetus, it is likely that this process regulates the growth and proliferation of blood vessels throughout the body.

I have demonstrated that exposure of fetal human lung explants to hyperoxic culture conditions results in a regression of the mesenchyme compartment relative to epithelium.

I hypothesise that the regression of mesenchyme is accompanied by a reduction of blood vessels in the tissue, in response to hyperoxia.

Methods

Tissue culture

Tissue culture and section preparation was carried out as described earlier in detail (Chapter 2, p75). Briefly, pseudoglandular human fetal lungs were minced and placed in culture within a collagen based serum supplemented semi-solid culture medium. Initially, explants were maintained at 37^{o}C in an air/5%CO_2 gas mixture. Samples were harvested 24 hours after establishment of culture (Day 1), and at 72 hours after placement in normoxic (air/5% CO_2) or hyperoxic (95% O_2/5% CO_2) conditions (N72 and H72 respectively). After harvesting, explants were paraffin embedded in blocks and were cut into 4um sections using a microtome, onto glass microscope slides.

Immunostaining

Paraffin tissue sections were immunostained for CD-31 for vascular endothelial cells. (Sigma-Aldrich,Poole,UK C-2931). Methodology used was as described in Chapter 2. Briefly, sections were deparaffinised before antigen retrieval was carried out with buffered citric acid under high pressure. Slides were incubated overnight at 4^{o}C with the primary antibody diluted in 0.05% bovine serum albumin/PBS at optimal dilution (mouse anti-human CD-31 1:30). Thereafter the slides were washed and incubated for 30min in goat anti-mouse biotinylated secondary antibody (Sigma-Aldrich,Poole,UK B9904) diluted 1:250. Avidin-peroxidase reagent (Vector laboratories) followed by diaminobenzidine produces a colour response, before sections were lightly counterstained with Ehlich's haematoxylin.

Image analysis

Analysis was carried out to express the proportion of tissue that stained positive for CD-31 relative to total mesenchyme. As my interest was to compare the effect of similar oxygen tensions on blood vessels, I standardised analysis to tissue at the periphery of my specimens. Sections were analysed for Day 1 specimens, and matched normoxic and hyperoxic samples at 72 hours (N72/H72).

For each specimen, eight pictures were taken at x100 magnification. These were taken in a standardised fashion to prevent biased sampling. The centre of the tissue was approximated, and then the photo would be acquired by moving from this point outwards in eight directions as denoted (Figure 3.1).

I wrote a macro program using the image analysis software package QWin. For each image, the software detected any brown staining and produced a binary overlay from this. I then drew a separate binary overlay utilising a graphic tablet and pen (Wacom plc) denoting airway lumen. Inverting this image onto an overlay of the sample and withdrawing its edges by the epithelial thickness obtained a representation of mesenchyme. The QWin package was able to calculate the size of each area of brown staining (termed a 'vessel body'), and the total areas of brown staining and of mesenchyme. The ratio of vessel staining to mesenchyme could then be calculated by transferring the results of the analysis to Excel (Microsoft, WA, USA).

Additional information was then obtained regarding the average distance from blood vessel to the nearest airway lumen. This was carried out using specialised properties in the image analysis package. The following steps were processed:- the airway lumen) is used as the template.

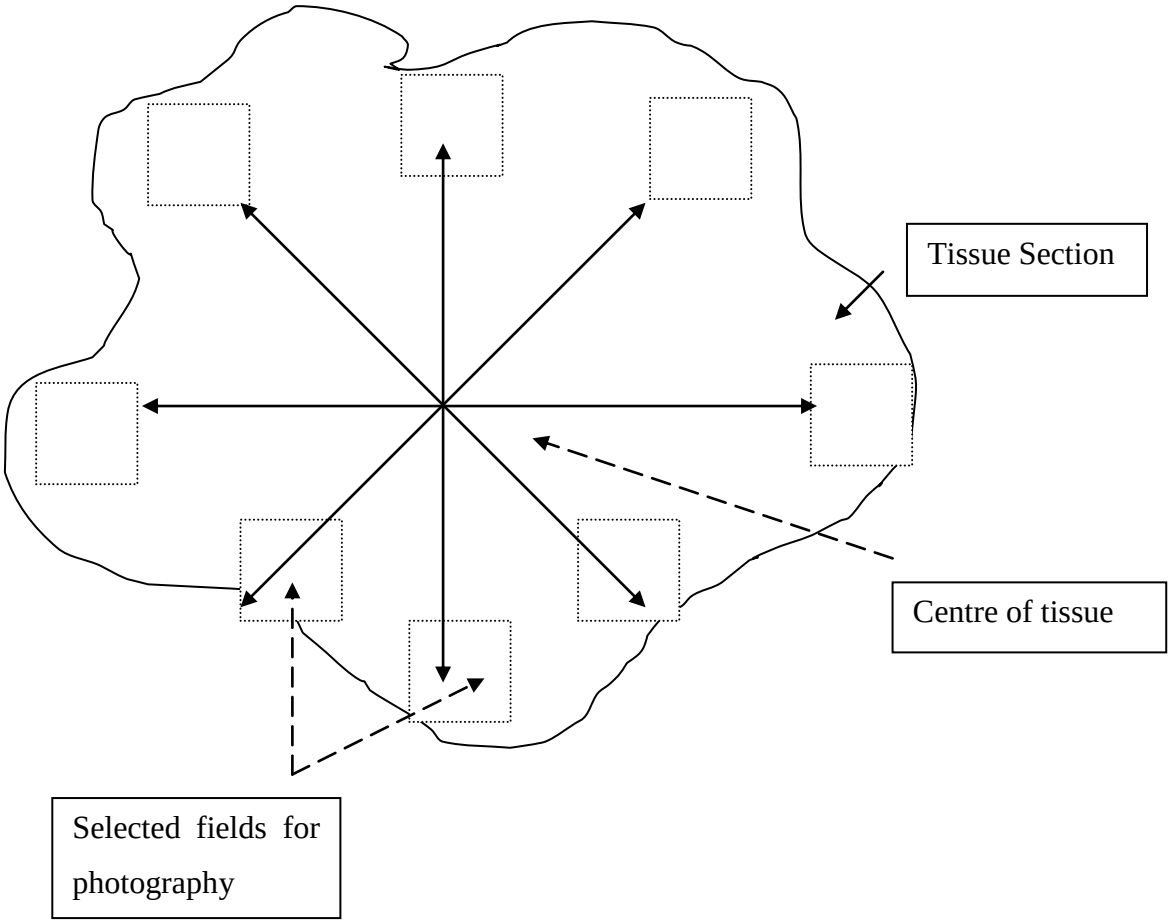


Figure 3.1: Representation of tissue sample. Eight areas are photographed using set directions from a central point. This enables periphery of the explants to be sampled in an unbiased fashion, allowing comparison between tissue specimens

1) The binary image depicting the section previously produced (excluding the airway lumen) is used as the template.

2) A process known as geodesic distance binary transformation was applied whereby every pixel within the image is given a value denoted by its relative distance from an edge. By restricting the process to an area within the centre of the image, it is ensured that the pixels analysed are measuring distance from airway lumen rather than from the edge of the tissue.

3) In order to test how far the vessels are from the lumen, I overlay the previously obtained binary image denoting blood vessels onto the 'distance map'.

4) A specialist measure function was applied where the values of the labelled pixels were collected, but with the blood vessel image used as a mask. As a result, the average value of the pixels represents the average distance of the blood vessel from the airway lumen.

This data was also transferred to Excel for analysis.

The program macro written to carry out these functions is provided in Appendix I.

Statistical analysis

Comparisons between Day1, normoxia and hyperoxia exposed matched lung sections were made with the SPSS software package using paired-t-test analysis for normally distributed data, and Wilcoxon signed rank paired analysis for non-parametric data. Significance for two-tailed tests were set at $p<0.05$.

Results

Descriptive

On inspection of whole sections, it could be seen that Day 1 tissue and normoxia cultured tissue (N72) had similar distributions of vasculature i.e. combinations of linear blood vessels between airways, and small bundles of cells staining positive for CD31 arranged in close proximity to terminal airway buds (Figure 3.2 A and B respectively).

Inspection of hyperoxic tissue revealed a relative paucity of endothelial cell bundles, particularly in the periphery of the tissue (Figure 3.3). The collection of vessels in these areas altered from clusters or rounded collections of vessels, to long, thin vessels (Figure 3.2C).

Morphometry

When comparing the proportion of mesenchyme occupied by vessels, it could be seen that there was an increase in percent coverage in 5 out of 6 samples. The median coverage was 3% (IQ 3-7%) in Day 1 tissue, rising to 7 % (5-8%) after a further 72 hours in culture under normoxic conditions. Hyperoxic culture samples had less vasculature in mesenchyme (4% (IQR 2-5%)) than paired normoxic samples 7 % (5-8%) ($p<0.05$). Thus CD31 occupied a greater proportion of the interstitium in normoxic cultured tissue when compared to hyperoxic cultured tissue (Figure 3.4).

Measurement of distance of vessels from the nearest airway lumen showed little change after 72hrs of normoxic culture from Day1 samples. Culture in hyperoxic conditions resulted in less distance between vessels and airways, which could only

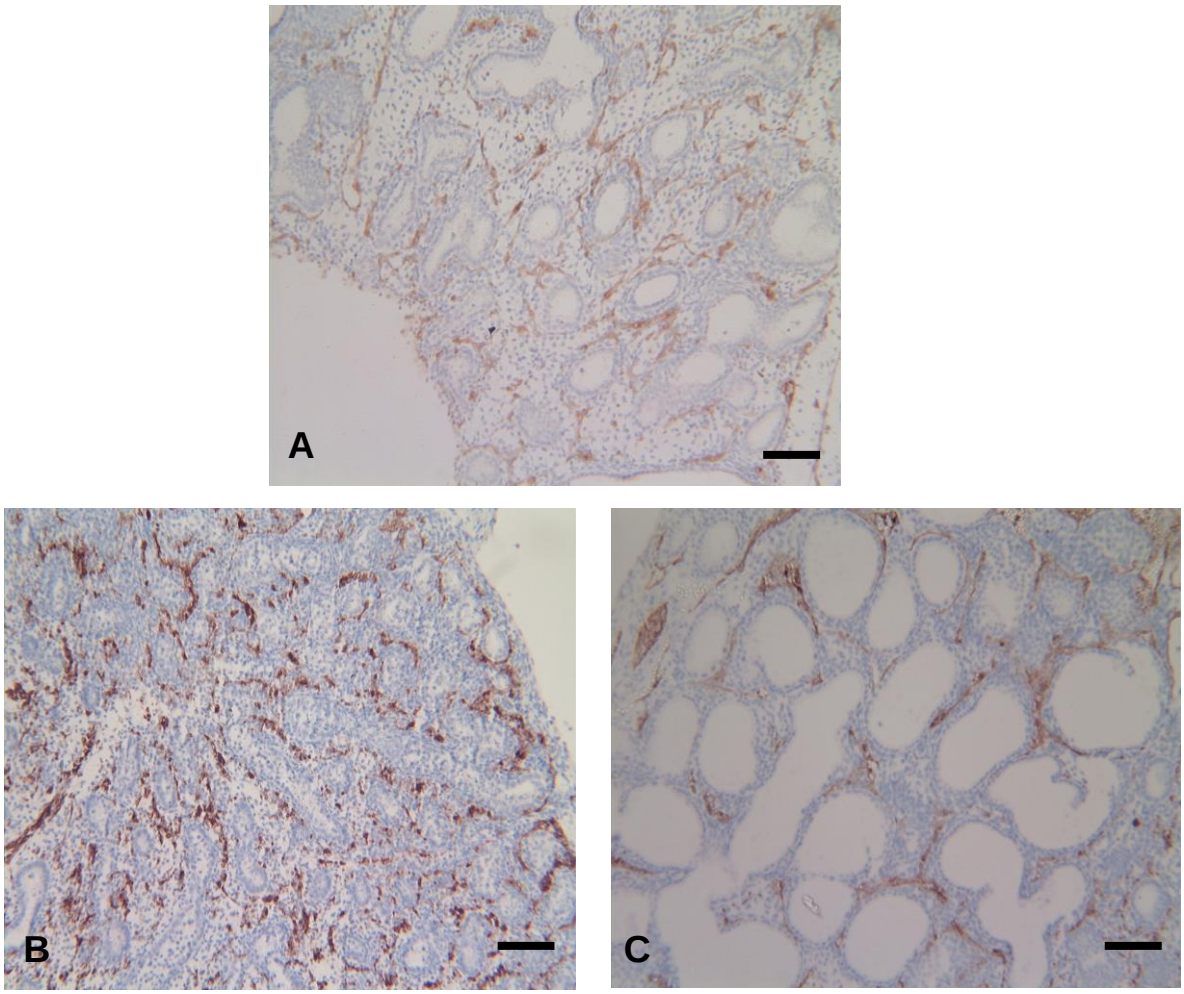
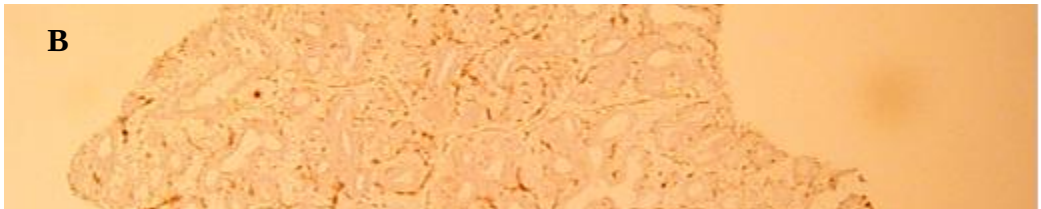


Figure 3.2: Immunohistochemical staining of cultured lung for CD-31 (brown) with haematoxylin background. Airways on Day 1 of culture (A) are surrounded by blood vessels that exist mainly as capillary plexus. There is little alteration in this appearance after subsequent culture in normoxic conditions for 72hrs (B). By culturing in hyperoxic conditions instead, there is a 'pruning' of vasculature. There are fewer capillary plexi seen, and more continuous bands of vessels (C). These denote vascular lakes around airways. Bar=100μm.

A

Figure 3.3: Culture of tissue under hyperoxic conditions leads to regression of vasculature from the periphery of the lung. Comparing Day 1 tissue (a) with H72 tissue (b) demonstrates the change in appearance with culture in oxygen. Bar =1mm



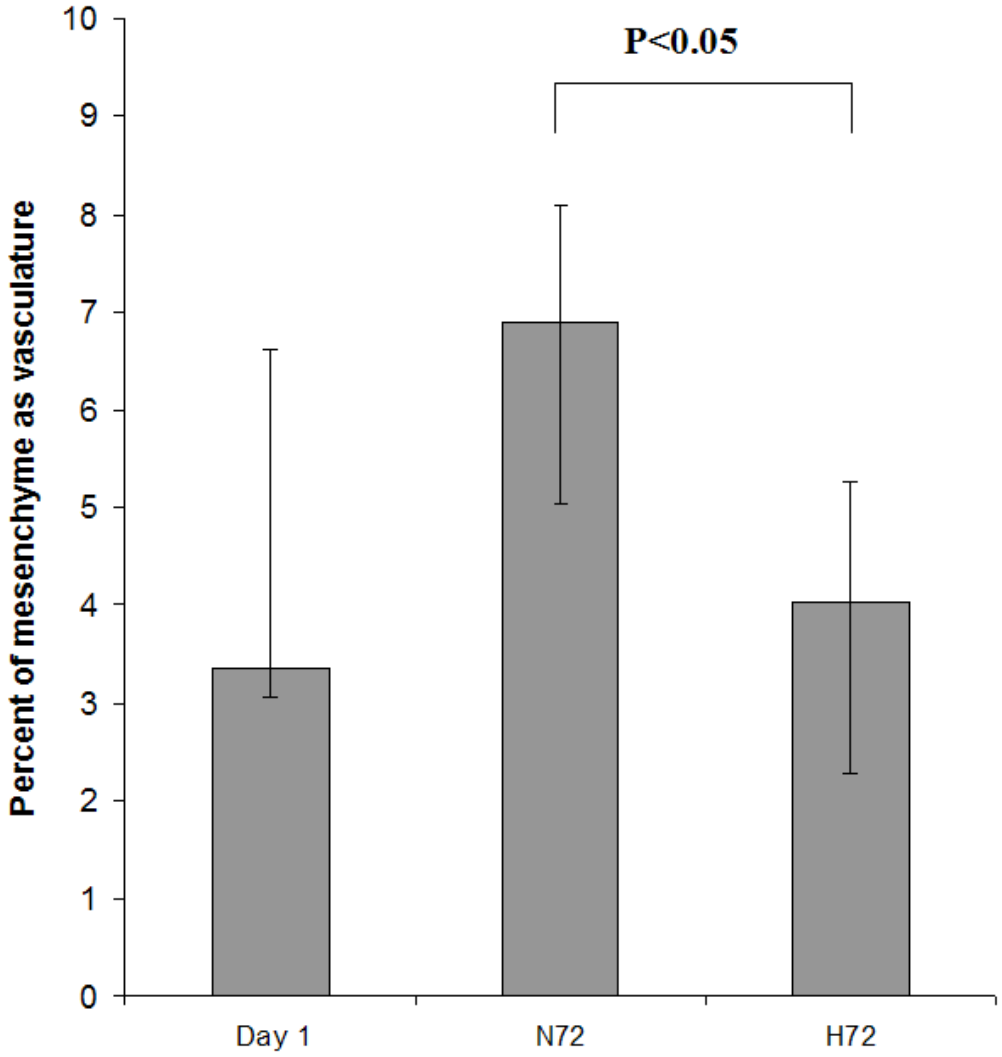


Figure 3.4: Culture under normoxic conditions resulted in an increased proportion of blood vessels within mesenchyme after 72hrs. Alternative culture in hyperoxic conditions resulted in less vasculature in mesenchyme when compared to normoxic controls.(Median 4 vs. 7%) Error bars=IQ ranges. N=6,$p<0.05$

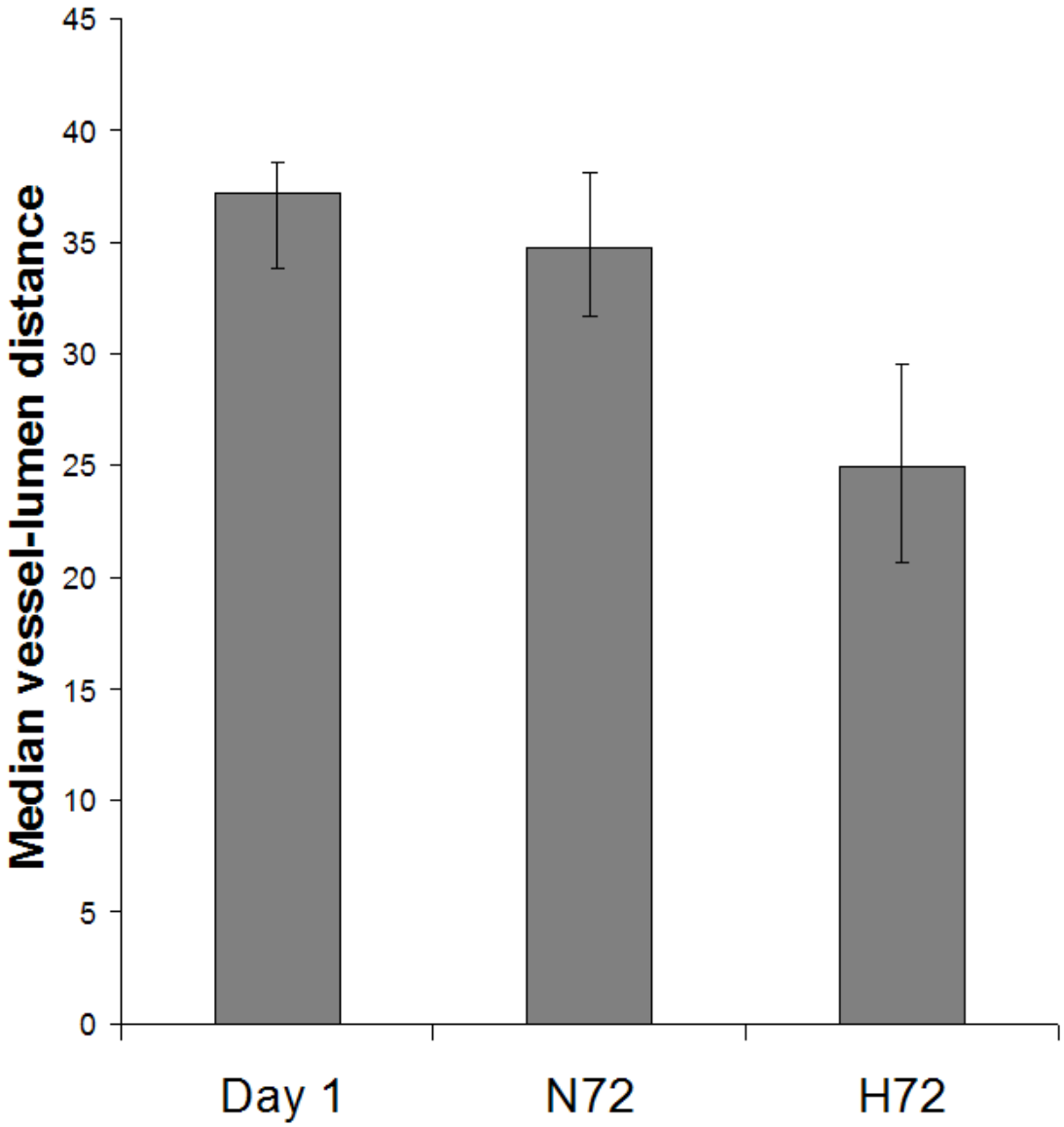


Figure 3.5: Distance between blood vessels and nearest airway lumen do not alter with culture under normoxic conditions. (Day 1=37μm, N72=35μm medians). Hyperoxia results in less distance between the two compartments (25μm,). Error bars=IQ ranges. N=6

partly be explained by thinner epithelium in hyperoxic airways. Median distance from vessel to nearest airway lumen was 37μm (IQR 34-39μm) in Day1 tissue. In normoxic cultured tissue (N72) the distance was 35μm (IQR 32-38μm), and in hyperoxic-cultured tissue (H72), the distance was 25μm (IQR 21-30μm) (Figure 3.5).

Discussion

My initial work using this model has demonstrated that the exposure of fetal human lung explants to culture in 21% oxygen (normoxia) results in the maintenance of the overall structure and relative proportions of epithelium and interstitium. The exposure of tissue instead to a 95% oxygen (hyperoxia) environment produced a decrease in the amount of interstitial tissue by processes that I shall investigate later(see Chapter 4).

The interstitium is comprised of a number of different cell types which may exist in varying degrees of differentiation or specialisation. Thus in the earliest embryonic lung, the mesenchyme is an amorphous collection of cells that most closely resemble fibroblasts. With time, groups of cells within this compartment mature into more specialised cell types. Thus, endothelial cells condense *de novo* to form blood vessels, smooth muscle cells form the sheath coverings of both airways and larger blood vessels, isolated myofibroblasts form within the maturing alveolar saccule enabling secondary septal formation, and fibroblasts functionally mature to produce the extracellular matrix components such as collagen and elastin.

The developing vasculature is specifically of interest as there is recognition of damage in the preterm infant exposed to a hyperoxic environment, and chronic remodelling of blood vessels in the chronic hyperoxic environment.

A brief description of the normal development of the pulmonary vasculature has been set out in Chapter 1. However, I shall outline key features to emphasise potential mechanisms driving these processes and thus help provide explanations for the pathophysiological changes seen in my experimental model.

The earliest demonstration of development of pulmonary vasculature occurs in embryonic lung. Two differing processes appear to occur leading to vessel development, these are angiogenesis and vasculogenesis. Angiogenesis is the formation of vessels by the sprouting of endothelial channels from pre-existing vessels. This process is believed to predominate in the proximal pulmonary vascular tree where as early as weeks 5-7, pulmonary vessels branch from the 6th pair of cranial ventral aortic arches to form the pulmonary arteries[3 15]. It is unclear how far along the pulmonary tree the process of angiogenesis continues.

Vasculogenesis is the *de novo* creation of blood vessels by condensation of angioblasts within the mesoderm which subsequently migrate to form minute capillary leashes. These are arranged in the form of plexi initially and tend to form in geographical association with terminal lung buds (Figure 3.6A). This process is believed to predominate in the distal vasculature and therefore probably becomes established later than angiogenesis within the pseudoglandular stage of lung development.

During the canalicular phase of lung development, the capillary plexi become arranged around and closely apposed to the dilating terminal airways (Figure 3.6B). With the marked dilatation of the airsacs during the saccular stage of development,

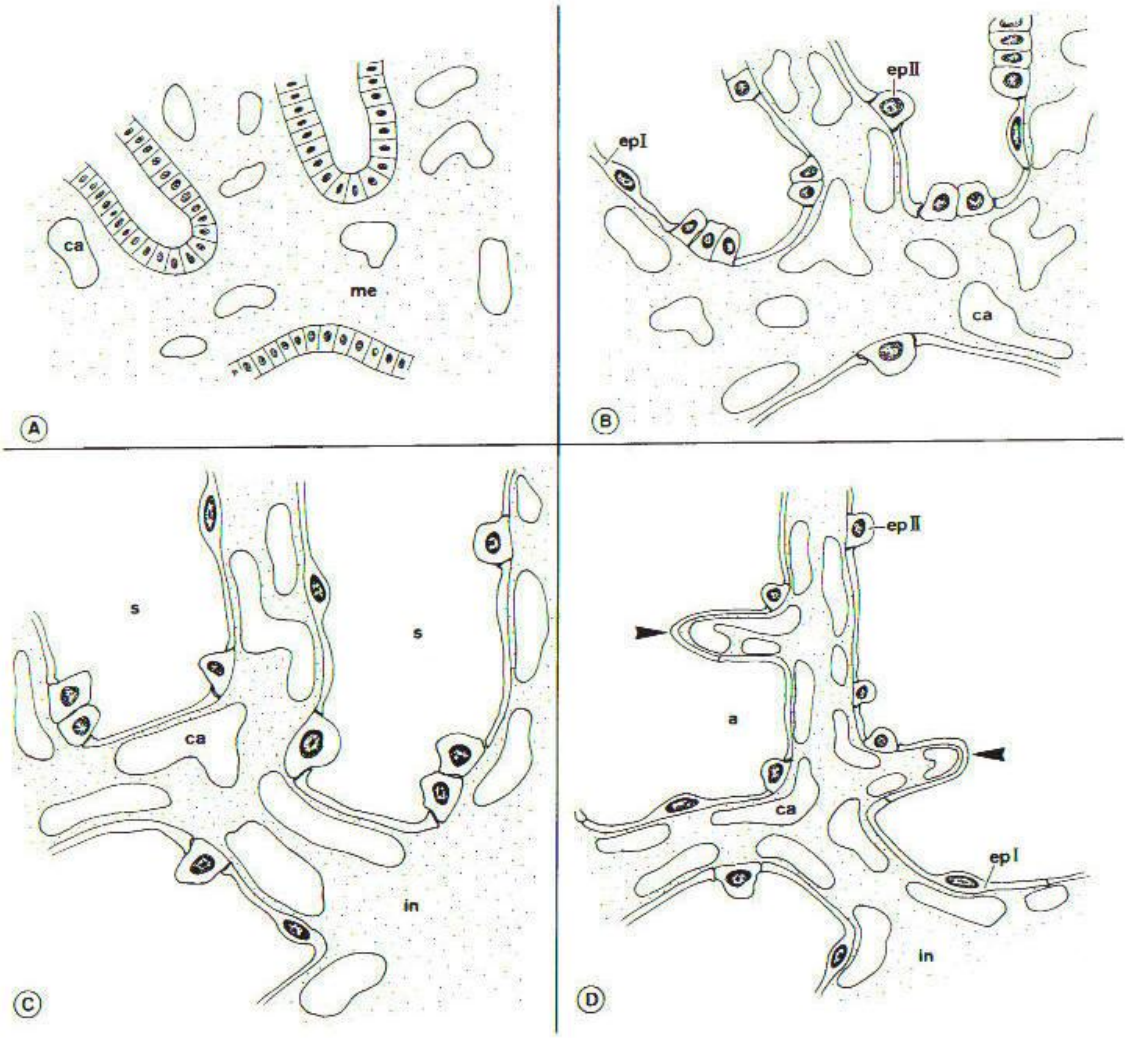


Figure 3.6: During the pseudoglandular stage, , capillaries form a loose network between the airways (A). During the canalicular stage (B), capillaries grow and form a more dense network, whilst becoming closely apposed to the airways. During the saccular stage, the vessels appear to form a sleeve around each airspace (C). At this stage, primary septae possess a capillary bilayer. In the alveolar stage (D), secondary septae form with up-folding of one of the capillary layers into the septum. Reproduced from Lung development and pulmonary angiogenesis, Peter Burri.

the squeezed capillary meshes organise into a capillary sleeve around the circumference of each saccule (Figure 3.6C). With the formation of alveoli by invagination of tissue into the saccules, there is a migration of an accompanying capillary double layer into the newly forming septum (Figure 3.6D).

Capillary plexi surrounding terminal lung buds have an absence of muscle sheaths and are therefore constructed for potential gaseous exchange and should allow a degree of capillary filtration. However, although the associated airway structures are initially distal structures, with time and the formation of subsequent airway branches, these capillary plexi become associated with relatively proximal air conduction tubules, rather than gas exchanging acinar structures. In the mature organism, these vessels would be expected to be arteries and arterioles with an associated muscle sheath. An explanation for this transformation is proposed by Hall et al [9] who studied serial reconstructions of human lung, stained specifically for markers of smooth muscle and endothelium. Their interpretation of these constructs was that there is *de novo* capillary plexi formation initially (vasculogenesis). These vessels then organised and coalesced with similarly formed vessels both proximally and subsequently, those created more distally. With this conglomeration, there is loss of mesenchyme interspersed within the plexi resulting in formation of larger bore vessels, primitive arteries. Simultaneously, there is believed to be migration of smooth muscle cells from the associated airways. These muscle cells which are rich in both actin and myosin and therefore capable of contraction even at this early stage. Migration of smooth muscle cells (SMC's) towards the blood vessels is followed by the formation of a muscular sheath and thus the vasculature is finally transformed from capillary plexus to arteriole.

The mechanisms that control both angiogenesis and vasculogenesis provide understanding of normal lung development, but more importantly, provide explanations for the abnormal vascular development seen in my lung model and the vascular maldevelopment seen in CLD. Vessel formation depends on the interaction of endothelial cells with their environment, namely other endothelial cells, the components of the extracellular matrix, and with diffusible growth factors.

I have demonstrated that culture of human fetal lung explants in relatively hyperoxic conditions results in a loss of pulmonary vasculature, over and above the regression of mesenchyme previously described under these conditions. In addition, I determined that these vessels became structurally apposed to the epithelium as occurs in the maturing canalicular lung as seen after 18 weeks gestation. I consider each of these observations separately, whilst relating them to normal and abnormal human lung development.

Loss of vasculature

The excess loss of vasculature in the presence of a diminishing mesenchyme implies either that there is a reduction in proliferation of endothelial cells or, more likely, that there is pruning of vessels due to cell death. This process may be mediated by direct toxicity as occurs in cell necrosis or in a programmed fashion by apoptosis either in response to non-lethal cell injury or via the initiation of specific cell signalling pathways. These processes are investigated and discussed further in Chapter 4.

<u>Lung vascularity during normal lung development</u>

Although there is relatively little vasculature in the pseudoglandular lung when

compared to the total parenchymal tissue, the canalicular period is characterised by a massive proliferation of blood vessels in an environment of interstitial regression[3]. The saccular and alveolar phases maintain the proliferative capillarisation of the lung but to a lesser degree and occurs in conjunction with new alveolar formation (Figure 3.6). It is not until the postnatal period that a degree of regression of capillary loading occurs. This is not as such due to a loss of vascularity of the lung, but by the merging of double layers of capillaries in alveolae septae into more efficient single layer capillary loops. Thus my findings of regression of interstitium in conjunction with a greater degree of vascular regression do not fit in with a simple picture of accelerated lung maturation. The only caveat to this would be to hypothesise that the changes in vasculature represent an early jump to the adult type maturation of vascularity. This would correspond to a jump of lung maturity from pseudoglandular stage lung (6-16 weeks) through to a post-40 week gestation equivalent lung. The morphological appearance of pruning however does appear in some respects to resemble the sheaths of capillary meshes that organise around the saccules during the saccular phase. Thus the appearances of the explant vasculature may represent maturation.

Loss of vessels seen in oxygen mediated acute lung injury

With the acute exposure of preterm lung to high oxygen tensions, rapid alterations in the structure of the vascular bed are noted. James Crapo summarised the morphological changes during pulmonary toxicity comprehensively in 1986[153] with reference to endothelial changes. The first morphological change within the lung exposed to lethal doses of oxygen (100%), was noted in the endothelium, and was denoted by subtle ultrastructural changes in these cells. With the inflammatory response recruiting a variety of cells to the vascular bed, soon neutrophils and

platelets are found to adhere to the endothelium wall, a precursor to migration of these cells into the perivascular compartment where damage and inflammation of the surrounding lung tissue ensues. This process is assisted by the increased expression of CD31 during hyperoxic lung injury[169]. Soon after the inflammatory phase commences, overt destruction of the endothelial compartment begins. This damage is wholesale and widespread with a demonstration of loss of 50% of endothelial cells in rats in the hours before death. Remaining cells display cell membrane injury, oedema and frank necrosis. Exposure to sublethal doses of oxygen (approximately 60%) does not produce an obvious destructive effect on the adult in the cell types other than endothelium. Again, upto 50% of endothelial cells may be destroyed over a longer period than in lethal oxygen toxicity. The difference is that the remaining cells tend to be relatively normal and are able to recover over the following days.

What must be taken into account however is that these changes are described in great detail in adult animals and term newborn animals most comprehensively. The preterm organism has a greater susceptibility to oxygen toxicity however, and thus the changes described can occur more acutely than in these more mature specimens[54].

These acute changes are generally ascribed to the direct effects of oxygen toxicity as has previously been discussed. This includes the removal of damaged cells by apoptosis and proliferation.

These features would correspond well with those observations I have made with my explant hyperoxic model, namely a loss of vascularity in lung interstitium in response to a hyperoxic stress. Obviously, the *in vivo* inflammatory responses are not seen with my lung model as these cells are not present within my explant tissue at this early

gestation.

Lung vascularity in Chronic Lung Disease of Prematurity

In CLD, the pulmonary vasculature displays a well characterised appearance as demonstrated by both experimental animal models and from the analysis of human preterm infant post-mortem tissue. In the human autopsy studies however, very little work has been carried out to demonstrate the degree of capillary vascularity in the lung parenchyma. All description of capillary histology have been morphological rather than morphometric. Hislop et al[170] in their examination of 17 premature infants of gestational ages 25 to 24 weeks who had died with CLD demonstrated that there was a reduced number of alveoli in the subjects, a reduced number of arteries whilst alveolar/arterial ratio was maintained. This implied a vascular arrest either as a consequence of failed alveolarisation of the lung, or alternatively raises the possibility that vascular arrest itself can impair alveolar development.

The use of the preterm baboon model by Coalson's group[171] has given more detailed analysis of vascular development with this model of CLD that as become accepted as the closest correlate to human infant CLD. They demonstrated a significant degree of capillary hypoplasia, again associated with impaired alveolar development. These findings of reduced capillary vascularity in the lung mirrors the changes I have demonstrated within my hyperoxic explant lung model. I believe that the vascular regression noted results from the loss of capillaries from the parenchyma by cell death over and above the loss of interstitium seen in my model. I believe that this may be due to a combination of factors, principally the direct toxic effects of hyperoxia on endothelium, but also indirectly by the loss of angiogenic factors from the lung such as VEGF. I postulated that hyperoxia suppresses these oxygen-sensitive growth

factors and will discuss the role of VEGF in vascular growth and vascular 'pruning' both in normal lung development and CLD subsequently in this chapter.

Decreased gap between pulmonary vasculature and airway lumen

My experiments included the analysis of distance of blood vessel to the nearest lumen under the conditions of hyperoxic and normoxic culture and related these findings to the baseline conditions after initiation of culture. I noted that culture in normoxic conditions maintained a similar vessel – lumen distance to the baseline Day 1 tissue with measurements of 37μm and 35μm respectively. It was the culture of lung explants in hyperoxic conditions that appeared to result in a reduction in this distance to a mean of 25μm. This distance was a measure of vessel from its closest point to the nearest airway, to the nearest luminal aspect of the adjacent airway. As this distance is inclusive of epithelial distance it could be postulated that part of this decrease was secondary to the thinner epithelium layer noted in hyperoxic tissue. However, this could only partially explain the differences as this difference in epithelial thickness was no more that 2-3μm (8.7μm in normoxia vs. 6.4μm in hyperoxia). Thus the remaining reduction in distance must be accounted for by alternative processes. The options available are migration of blood vessels towards airways through interstitium, growth of vasculature towards airways, loss of intervening interstitium by cell death or by loss of extracellular matrix.

Our observation of the structure of the vessels that were stained does not support the growth of vessels as a likely mechanism as I noted a simplification of the vasculature near airways resembling a pruning of the structure to a straight line. This 'pruning' of vasculature is observed during vascular development of certain organs when exposed

to varying degrees of oxygen tension *in vivo*, namely the retinal vessels[167].

Our supposition is that a loss of mesenchyme between the vessels and the airways provides the most likely mechanism and is further explored in the following chapter.

Vessel-airway distance during normal lung development

During the development of the human lung *in vivo*, the pulmonary vasculature becomes intimately associated with the airway epithelium during the canalicular phase of morphogenesis. At 14 weeks gestation, developing vessels lay within the mesenchyme with tissue separating these vessels clearly form the nearest airways. Between 16 and 22 weeks gestation, the vessels gradually near these airways until 23 weeks of gestation where the endothelium manages to become so closely involved with the airway that endothelial cells are found in between airway epithelial cells on inspection with electron microscopy. The apposition of airway and vasculature is a prerequisite for the survival of the prematurely delivered human infant. I have demonstrated that culture of lung in hyperoxic conditions has an effect on vessel-airway distance that resembles a maturing of lung to the canalicular stage rather than the pseudoglandular stage at which culture began.

Vessel-airway distance in acute lung injury

As previously described earlier in relation to Crapo's description of the morphology of oxygen toxicity to the lung, the main process that may influence the distance between vessel and airway in acute lung injury is secondary to accumulation of fluid exudate and cellular infiltrates into the interstitium[172]. This is not associated with an acute loss of fibroblasts acutely, thus there is a potential increase in distance from airway lumen to vasculature thus impeding gaseous exchange. In newborn preterm

infants, this effect on gaseous exchange is exaggerated by the movement of this fluid and cellular infiltrate into the airway lumen and alveoli, thus the gap to air is transferred beyond the epithelial surface to well within the alveolus.

This increase in distance is in contrast to my findings implying that at least in regard to vessel-airway distance, my hyperoxic model does not fit with the changes seen in acute lung injury secondary to oxygen toxicity.

Vessel-airway distance in Chronic Lung Disease

The histological description of intervening gap between vessel and epithelium is not clearly defined for infants with CLD. Findings are somewhat dependant on the type of CLD i.e. old vs. new. The 'old' CLD found in babies ventilated particularly in the 1970's finds an increased gap in areas as a result of extensive fibroproliferation within the lung parenchyma, but that these pathological changes had a more pronounced effect on the arterioles of the pulmonary circulation with medial hypertrophy and adventitial fibrosis. Extension of these muscular vessels appear to occur beyond their normal positions and further into the lung parenchyma thus further impeding gaseous exchange whilst providing increased vascular bed resistance which in itself is a contributor to right ventricular hypertrophy and finally cor pulmonale[170 173].

With the 'new' CLD seen in more immature infants born in the canalicular phase (rather than canalicular-saccular border of old BPD), the vascular changes are far less pronounced. An increased gap between vessel and lumen has however been described by the demonstration of subepithelial and centrally placed capillaries in focal locations within the lung[174]. These changes were also able to be demonstrated in baboon models of extremely preterm chronic lung injury adding credence to this

description[171].

Once again, neither of these descriptions fits the findings that I demonstrated within my hyperoxic lung explant model.

Role of VEGF in regulation of vascularity

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor promoting vascular development in the newborn infant and exists in four isoforms: VEGF -121, -165 (diffusible), -189 and –206 (bound almost exclusively to the extracellular matrix) [160] [161]. The -165 isoform appears to be the predominant member. Many cell types including epithelial cells, fibroblasts, glial cells, macrophages, smooth muscle cells, osteocytes and tumour cells produce VEGF[168] [175] [176] which binds to its receptors VEGF-R1 and R2 (tyrosine kinase proteins previously termed flt-1 and KDR respectively) [177-179] resulting in proliferation of endothelial cells, increased vascular permeability and increased production of enzymes to assist breakdown of surrounding extracellular matrix e.g. collagenases.

In hypoxia, VEGF has consistently been shown to be increased and in raised oxygen tension environments its production is decreased[144] [180] [181]. A study of preterm infants showed increasing amounts of VEGF in tracheal aspirates during the first postnatal week, a finding that was less marked in infants destined to develop CLD[182].

The effect of VEGF in tissues is to stimulate vascular growth (angiogenesis) by the sprouting of new branches from existing vessels. The grafting of heparin-bound VEGF beads onto whole mouse fetal lung explants has been shown to stimulate a neovascular response within 48 hours in culture. Although the oversecretion of VEGF

can have local complications, there are also consequences from an absence of this angiogenic factor. A number of factors have now been deemed 'survival factors' whereby the absence of their signal to a cell results in a programmed cell death by the target cell[183]. VEGF is classed as such a factor, thus where there is a lack of VEGF, susceptible endothelial cells simply 'disappear' by a process of apoptosis.

The question has thus been raised whether these potent effects of VEGF have a role to play in the normal and abnormal development of the immature lung. Certainly, the developing lung expresses both VEGF and its receptor in the blood vessels of fetal lung during vascularisation[144 184]. Healy looked at the immunolocalisation of VEGF in developing murine lung[185] and identified a gestation related distribution. During early gestation, VEGF was located uniformly throughout the airway epithelium. At later time-points, VEGF became localised to the branching tips of airways in the distal lung, specifically in the subepithelial matrix. It is this localisation that may well prove to be the stimulant for formation of capillary plexi associated with the forming airway terminal buds during late pseudoglandular and canalicular lung development.

The loss of endothelial cells and vasculature seen in acute lung injury secondary to hyperoxia may not only be due to the direct toxic effects of oxygen as earlier implied. Animal studies have demonstrated that hyperoxic lung injury is associated with a decreased expression of VEGF mRNA and decreased production of VEGF in lung[186 187].

The recovery of lung after hyperoxic lung damage may result in CLD if the subsequent development of the lung is in any way disordered. Again, VEGF may play a part in this by affecting the revascularisation of this lung. In animal models of

hyperoxic lung injury, recovery was associated with an upregulation of VEGF expression[187]. In preterm infants, again recovering from the acute phase of lung disease, lavage from lungs demonstrates that infants who go on to develop CLD have lower concentrations of VEGF when compared to those who go on to recover completely[182]. Thus the ability to mount a response during recovery from acute lung injury with VEGF appears to be associated with normal growth of the lung and avoidance of CLD. Thus VEGF does not appear to have an effect limited to vascular growth in the lung, but also general growth and alveolarisation.

The role of VEGF in alveolar and vascular development of fetal lung has made this protein a target for investigation within this model. Using the model I have developed within this thesis, our group have subsequently analysed the changes in expression of VEGF mRNA caused by hyperoxic culture. VEGF mRNA was detected using standard RT-PCR techniques[188] and using primers to all isoforms of VEGF mRNA in three fetal lung explants cultured in normoxia and hyperoxia for 24 hours. To enable comparison between experiments, normoxia was designated 100% in each experiment and the relative intensity is shown for VEGF mRNA detected in explants exposed to hyperoxia. β-actin mRNA was shown to be similar expressed in all experiments (data not shown).

We detected VEGF mRNA by RT-PCR in the explants exposed to hyperoxia and normoxia and noted a decrease in VEGF mRNA in explants exposed to hyperoxia when compared to normoxic exposed lung tissues (Figure 3.7). These results provided biological evidence for changes associated with increased tensions of oxygen within our hyperoxia exposed tissues.

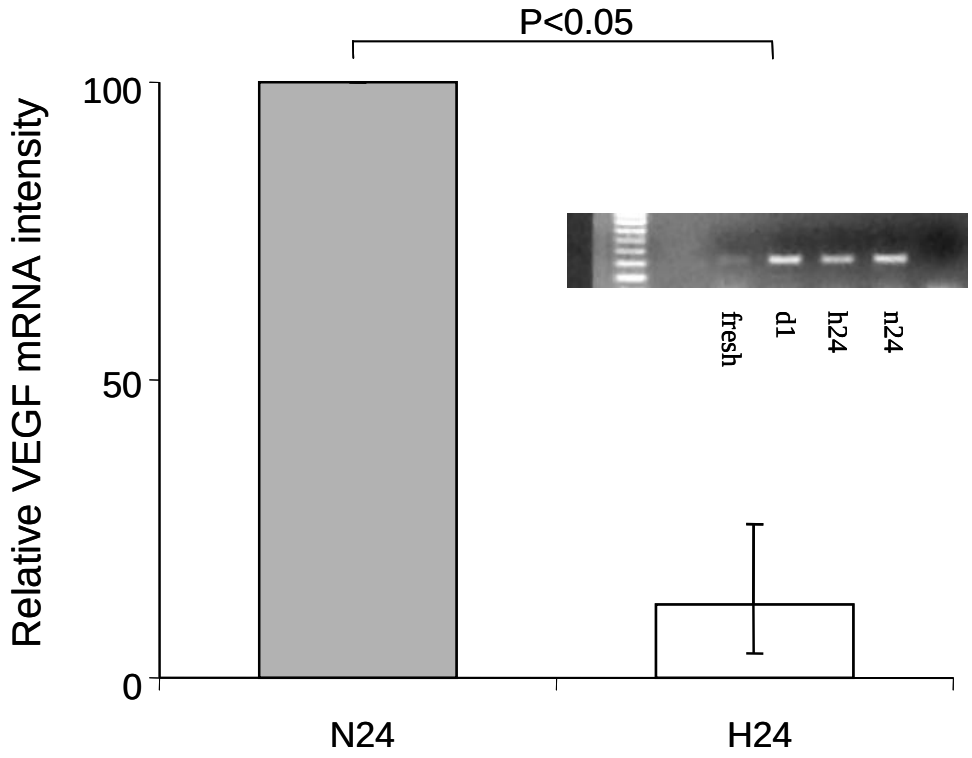


Figure 3.7: Significant decrease in VEGF mRNA was noted when explants were exposed to hyperoxia for 72 hours (H72) when compared to those explants exposed to normoxia for 72 hours (N72), $p<0.05$. The results are from three separate experiments. The inset shows a representative explants amplified for VEGF mRNA by RT-PCR.

Role of angiogenesis in alveolarisation

The evidence appears to support a mechanism whereby angiogenesis itself promotes normal development of alveoli. A combination of anti-angiogenic drugs acted to prevent normal alveoli formation in developing rat lung. One of these drugs acted by inhibition of VEGF receptors, but the others had a VEGF independent mechanism[189].

Certainly this supports the observation that secondary alveolar septae develop at sites of capillary up-folding and thus these capillaries are the driving force for the development of mature alveoli (Figure 3.6d).

Summary

Culture of human lung explants in hyperoxic conditions results in morphological changes in the vasculature characterised by pruning of the blood vessels and apposition of these vessels towards the airways. The apposition of vessels to airway most resembles the accelerated lung development seen with other aspects of the explant model i.e. saccular dilatation, epithelial thinning and surfactant expression. The pruning of blood vessels appears to more resemble the loss of vasculature seen in both acute lung injury and CLD as a result of direct oxygen toxicity and loss of angiogenic factors such as VEGF. However aspects of this pruning produce an appearance of maturation of the vasculature rather than frank destruction. Overall therefore, I hypothesise that hyperoxia leads to removal of blood vessels due to pruning, representing a maldevelopmental maturation of lung as seen in CLD after initial Acute Lung Injury.

Chapter Four: Mechanisms involved in the remodelling of cultured human lung

Introduction

The elucidation of the role of oxygen in altering lung development requires not only an observation of its effects on tissue structure, but also an investigation of the underlying processes that result in the ensuing structural changes. This investigative process applies equally to *in vivo* and in vitro experiments, however the *in vitro* environment provides the optimal scenario to analyse these mechanisms.

The work carried out so far in my investigations have revealed that by maintaining pieces of human fetal lung explants in culture in a semi-solid collagen base, tissue viability can be maintained over a 96 hour period. The exposure of this lung tissue to a relatively hyperoxic environment results in remodelling of the lung tissue. This remodelling is histologically characterised by regression of the interstitium, expansion of the airway compartment and pruning of the vasculature within the interstitium. In addition, the epithelium of hyperoxic tissue alters from a columnar appearance, to a more flattened morphology, resembling maturation into cuboidal Type II cells, and approaching the appearance of Type I cells.

The changes in histology noted can be explained potentially by a relatively limited number of processes. These are the addition of new cells to the tissue (proliferation), the removal of cells (cell death), changes in the size of the cell (hypertrophy or atrophy), change in the shape of the cell as part of a maturational process (differentiation) or by functional changes to the cells (e.g. fluid production, surfactant production, intercellular matrix formation). Within these categories exist specific subsets, in particular, cell death is known to occur by two potential processes-

apoptosis and necrosis.

Evidence for active proliferation and apoptosis in cells can be sought in a variety of ways. An attractive methodology is by the labelling of specific cells during immunohistochemistry with antibodies raised to Ki67 and activated caspase 3. These nuclear proteins are markers of proliferation and apoptosis respectively.

I proposed that high oxygen tensions in my human fetal lung explant model lead to alterations in structure determined partly by alterations in the rate of apoptosis and proliferation in the epithelium and mesenchyme.

Methods

Tissue culture was carried out as described earlier (see Chapter 2, page 75). Samples were harvested 24 hours after establishment of culture (Day 1), and at 24, 48 and 72 hours after placement in normoxic or hyperoxic conditions (N24, N48, N72 and H24, H48 and H72 respectively).

Harvesting of samples

For immunohistochemistry, samples were dissected from the collagen gel, and then dropped into 10% formaldehyde at 4^{o}C for 24hrs. Subsequent to this, tissue was kept in 70% IMS until processing was possible. Paraffin embedding was carried out on my behalf by the Pathology Department at the Leicester Royal Infirmary. I cut paraffin embedded specimens into 4um sections using a microtome, as previously described (Chapter 2, page 80)

Immunostaining

Paraffin tissue sections were immunostained with mouse anti-human Ki67 antibody (Pharmingen), and rabbit anti-human activated-caspase-3 antibody (R and D systems, UK). Methodology was as set out previously (page 80). Briefly, sections were deparaffinised in xylene, then rehydrated. Antigens were exposed using heated citric acid retrieval. Endogenous peroxidase was inhibited and non-specific binding sites were blocked with non-serum protein block. Slides were incubated overnight at $4^{o}C$ with the primary antibody diluted in 0.05% bovine serum albumin/PBS at optimal dilution (Ki67 1:200 dilution, activated caspase-3 1:500). Thereafter the slides were washed and incubated for 30min in goat anti-mouse biotinylated secondary antibody (Sigma-Aldrich,Poole,UK B9904) diluted 1:250 (anti-rabbit secondary at 1:400 dilution for activated-caspse-3). Sections were developed using avidin-biotin peroxidase (Vector ABC) and DAB chromagen (DAKO). Counterstain was with Ehlich's haematoxylin.

Image analysis

Analysis of Ki67 and act-caspase-3 were both carried out in the same manner. All slides were blinded to the investigator by coded labelling. Each section had 8 peripheral fields photographed digitally as previously described (see page 106) at x400 magnification. These images were then analysed using the QWIN imaging analysis program (Leica).

For Ki67, a macro was written which initially detected all cells in the field of interest. The user was then invited to isolate those cells that were epithelial using a graphic tablet (airway map). The routine was thus able to count numbers of epithelial and

mesenchymal cells respectively. The program then detected all positively immunostained cells by detecting all brown-labelled cells. These were counted by the program and separated into epithelial and mesenchymal components using the previously laid out airway maps. This routine is provided in the appendix.

For activated caspase-3, a similar routine was written for QWIN. This differed in that manual detection of positively labelled cells was conducted. This was performed so that cells that appeared to have lost normal cellular morphology were excluded, as were positive cells extruded from epithelium into the airway lumen. Lumen extruded cells and degraded cells were assumed to be cells that had undergone apoptosis, and not been cleared by phagocytic mechanisms. Thus selected cells were those assumed to be undergoing apoptosis. Again, this routine is provided in the appendix.

Statistical analysis

Comparisons between Day1, normoxia and hyperoxia exposed matched lung sections were made with the SPSS software package using Wilcoxon signed rank paired analysis for non-parametric data. Significance for two-tailed tests were set at $p<0.05$.

Results

Cell types

The relative contribution by epithelial cells was similar in the day 1 [median 30% (IQR: 25-31%)] and normoxic tissues at 72 hours [29% (IQR: 26-34%)]. In contrast, the hyperoxic-exposed tissues were comprised of a higher percentage of epithelial cells [38% (IQR: 33-46%); $p<0.05$ when compared to day 1 and N72 conditions] (See

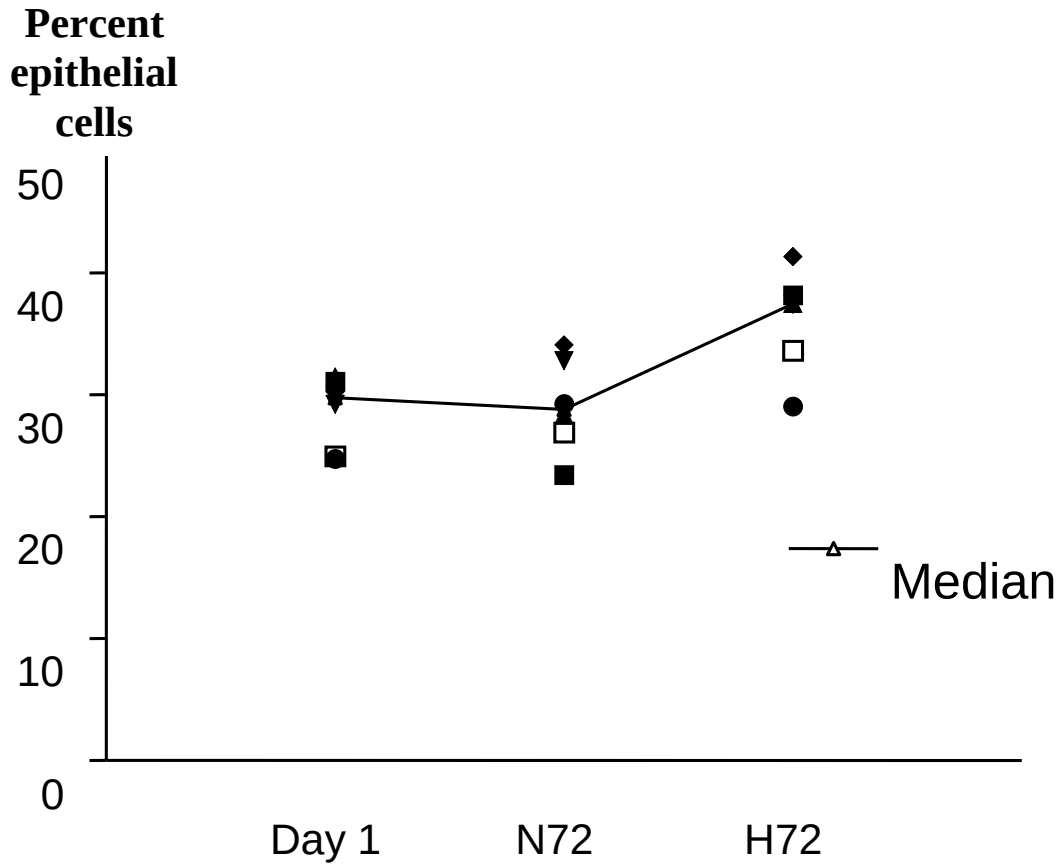


Figure 4.1: Analysis of the proportion of epithelial cells to total cells (epithelial + mesenchyme) shows in the fields analysed, a relative increase in epithelial cell numbers in tissue cultured in hyperoxic conditions.

Figure 4.1).

Proliferation indices

Inspection of tissue for Ki67 immunostaining inferred high rates of proliferation in both mesenchyme and epithelium in Day 1 tissue (Figure 4.2). Normoxic and hyperoxic cultured tissue appeared to lose Ki67 reactivity in mesenchyme, although epithelium staining was maintained. Dilated airways in particular, had high rates of proliferation.

The proportion of Ki67 immuno-reactive cells was higher in day 1 explants [12% (IQR: 9-15)] than in fetal lung explants maintained in culture for a further 72 hours in either normoxia [5% (IQR: 4.5-8)] or hyperoxia [8% (IQR: 5-11). These differences were not statistically significant however (n=6).

Epithelial cells

Analysis of epithelial cells showed that at Day 1 in culture, 13% (IQR: 9-16) of cells were positive for Ki67 (Figure 4.3). After a further 72 hours in culture in normoxic conditions, the median Ki67 index was 10% (IQR: 4-16). Culture in hyperoxic conditions for 72 hours maintained a median Ki67 index at 13% (IQR: 10-16%). These differences were small, and were not statistically significant. Thus culture in normoxia or hyperoxia had no discernible effect on epithelial cell proliferation in human lung explants.

Mesenchyme cell

At Day 1 in culture, 12% (IQR: 9-13%) of mesenchyme cells were positive for Ki67

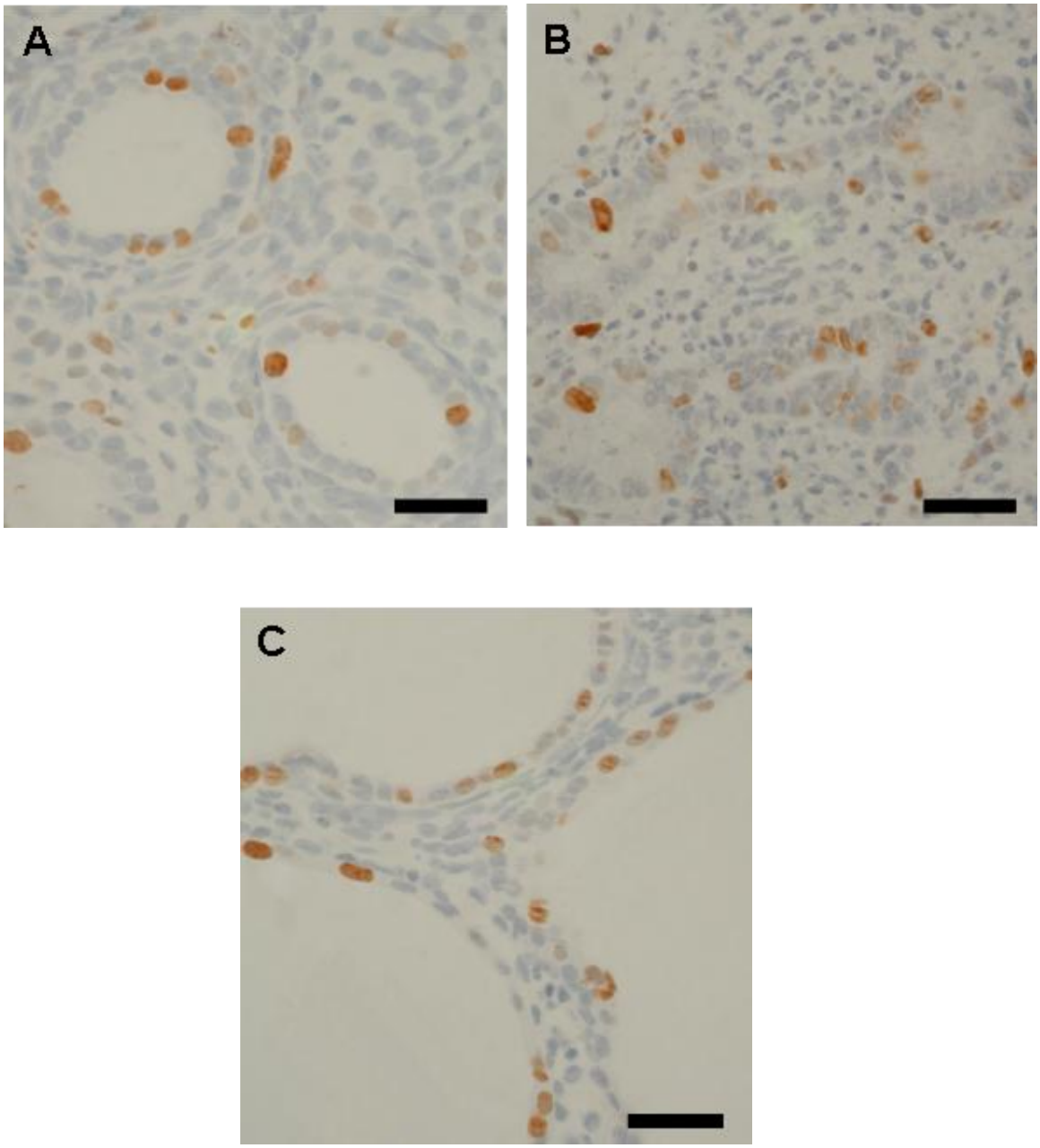


Figure 4.2: Tissue stained for Ki67 shows Day 1 tissue (A) has proliferating cells in both epithelium and interstitium. In contrast, normoxia (B) and hyperoxia (C) cultured tissue has positively staining cells predominantly in the epithelium only. Bars represent 20μm

median values for n=6 sets of lungs). Culture in normoxia led to a reduction in Ki67 index in all six pairs with a median value of 4% (IQR 2-5%) ($p<0.05$). Median proliferation index in hyperoxic tissue was 3% (IQR: 2-4), again statistically less than Day 1 proliferation results. There were no significant differences between normoxic and hyperoxic cultured rates of proliferation in mesenchyme tissues (Figure 4.3).

Combined analysis

Individual cell type proliferation indices were combined with cell type predominance to formulate a cell type specific proliferation graph (Figure 4.4). In this it can be seen that culture in normoxic conditions results in a decrease in overall proliferation over a 72 hour period (12% to 5%). The predominant contributor to this decrease was in mesenchyme due to a reduction in proliferation in this cell compartment. The reduction in hyperoxic culture proliferation was less marked compared to Day 1 cultured tissue, with an overall decrease from 12% to 8%. Mesenchyme proliferation was reduced; however, a relative reduction in mesenchyme cells in tissue reduced the overall effect. There was a relative increase in proliferating epithelial cells in tissue. This was due to a maintained epithelial proliferation index combined with a greater relative epithelial contribution to tissue.

Overall, culture results in maintained epithelial proliferation, but reduction in mesenchyme proliferation. Culture in hyperoxia has a similar effect on proliferation, thus the relative changes in cell type under hyperoxic conditions are not due to differences in proliferation.

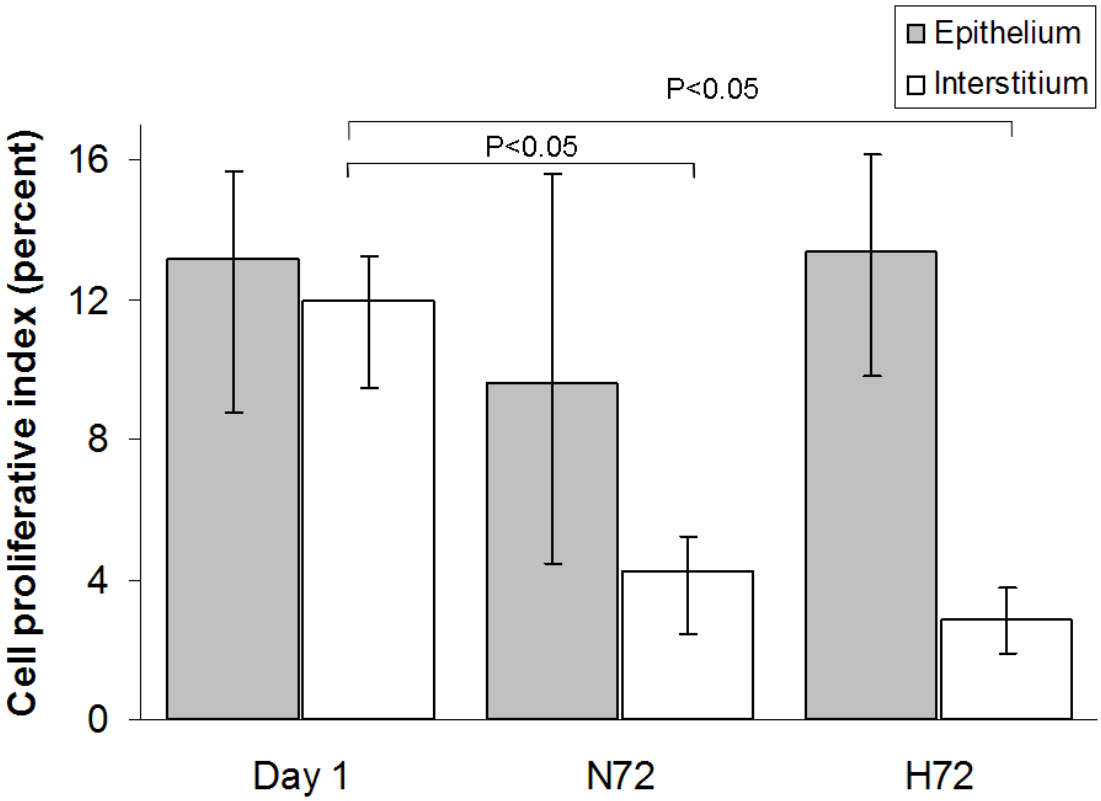


Figure 4.3: Culture in normoxic or hyperoxic conditions produces no significant change in proliferation rates in epithelial cells. However mesenchyme cells in both normoxia and hyperoxia explants display reduced proliferation when compared to Day 1 tissue (n=6).

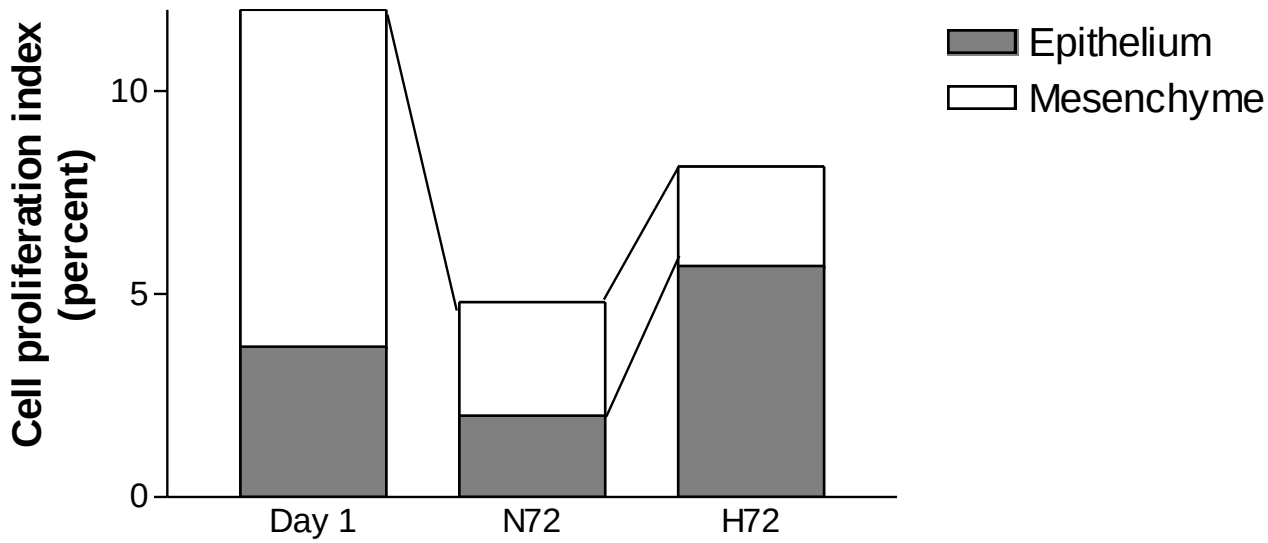


Figure 4.4: Overall rates of proliferation (epithelium and mesenchyme) tend to be lower in normoxic (N72) and hyperoxic (H72) tissue when compared to Day 1 tissue, however these differences are not significant (n=6).

Apoptotic indices

General

Inspection of tissue for activated caspase-3 staining shows obvious differences between normoxic and hyperoxic cultured tissue with significant staining present in the mesenchyme of hyperoxic cultured tissue (Figure 4.5).

After 24 hours in culture, there was a relatively low level of apoptosis in the explant. Median number of cells that stained positively for activated-caspase-3 was 2% (IQR:1.6-4.8%) . Further culture in normoxic conditions for 72 hours, results in 4% median apoptotic index (IQR:4-4.6%) , however this result was not statistically significant. Alternative culture in hyperoxic conditions for 72 hours led to a greater increase in apoptosis to 9% (IQR: 5-12%). This was a statistically significant rise when compared to Day 1 samples ($p<0.05$) (n=6) (Figure 4.7).

Epithelium

Analysis of epithelial cells in culture show that at 24 hours in culture, 1% of cells were positive for activated-caspase-3 on immunocytochemistry (IQR:0.5-2.7%). Culture for a subsequent 72 hours in normoxia resulted in an increase in apoptotic index in 5 out of 6 tissue specimens. The median apoptotic index was 4.8% (IQR: 3.9-5.6%) however, due to small numbers, this value was not statistically significant (Figure 4.6).

Culture in hyperoxia for 72 hours resulted in a median apoptotic index of 2% (IQR:1.3-2.9%), not significantly different from Day 1 culture. Hyperoxic samples

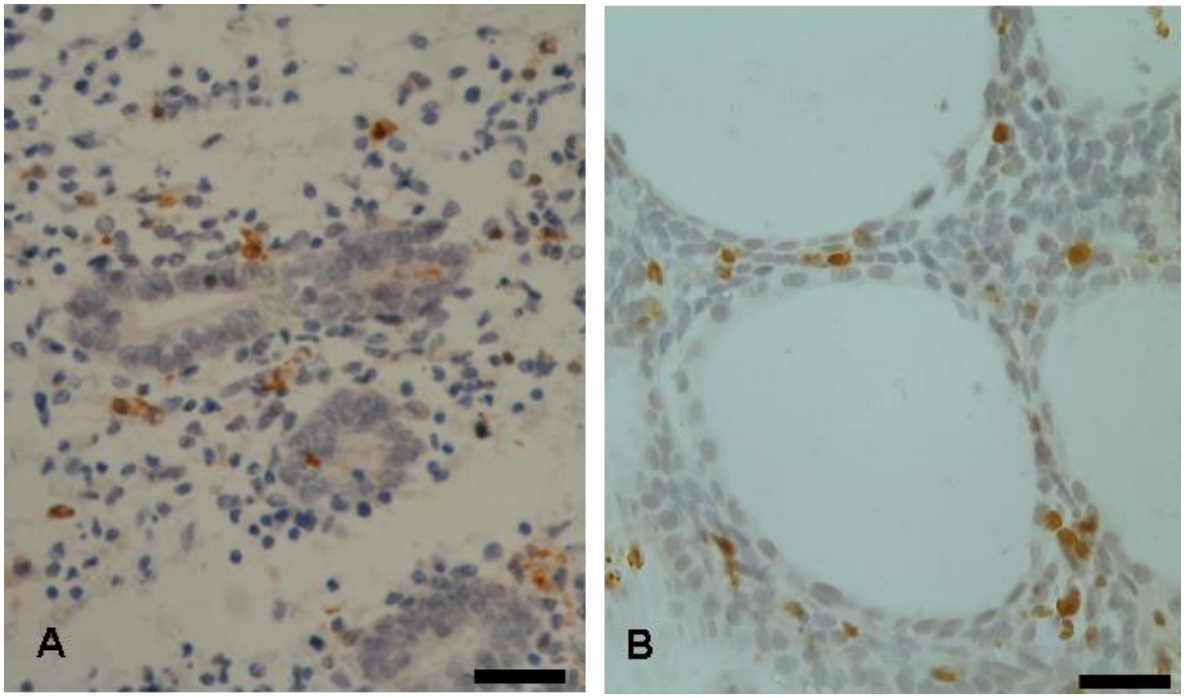


Figure 4.5 : Tissue stained for activated caspase-3 showed occaisional apoptotic cells in the interstitium and epithelium in normoxic cultured tissue (N72) (A). Hyperoxia cultured tissue (H72) had numerous apoptotic cells within the interstitium (B). Bars represent 20mm

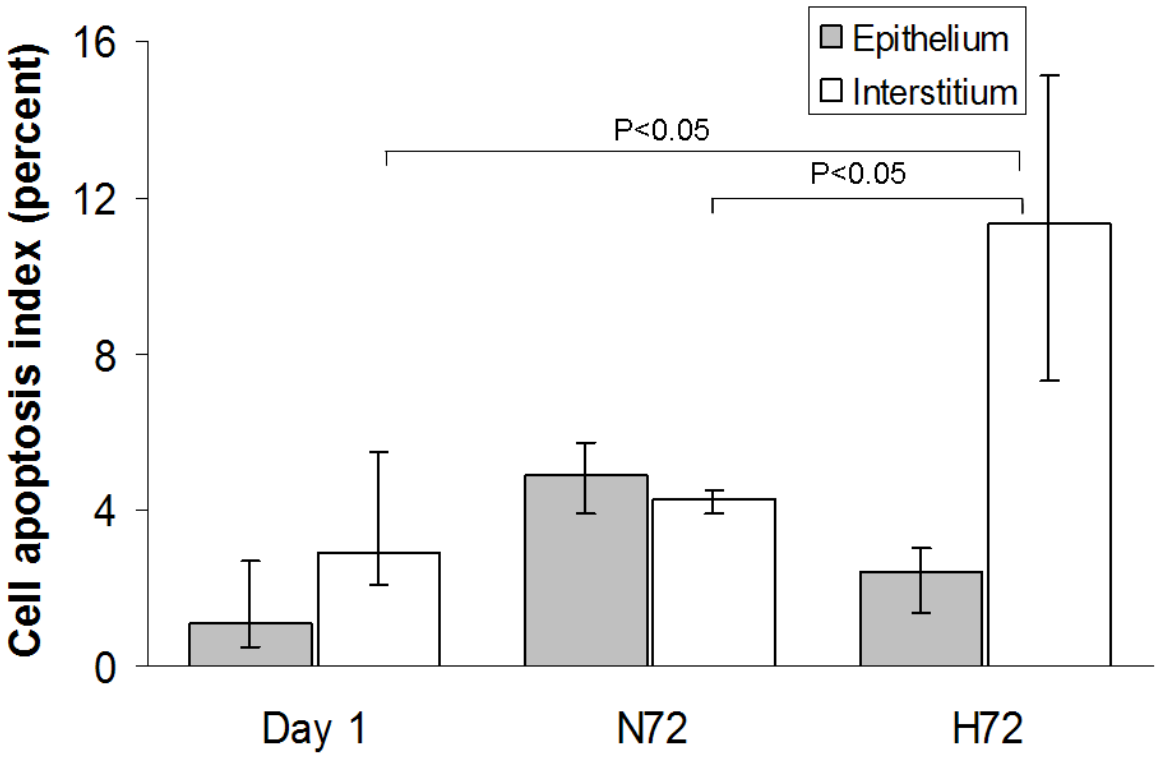


Figure 4.6: Epithelial apoptosis was unaffected by culture in normoxia (N72) or hyperoxia (H72). Interstitial apoptosis was increased in N72 tissue when compared to Day 1 tissue. H72 interstitium demonstrated a further increase in apoptosis ($p<0.05$). (n=6)

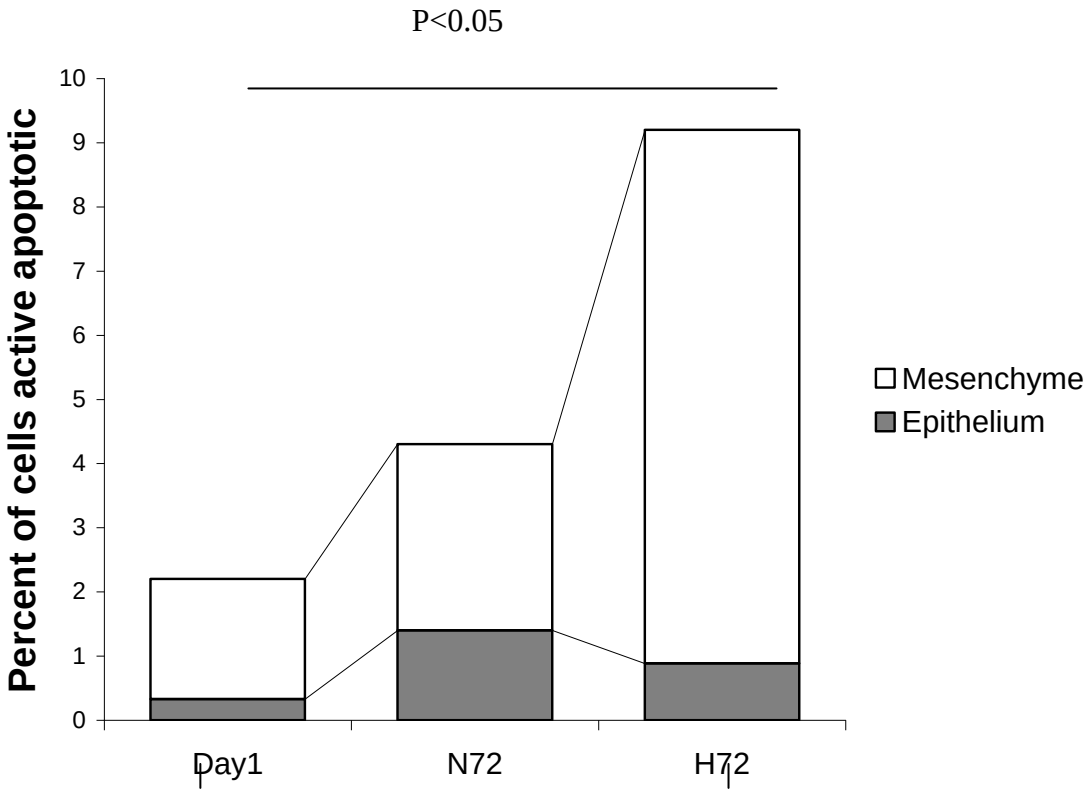


Figure 4.7: Combined epithelial and mesenchyme indices demonstrate that apoptosis rates are highest in hyperoxic cultured lung (H72). This is mainly due to increased activated caspase-3 activity in the mesenchyme (n=6)

had fewer apoptotic cells than normoxic tissue in 5 out of 6 specimens. The difference between normoxia and hyperoxia was, again, not statistically different.

Mesenchyme

Apoptotic indices were similar in Day 1 cultured lung and 72 hours normoxic cultured lung (Medians 3% (IQR:2-5.5%) vs. 4.3% (IQR:4-4.6%) respectively, p=NS). Culture in hyperoxic conditions resulted in significantly higher apoptotic indices than both Day 1 tissue and normoxic 72 hours tissue, with a median of 11% (IQR: 7-15%) of cells undergoing active apoptosis ($p<0.05$) (n=6) (Figure 4.6).

Combined apoptotic results

It can be seen from the above results together with the graph derived from individual cell type proliferation indices combined with cell type predominance that explant culture results in an increase in apoptosis. This is contributed by slight increases in both epithelial and mesenchyme apoptosis.

Hyperoxic culture apoptosis was significantly increased compared to both Day 1 tissue and normoxic 72 hour cultured tissue (9% vs. 2% and 4% respectively). This was due to hyperoxia mediated mesenchyme apoptosis. Epithelial apoptosis was similar to normoxic culture conditions (Figure 4.7).

Discussion

My previous observations of dilatation of airway structures and regression of mesenchyme may reflect changes in the relative numbers of cells in each

compartment. The increased proportion of lung occupied by airway lumen in hyperoxic conditions may reflect excess epithelial proliferation relative to mesenchyme. In contrast an excess of mesenchyme cell loss may also result in this remodelling. My analysis provides evidence of both.

Proliferation

Using Ki67 as a marker of proliferation, I was able to demonstrate the effect of culture and hyperoxic conditions on proliferative rates in both epithelial cells and mesenchyme cells. There was an overall decrease in proliferating cells during explant culture from Day 1 samples. The effects of normoxic culture where analysed according to cell type demonstrating that there was a significant decrease in proliferation in the mesenchyme compartment, but that epithelial proliferation was preserved. The addition of hyperoxic conditions to culture had no discernible effect on proliferation indices in either compartment. In discussing this I shall relate these findings to those from related studies whilst considering the overall role of proliferation in lung development and attempt to justify my methodology of assessment of proliferation.

The presence of Ki67, was used to denote cell proliferation. This nuclear antigen is expressed in cycling cells outside the G0 phase[190] and its presence is necessary for proliferation to proceed[191]. Its short half-life means that arrest of the cell cycle at the G1/S interface, described under hyperoxic conditions[147], would result in loss of signal.

The use of Ki67 to denote proliferating cells is but one of a number of techniques available to this end. Alternative methods such as PCNA staining and thymidine uptake have been discussed in the Introduction (page 26). The use of Ki67 or PCNA

have the advantage that they do not require addition during the culture process, thus enabling post-hoc analysis of tissue. They are also independent of timing artefact. Hence, delay of harvesting of explants after addition of thymidine, leads to an increased prevalence of positive staining cells; whereas Ki67 staining reflect proliferation rates at the point of tissue harvesting and fixation. A strength of thymidine uptake staining is that that the marker is not specific to particular aspects of the cell cycle, and therefore will not miss cells about to commence or just completing mitosis. I chose not to use this technique however as I was unsure of the ability of thymidine to diffuse freely through the collagen based gel culture medium into the tissue. Similarly, diffusion of the markers throughout the tissue evenly could not be guaranteed making proliferation rate comparisons between the centre of the tissue and the periphery unreliable should that become a proposed outcome. The use of Ki67 over PCNA was preferred after trial staining for both antibodies on samples tissue specimens. I determined a very low positive staining of cells using PCNA on paraffin embedded, fixed tissue. Using Ki67 staining revealed specific cell staining with low background noise and reproducible results.

Effect of cell and tissue culture on proliferation

I found in my experiments that rates of proliferation appeared to alter from baseline within my control environment (normoxia). In determining the effect of oxygen tension on proliferation rates in culture material, I need first to recognise that other factors inherent in culture methodology may alter the tendency of cells to undergo division. I noted earlier that May[24] used stillborn fetuses as controls for the assessment of proliferation in ventilated preterm infants. Approximately 2/1000 cells stained positive for Ki67 between 22 and 36 weeks gestation, although no comment

was made regarding the location of these cells. This is far lower than the baseline proliferative rate in my pseudoglandular explant lung (12% of all cells at Day 1 culture). Although the data is not provided in this thesis, staining of preculture pseudoglandular tissue was carried out a part of control immunohistochemical evaluation. Rates of proliferation appeared at least as high as at Day 1 culture, supporting the premise that proliferation is most active in the early stages of lung development, then reduces during the saccular and subsequent stages.

Although epithelial cells in my lung tissue did not demonstrate altered rates of proliferation after 3 days of culture when compared to after overnight culture had been established, I found that mesenchyme tissue showed a significant reduction in proliferation when compared to baseline conditions (4% and 12% percent of total mesenchyme cells positive for Ki67 respectively).

A number of factors inherent to the culture environment may be responsible for this decrease.

Jyonouchi's experiment with small airway epithelial cells[18] showed that degree of cell density in cell culture could affect proliferation rates as demonstrated by thymidine incorporation. Confluence of cells resulted in proliferation rates to approximately one third that found in subconfluent cultures. My culture methodology did not result in increased cell density when compared to baseline conditions and thus I do not believe this was a major contributor to my observations.

The use of a gel matrix as opposed to standard plate culture enables a 3D cell culture environment rather than the traditional 2D model. Mio[165] looked at proliferation rates of fibroblasts in either methodology and found significant reduction in proliferation in

the 3D matrix with reduced responses to a number of mitogens, although not to all. It is unclear whether this effect is due to the immersion of the cells in a collagen based solution[192 193]. In my model however, the 3d environment is a continuation of the in vivo state of the tissue and only the surface of the tissue is in contact with the collagen based semi-solid gel. It is thus unlikely that this itself leads to the reduced mesenchyme proliferation seen.

Use of growth factors or serum in culture experiments has been extensively investigated to determine effects on proliferation response. In the majority of the time, the aim of the experiment is to indicate the effect of the hormone/factor on cell responses, however at times differing use of factors are utilised to improve culture methodology. The use of serum, particularly from fetal animals, is often used to promote proliferation in cells within a culture system as it is believed to be replete in all the major growth factors[194-196]. Where individual factors are the subject of interest pertaining to their role in maintaining cultured cells or inducing proliferation, these factors can then be added to serum-free media and their effect analysed. Those factors that have been determined to be most important in cell culture include growth factors such as insulin like growth factors (IGF) and fibroblast growth factors[197 198], hormones such as corticosteroids[199], and cytokines such as TGF-beta[200 201].

Effect of lung injury and hyperoxia on proliferation

Our research concluded that hyperoxia had no additional effect on proliferation rates in fetal lung explant cultures when compared to normoxic conditions. This pertained both to the epithelial and mesenchyme cell compartments. These findings contrast with and should be interpreted alongside a number of conflicting results in the

literature.

Studies in historical human subjects looking at proliferative responses in hyperoxia exposure are scant. May et al[24] examined post-mortem specimens from stillborn infants and compared these with infants born prematurely who succumbed after ventilation in supplemental oxygen. Using Ki67 to identify proliferating cells, and double labelling with cytokeratin to subgroup cells into epithelium and mesenchyme, there was found to be a doubling of proliferation rates in lungs from ventilated babies when compared to stillborn controls. This effect appeared to be time-dependant with a greater increase in proliferation in those lungs where ventilation was prolonged before death. The alterations appeared to be distributed between both epithelium and parenchyma, although data is not provided to support this observation. This therefore contrasts with my findings where I could not demonstrate an oxygen effect.

Animal studies provide a relatively controllable environment when compared to retrospective human studies. Warner et al[68] examined the effect of prolonged hyperoxia (85% oxygen for upto 28 days) on proliferative responses assessed using BrdU incorporation. Cell type was not identified in this study, but it was noted that when compared to controls, there was an acute depression of proliferation rates, but that this normalised over the 4 weeks of exposure. Maniscalco et al[202] carried out similar experiments but utilised premature baboons. These animals have become accepted as the gold standard for animal models of 'human CLD', but are seldom used due to their difficulty in acquisition and maintenance in experimental laboratories, together with the significant expense associated with their use. Exposure of preterm baboons to oxygen concentrations of approximately 40% for prolonged periods (upto 21 days). Once again, as seen in the Warner paper, proliferative

responses were time dependant. Over the first two weeks of exposure, marked increase in proliferation was noted in the epithelial compartment. Continued exposure however results in suppression of proliferation of epithelium with concurrent increased proliferation in the mesenchyme. Thus in these two species there was a very different response to hyperoxia where one had a depressed rate of cell division whereas the other had a marked increase. Although this might represent differing species specific responses, it may well be that this represents responses to differing oxygen tensions.

Pandya[17] demonstrated, as if to illustrate this point, that degree of oxygen tension can influence proliferative responses in cultured smooth muscle cells. Using a series of oxygen tensions ranging from 5 to 50kPa, they assessed proliferation by a number of methods including direct cell counts, assessment of DNA synthesis and the mitochondrial mediated reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). A bell shaped curve was demonstrated with proliferation of cells being most prolific at approximately 10kPa, and with a gradual tapering away of rates as oxygen tensions either increased or decreased in each direction. Hence, it could be postulated that differences in proliferation in the varying experiments may reflect differing degrees of hyperoxia, and potentially differing sensitivities to these tensions in the various species. An additional finding of this paper was the demonstration that these oxygen related proliferative responses were dependant on the presence of certain growth factors, namely fibroblast growth factor, platelet derived growth factor and fetal bovine serum. This last point had been discussed in the previous section, but reminds us that the degree of maturity of the experimental subject may influence the outcome of a study in that growth factors are

highly abundant in the fetus when compared to the full term of adult animal.

In contrast to Pandya's experiments with cells of mesenchymal origin (smooth muscle cells), Jyonouchi's[18] exposure of small airway epithelial cells to hyperoxic conditions (95% oxygen) demonstrated a suppression effect of hyperoxia on proliferation when compared to 21% oxygen when assessed by thymidine uptake.

Finally, work with rat explant cultures by Chetty and Nielsen[203] also analysed the effect of hyperoxia on cell proliferation. Utilising whole lung explants from neonatal, rats it was determined that the observed increase in cell proliferation was in part mediated by the increased expression of Insulin-like Growth Factor-1 within the airway epithelial cells. This was corroborated by the addition of anti-IGF antibody to the feeding medium, which reduced the uptake of thymidine when hyperoxic conditions were introduced.

It can this be seen that proliferative responses of lung tissue to hyperoxia is not entirely straightforward. The literature has demonstrated that epithelium and mesenchyme have potentially independent responses to this stimulus; however these responses are inconsistent and appear to be dependant on secondary factors. These include the nature of the model i.e. cell culture, explant culture or in vivo studies. Presence of serum or growth factors may have a permissible effect of oxygen tension within a lung model, and by inference, the maturity of the subject thus becomes an important factor due to the gestation dependant presence of growth factors within serum. Of particular interest is the observation that oxygen tension does not have a linear response as shown by Pandya, but more a bell-shaped response, at least when studies in airway smooth muscle cells. Linked to this is the knowledge that even my

and others' definitions of a 'normoxic' model, do in fact constitute another less-extreme form of hyperoxia when compared to the fetal hypoxic environment. This may go some of the way to explain why I was able to demonstrate a suppression of proliferation in mesenchyme both in normoxia and hyperoxia branches of my experiments.

Apoptosis

Our analysis of apoptosis was performed using immunodetection of activated caspase-3. Using this technique, I demonstrated that culture under normoxic conditions maintained rates of apoptosis both in epithelium and interstitium. Hyperoxia had no influence on apoptosis rates in epithelial cells, however there was a marked increase in mesenchyme apoptosis. I have therefore demonstrated that these two cell types have differing apoptotic responses to hyperoxia. In discussing this observation, I shall examine the role apoptosis has in normal lung development, abnormal lung development, and analyse the mechanisms by which oxygen may influence this process in cell populations.

Evidence for a role for hyperoxia in regulation of apoptosis

That hyperoxia *per se* can lead to cell death has long been recognized. Oxygen toxicity has a dose dependant effect, which is correlated to both duration of exposure and concentration of oxygen. Indeed, 21% oxygen itself has oxidizing properties that cells have evolved antioxidant defences to.

In 1975, Bonikos[49] investigated the effects of 100% oxygen on newborn mice. There was a survival of 75% of animals by 7 days of life after continuous exposure to the

oxygen. Death when it occurred was generally between days 3 and 4 with a clinical picture compatible with pulmonary oedema and pulmonary haemorrhage. Cell death was not characterised into either apoptosis or necrosis as detection of the former was not widespread in laboratory practice.

The pathways to cell death were more closely characterised by Barazzone et al with the exposure of newborn mice to 100% oxygen for 4 days[20]. The awareness of cell death by both necrosis and apoptosis was closely analysed to define which of the two pathways were seen in the lung, and strategies to block aspects of apoptotic signalling were attempted. Apoptosis was identified using TUNEL staining, and indirect quantification obtained by electrophoretic DNA laddering. Necrosis was sought by the appearance of smearing in the electrophoretic DNA fields. Although hyperoxia resulted in increased frequency of apoptosis and necrosis, there were conflicting results when analysing the signalling pathways of apoptosis. The promotors of apoptosis p53 and bax were increased manifold, however the Fas pathway appeared not to be involved, as demonstrated by only a small increase in Fas mRNA levels, no alteration in Fas ligand, and the observation that Fas null mice had equivalent if not greater amounts of cell damage in response to hyperoxia. More unusually, the executioners of apoptosis i.e. the caspases (namely caspase 3) did not appear to be increased in hyperoxic subjects when protein extracts were assayed for these proteases. Thus although apoptosis could be demonstrated, the pathways remained obscure during these experiments.

Thus, although *in vivo* experiments demonstrate evidence for apoptosis in response to hyperoxia, there is some difficulty elucidating the pathways involved, and the teasing out of apoptosis from necrosis. It is with *in vitro* studies that the mechanisms involved

in hyperoxic cell death become clearer.

Cell culture with lung cell lines is established primarily with epithelial cells, fibroblasts and smooth muscle cells. Although the effects of hyperoxia on smooth muscle cells in culture have demonstrated altered rates of proliferation[17], apoptosis in these cells have not been characterised clearly.

Epithelium is difficult to culture, thus much of the work carried out with these cells has been performed with the easier to culture adenocarcinomatous cells such as the A549 cell line. Work with the A549 cell line has demonstrated cell death in response to hyperoxia via necrosis primarily. When used as a control cell line for experiments on human small airway epithelial cells (SAE's) [18], it was noted that the SAE's had a more pronounced apoptosis response. Necrosis in these epithelial cells was noted to be minimal despite environmental oxygen conditions. In these experiments, necrosis was assessed primarily by lactate dehydrogenase (LDH) levels in supernatant, and apoptosis by nuclear morphology on light microscopy or by TUNEL staining. In addition, it was found that the apoptosis induced by hyperoxia in these SAE cells could be reduced by the administration of either Vitamin C or Vitamin E. Both of these agents act as antioxidants, thus it is inferred that hyperoxia induces apoptosis via the generation of Reactive Oxidant Species (ROS), also known as Reactive Oxidant Intermediates dependant on the specific agent and the group describing them.

It can thus be seen that exposure to increased oxygen concentrations can result in increased rates of apoptosis in lung independent of the effects of preterm delivery. This phenomenon is dependant on the type of cell being utilised and the specific source of the cell e.g. adenocarcinomatous epithelial cells versus small airway

epithelial cells.

The pathways by which apoptosis is induced have been partially discussed here, however I now wish to consider the mechanisms by which hyperoxia or oxidant stress each effect apoptosis and whether they act independently or through each other.

The role of oxidant stress in apoptosis

I did not attempt to quantify the degree of oxidant stress within my model. However, I bore in mind that the role of ROS has in regulating cell death is extremely complex. The mechanism by which hyperoxia is believed to exert its toxic effects is by the generation of excess ROS in cells, which then act as the mediators of injury and intracellular signalling activation[69].

The specific role of these ROS have been examined in hyperoxic lung injury as there are many different species, and these may have differing modes of action. In particular, from a pharmaco-therapeutic standpoint, each may have its own specific intervention in an attempt to reduce the effects of this injury in humans.

The finding of DNA fragmentation in cells exposed to hyperoxia can be explained by two mechanisms. The first is that the direct oxidative damage to cellular DNA can subsequently lead to cell death without apoptosis, but that the DNA nicking associated with this damage can be misinterpreted as apoptosis on TUNEL staining. Alternatively, the generation of ROS leads to oxidative damage to the mitochondria amongst other organelles, leading to intrinsic apoptotic pathways being activated. It is believed that the oxidation of mitochondrial pores by ROS contribute to cytochrome c release by disrupting the mitochondrial membrane potential[204]. This apoptotic pathway can be potentially exaggerated by upregulation of TNF expression as part of

an inflammatory response to hyperoxia, leading to signalling in the extrinsic apoptotic pathways.

Auten et al[205] in 2001 investigated these alternatives in their hyperoxia injury newborn rat lung model. By exposing these animals to 95% oxygen for the first 8 days of life and comparing with controls, a basic comparison of hyperoxic lung injury was possible. This was taken further by the administration to some of these animals of anti-cytokine-induced neutrophil chemoattractant (CINC) in an attempt to block neutrophil recruitment. It is the recruitment of these cells that is a major player in the establishment of inflammation and these cells are a rich (and probably prime) source of ROS. Their finding that hyperoxia resulted in increased rates of TUNEL positive staining in tissue implied initially that apoptosis was being observed, and that administration of anti-CINC prevented this apoptotic response. Concomitant staining was carried out for other markers of apoptosis in particular bax, bcl-2, and M30. M30 is a very specific marker looking for digestion of cytokeratin 18 that occurs by caspase 6 during epithelial cell apoptosis. The observation that these latter markers of apoptotic activity were unchanged during hyperoxic lung damage led them to believe that the change observed during TUNEL staining was secondary to oxidation damage of DNA rather than actual apoptosis. This therefore implies that the generation of ROS by hyperoxia is not the driving agent of in vivo apoptosis, and indeed that apoptosis has not been satisfactorily demonstrated in their model.

Similar hypothesises were tested by Budinger et al[206] on an in vitro model of hyperoxic injury of fibroblasts. They were able to demonstrate apoptosis in response to hyperoxia in a culture model of rat fibroblasts as determined by activation of caspase-9, nuclear fragmentation, and release of cytochrome c. These changes were

prevented by over-expression of *bcl-xl* (an antagonist of apoptosis), and by the use of *bax* or *bak* null rat fibroblasts (both of which are agonists in intrinsic apoptotic pathways). A variety of antioxidants were unable to prevent the apoptotic response, however the use of human fibrosarcoma cells lacking mitochondrial DNA, and thus unable to generate ROS under hyperoxic exposure, did not undergo apoptosis.

Thus I note contrasting findings in the investigation of the role of ROS in inducing hyperoxic mediated apoptosis. Some of this confusion may be explained by the differences noted between in vivo and in vitro apoptosis detection. The main evidence for apoptosis in cells where multiple agents have been used to identify this form of cell death have come in studies involving cell culture. Far greater difficulty has been encountered in the investigation of apoptosis in *in vivo* models. This has now led to greater support for the belief that apoptosis as a 'pure' mechanism for cell death does not exist in the in vivo environment. Instead, it is postulated that cell death is not either necrosis or apoptosis, but these are extremes on a spectrum of cell death and that most cells die somewhere along this spectrum by a process coined 'necroptosis'[207].

Also providing an explanation for these contrasts are the observations that ROS may have does dependant effects in governing apoptosis. Although they have a role in the signalling that enables apoptosis, they may also have an antiapoptotic effect[208].

To add even more confusion to this arena, is the alternative hypothesis that ROS are the prime instigators of apoptosis, and that hyperoxia, whilst in itself causing non-apoptotic cell death, can inhibit subsequent ROS mediated cell death in cells. Franek et al[209] pre-exposed lung epithelial cells (A549 adenocarcinoma) to 95% oxygen

before exposing the same cells to lethal doses of the oxidants hydrogen peroxide. The results demonstrated that hyperoxic pre-exposure protected the cells from apoptosis and was presumed to be secondary to a noted activation of NFκB. This transcription factor has been shown to protect cells from apoptosis by regulating bcl-2 proteins and caspases themselves[210 211].

In considering antiapoptotic strategies, attention has focussed on antioxidants. Catalase is a potent antioxidant enzyme present in the cellular peroxisomes which acts to convert hydrogen peroxide to water and molecular oxygen without the formation of radicals. The addition of catalase to neutrophils reduced rates of spontaneous apoptosis, and reduced the apoptotic effects of supplementary hydrogen peroxide[212].

Glutathione is an intracellular antioxidant and has been to shown to prevent fas receptor mediated apoptosis in cells whose glutathione concentrations have been augmented by strategies such as exogenous supplementation or by the addition of N-acetylcysteine[213].

In summary, excessive production of Reactive Oxygen Species is likely to be responsible for causing apoptosis in cells, either when administered or when produced endogenously by the injured mitochondria. These same species however have a role to play in normal intracellular signalling and can inhibit apoptosis under the tight circumstances, probably via activation of NFκB. Whether hyperoxia causes cell death by causing increased generation of these ROS in cells, or by the oxidative damage to DNA it directly causes is dependant on the type of cells, whether the model is in vivo or in vitro and may not be of a clear-cut apoptotic process.

Our attempts at detection of apoptosis had varying results. Initial attempts were made

using TUNEL staining on paraffin embedded histological sections. Although test specimens that were cytological rather than histological produced good results with differentiation of positive control cells from normal cells, I found that staining of my tissue was problematic in interpretation. The brown staining indicative of cleaved DNA was not only found in nuclei, but significant staining was found dispersed throughout the extracellular matrix, and some associated with damaged or smeared cells. my interpretation was that some necrotic cells were staining positively as has been described, and that cells that had undergone lysis has spilled contents into the surrounding area making differentiation of positive and negative cells difficult.

Bax staining was again successful with positive control sections for which I utilised bowel carcinoma tissue. Here staining was discrete and easily identifiable, but was not reproducible on explant lung tissue sections. It is possible that *bax* staining was not positive as this was not the augmented pathway, however it would be surprising if the intrinsic pathway was not utilised in my model. The likelihood of mitochondrial damage as being the mechanism of cell damage is far more likely in the hyperoxic environment than the extracellular signalling that would be required for cell receptor induction of apoptosis. This is all the more likely in a model where signalling was restricted to cell-cell direct interactions as I did not have a functional circulation in my tissue.

Other potential causes for apoptosis

The assumption during my investigations was that the differences seen between the control specimens and the hyperoxia exposed specimens implicated oxygen tension as the culprit for apoptosis. However it must be bourn that I witnessed relatively high rates of apoptosis in my tissue when compared to reported in vivo lung from

stillborn infants. It may therefore be that the apoptotic rates were superimposed on an environment conducive to proapoptosis.

Raff[183] recognised that apoptosis was the default pathway taken by cells if specific survival factors were absent from their environment. I have already reported that previous work with organ culture has shown many of the morphological changes I have described in hyperoxic conditions have occurred but at relatively low oxygen tensions (21% oxygen). What is noted is that all these experiments were carried out with culture media that was not supplemented with serum.[35 160 162 163]

Serum has been recognised as possessing factors that impair apoptosis in culture, providing a rich source of various growth factors which may have specific effects on the maturation or differentiation of the various cell types within culture[214-218]. I believe my inclusion of serum within my culture medium enabled my explants to relatively maintain their preculture characteristics under normoxic conditions, and it is this that allowed us to examine the effects of hyperoxia independently of this serum effect.

In summary, my work looking at the contribution of apoptosis to the changes seen within my hyperoxic lung culture model appear to confirm that hyperoxia induces a 'wave' of apoptosis, limited to the mesenchyme compartment and that this is in part responsible for the regression of this compartment that I have noted. I have provided evidence that confirms that oxygen has been recognised as a factor that can induce apoptosis by a number of mechanisms. I have also explained that apoptosis can be found in a number of biological scenarios. This observation is found in acute lung injury[20], as a prelude to chronic lung injury[36], and is also recognised as a part of

normal[31] and accelerated lung development after delivery[34 35].

I suggest that apoptosis induced during acute lung injury is reactive, and that the apoptosis seen in accelerated lung development is adaptive, enabling the preterm infant more effectively exchange gas via loss of mesenchyme. Both are contributors to the process of CLD where even in the absence of obvious acute lung injury, I note the development of maldeveloped lungs in extremely preterm infants. I believe my explant model of hyperoxia may represent aspects of both ALI and acceleration, particularly by reference to the findings in the vascular compartment in Chapter 3.

Chapter Five:

Technical considerations

A number of difficulties were encountered whilst refining the methodologies for morphometric analysis of immunohistologically stained explant tissue sections. I shall consider the preparation of the stained sections and the analysis of these samples separately. In doing so I shall discuss the basis for these techniques and my choice of specific variants.

Immunohistochemistry

Specific difficulties

During the development of my culture methodology, I decided that tissue samples should be frozen after harvesting for subsequent analysis. I had initially considered that immunohistochemistry of frozen tissues would be advantageous. Frozen tissue requires minimal antigen retrieval to locate the antigen of interest using commercial antibodies. The fixation of tissue for paraffin embedding, using formaldehyde, produces cross-linking between neighbouring proteins. While formaldehyde fixation of tissue provides structural stability over prolonged period of time, the treatment of this tissue to reveal these antigens may damage the cellular structure of the sections. Unfortunately, I encountered a number of difficulties with frozen specimens. Sectioning of tissue samples was particularly difficult. A cryotome was utilized for the purpose, a specialized apparatus for sectioning frozen specimens. These have a minimum section depth of 10μm to achieve a degree of reliability in sectioning. Any thinner than this resulted in the majority of sections tearing. This depth produced a high degree of cell overlap on microscopy making morphometric analysis of sections

more difficult. I generally found sectioning of tissue to be slow and unreliable and a significant percentage of harvested material was discarded due to poor quality. The quality of sections was far better with paraffin embedded tissue, and serial sections were more likely to be achieved using this methodology.

The next difficulty arose with immunostaining. Although, in theory, immunocytochemistry of frozen tissue should be simpler than with paraffin-embedded tissue, many antibody manufacturers had developed antibodies to specifically work on these latter tissues. Cross reactivity with frozen specimens was sub optimal in these scenarios. Thus after some months utilizing frozen specimens, I changed my methodology to utilize fixed, paraffin embedded tissue.

Theory of immunohistochemistry

The procedure of immunohistochemistry (IHC) has become an invaluable technique for researchers interested in tissue structure. This process utilises antibodies raised to specific structural proteins, to denote their presence in histological specimens[219].

Basic histology has traditionally relied on histochemistry. This takes advantage of certain tissues or cellular structures to have differing affinities to various dyes. The widely used haematoxylin and eosin technique depends on the acidophilic properties of haematoxylin. This blue dye thus labels cellular nuclear structures. In contrast, the pink dye eosin has basophilic tendencies, staining cytoplasmic structures and, to a lesser extent, intercellular matrix. This enables histologists to examine the structure and organisation of tissues and still forms the basis of most clinical histology.

Primary antibody

More detailed analysis of tissues became possible with the advent of labelled antibodies. These were made to bind to specific proteins to which they had been raised. Polyclonal antibodies are produced by the injecting of antigen into an animal differing from the species responsible for the antigen e.g. injecting fragments of recombinant human actin into mice. The blood from these mice is then removed and plasmapheresed to remove the large amount of anti-actin antibody produced as part of an immune response.

Monoclonal antibodies bind to a very specific epitope on the chosen antigen. This is carried out by the use of recombinant technology to fuse plasma cells with tumour cells. These plasma cells are preselected for their production of specific antibodies, and injecting these rapidly dividing cells into the peritoneum of mice. These then rapidly divide producing large amounts of ascitic fluid rich in this antibody.

The decision to use monoclonal or polyclonal antibodies is dependant on their individual advantages. Monoclonal antibodies compete for a single epitope on an antigen but are raised to predetermined polypeptides, which are highly specific, reducing non-specific binding. Polyclonal antibodies may bind to a number of sites on an antigen, thus amplifying the resultant signal. This increases the sensitivity of the antibody, but may reduce specificity.

Specific antibody binding is carried out by a specialised end-portion of the molecule known as Fab (Fragment ab). Fragment c (Fc) provides the structural backbone of the antibody protein. Although affinity for binding is greatest between the Fab portion and the matched antigen, there is also a low affinity binding between Fab segments

and non-antigenic substances. This binding is known as non-specific binding, and impairs the localisation of antibody to the molecules of interest.

Attempting to occupy the areas on a slide that have a protein affinity can decrease non-specific binding. Hence, before the addition of primary antibody to a section, a binding protein is applied. This may comprise a solution based on the casein protein. This is commercially available as a protein blocker.

Experimentation has shown that further reduction in non-specific binding can be encouraged by bringing utilising antibody at cool temperatures ($4^{o}C$) for long periods of time-usually overnight. I utilised this technique throughout my experiments.

Antigen retrieval

To be able to bind to antigen, there needs to be adequate exposure of the antigen to the environment. In frozen sections, this is rarely a problem, however, the fixation involved in formalin fixed, paraffin embedded tissue results in crosslinking of proteins. This effectively masks the antigen of interest preventing combination with antibody.

The process of unmasking antigens is termed antigen retrieval, and a number of techniques are utilised, each being particularly suited to specific tissues and antigens. These include the use of proteases such as trypsin to partly digest proteins and break the disulphide bonds that crosslink differing proteins as part of the fixation process. Excessive digestion results in loss of antigenicity and may reduce specificity of antibody binding. I attempted the use of protease antigen retrieval early in my endeavours, and found that excessive degrees of background staining resulted

preventing us from clearly defining the tissues of interest.

Heat has been found to unmask proteins by breaking crosslinks. This is optimally achieved with the use of a pressure cooker to reach temperatures above 100^{o}C. Immersion of tissue sections in buffered citric acid acts synergistically for antigens that are particularly difficult to expose, often by nature of their intercellular location.

It was by the use of a microwavable pressure cooker enabling us to achieve temperatures of 120^{o}C whilst immersed in buffered citric acid that I was able to achieve optimal staining for CD31[139]. I first utilised this method after communicating with a number of different investigators in this field. I are grateful to Dr Alison Hislop and Dr Su Hall at the Institute for Child Health in London who permitted me to visit their laboratory and carry out test staining of my tissue with their protocol and equipment. After successfully carrying out this test run, I subsequently applied this method to all my immunohistochemical staining using similar equipment in my own laboratory. It must be emphasised that the pressure cooker utilised is of porcelain clay to allow its use in a microwave, as opposed to the traditional steel pressure cookers.

Secondary antibody and substrate visualisation

To be able to visualise bound antibodies, they need to possess a label, which can produce or induce a colour response. Primary antibodies are available which possess these properties; however the use of secondary antibodies is favoured.

Secondary antibodies are polyclonal antibodies raised by the injection of immunoglobulin of one species into another. The host species thus produces an antibody pool that has a high affinity for the antibodies of the other species. By

injecting antibodies from the species responsible for the primary antibody, the resultant secondary antibody is produced.

Commercial secondary antibodies are linked to a label, most commonly the biotin molecule. This protein is used for its recognised prodigious affinity for the avidin protein. Target substances can subsequently be directed to secondary antibodies by combining them with avidin. The target substances in question possess properties allowing visualise the protein-antibody complex.

The combination of avidin with the enzyme peroxidase is the most commonly used enzymatic agent in immunohistochemistry. The peroxidase enzyme catalyses a colour change in the 3,3-diaminobenzidine (DAB) molecule. It is this substrate which, when finally added to the tissue section, is converted to a brown pigment which can then be seen by light microscopy.

Counterstain

In the majority of scenarios, the localisation of a protein by 'DAB-brown' reveals little information unless the background tissue can be seen for contrast. For this reason, a counterstain is used which identifies the tissue structure without masking the 'DAB-brown'. I have utilised the haematoxylin stain for this purpose, due to excellent differentiation from brown.

Haematoxylin stains are available in a number of differing forms e.g. Ehrlichs's, Mayer's and Harris'. These are divided into two main groups, the progressive stains and the regressive stains. The progressive stains of which Mayer's is one of the most popular have a high specificity for nuclear structures and not having a tendency to

over stain tissues, but have the disadvantage of requiring a long period of time before optimal staining is achieved. Regressive stains have a much more rapid action but in doing so may over stain tissue, and may not selectively stain the nuclei with blue staining occurring elsewhere[220]. For this reason, regressive stains such as Ehrlich's, required differentiation where after briefly placing in the haematoxylin for a few seconds, the section is the briefly dipped in and acid alcohol solution which removes the excess stain from unwanted regions. This permits a much faster optimal result when compared to progressive stains. I utilised both techniques, but found that the regressive staining method achieved with Ehrlich's solution produced a lighter more distinct result enabling easier cell counts to be carried out, and optimised computer differentiation of blue counterstain from brown immunolabelling. For optimal results with Ehlich's stain, the process of blueing must be carried out at the end of the process by placing the slides in tap water for unto five minutes, changing the acid mediated reddening of the stain back to a blue.

Controls

The success of immunohistochemistry can be relatively easy to assess when a protein is searched for that has well defined spatial characteristics. For example, cytokeratin is known to reside exclusively within the cell structure of epithelial cells. One can therefore be assured that an immunohistochemical procedure has been successful when the cell lining of airway structures are exclusively dyed. If however, one is studying tissues where the cells of interest are not easily discernible from other cell types, it is more difficult to assess if the highlighted cells are indeed correct for example when looking for surfactant producing cells in tissue where they would not necessarily be expected to exist such as pseudoglandular lung. For this reason,

positive controls are included within studies. Positive control slides are sections of tissue where, in this example, the cell of interest is easily recognisable and expected to be found e.g. surfactant producing pneumocytes in adult lung. This provides confidence that the procedure has been successful, and the cells denoted brown in the study sample are those being sought out. The use of positive controls reduces the possibility of false negative staining in a tissue.

A number of positive controls are available for each antibody type. Some may need to be generated by the investigator. For example, when staining tissue for markers of apoptosis, positive controls are generated by treating a tissue sample with agents that induce apoptotic change in large numbers of cells. This is important since the study tissue may have infrequent cells of this nature. Thus, if the control tissue has numerous positively stained cells, but the study tissue has none, it is more likely the result is valid, rather than a particular step in the process having been omitted.

Similarly, negative controls need to be generated to ensure the brown stain detected is not an artefact due to false positive staining. It is possible that a primary antibody may bind to a substrate that is not the protein of interest; this is investigated by utilising this primary antibody on tissue where this protein should not be present, once again by staining a control slide of human colon for surfactant, positive staining should not be seen at all. If present, this leads the researcher down an investigative pathway as the staining may be due to primary antibody cross reactivity, or due to non specific binding by other elements in the process such as the secondary antibody. I repeatedly used positive and negative controls in my work, in particular to optimise the concentration of the antibodies utilised. Excess concentration of an antibody would lead to false positive staining where a tissue could look universally brown, or where

too dilute a antibody concentration, too short an incubation time or inadequate antigen retrieval resulted in a false negative result.

Overall, the process of immunohistochemistry proved to be initially easy to learn as a process when commencing with a protein easily detectable e.g. actin, but proved to be a major challenge with utilisation of excessive time and materials when the proteins of interest were more difficult to stain adequately. I have found the experience of the problem solving process itself invaluable however and am confident that I will be able to apply this process to future research with a determined, logical and analytical approach.

Image analysis

When carrying out investigations that produce a visual result, in contrast to a numeric value, it must be decided how this result is best reported. Investigations that fall within this category are numerous and include tissue histology, cytology and gel electrophoresis. The method employed is dependant on the investigation, and the nature of the question posed. This discussion shall be limited to tissue histology as that is the analysis performed in my studies.

General approach to morphometry

The earliest means of assessing tissue histology was that of qualitative inspection. Experienced histologists inspect stained tissue sections under light microscopy. The appearance of these is compared with other examples available to the investigator. Thus, for example, a study looking at the effect of a toxin on the development of desquamation of the villous lining of the ileum in mice would involve looking at this

tissue in a number of exposed animals and commenting on the presence or absence of these structures. To make a reasonable assessment, a reference is required for comparison. In this circumstance, control mice are utilised, enabling a comparison to be made. Investigator bias may become an issue due to the subjective nature of this analysis. For this reason, investigators are blinded to the study or control status of the subject. This method of comparison requires expertise in pathology, and is best reserved for gross changes in histological appearance.

A more rigorous approach to histological comparisons is that of morphometry. This refers to the quantitative analysis of tissue specimens. Much of the work carried out in morphometry has been applied to respiratory diseases, due to the complex branching patterns of the human lung.

The nature of the analysis is determined by the clinical question posed, and a range of tools are available to assist the researcher answer these questions.

In my experiments, the major questions I was interested in fell into the following categories:

1) Comparison of cell counts where two identifiable populations exist.

   I used these techniques to assess the proportion of cells in the explant tissue were epithelial, and what percentage of explant cells were apoptotic or proliferating.

2) Comparison of relative surface areas of sections occupied by various tissue types.

   I used this to determine the percentage of tissue occupied by airway lumen in my explant sections, and the percentage of mesenchyme occupied by vasculature. I

also determined the percentage of tissue from infants which was occupied by smooth muscle.

3) Determination of distance.

This was undertaken when calculating the epithelial cell thickness in explant tissues and calculating the distance of vasculature from airway lumen in these same samples.

I shall discuss the various methods applied to morphometry and relate these to the methods I chose to utilise in answering the above questions.

Morphometry has moved beyond the simple process of measuring lengths of objects and became rationalised into a collection of tools collectively termed 'stereology'[138]. These tools are linked in their formation of geometric inferences of three-dimensional structures based upon two-dimensional images.

Initially, the methods used were carried out under the assumptions that by the sampling of certain images, these would equate as being representative of the whole organ being sampled. However, over recent years, it has become realised that for prevention of bias, certain rules needed to be followed to ensure that it was not necessary to assume the overall structure of the object was behaving as the investigator hoped. This led to a move from 'classical stereology' to 'design-based stereology' where the nature of the sampling followed strict predetermined criteria. I shall discuss the approaches of 'design-based stereology' subsequently in justifying the rationale for my own approach to morphometry employed.

Cell counts

In classical stereology, the method employed to identify the number of cells of one particular population within a tissue would be to examine a field of known size and count the cells manually in that field. This at its most basic could be by overlaying a grid onto an image, either by computer or by having a specially adapted lens graticule.

If the grid is of known surface area, the density of cells per unit area can be provided. This is demonstrated in figure 5.1 where a simple overlay with 4 grids of known size are placed over the random section.

The problem with this approach is that even though there may no bias in selecting the image, there is an assumption that this tissue sampled represents the whole tissue.

With my work, cell counting was integral to my methodology. Not only was I counting cells, but I was subdividing these into subgroups e.g. epithelial, interstitial, apoptotic, proliferating etc. I was interested in only the periphery of the explant lungs and wished to compare like for like. For this reason I set out stringent sampling techniques i.e. 8 fields at the periphery of the sections taken at equal angles of 45^{o} from a central point in the section. Having collected comparable pictures from each tissue, I then set about maximising the volume of grid area utilised for sampling for each section. With manual counting, it is necessary to select random small areas to analyse to make this process feasible. I however used computer assisted image analysis and so were able to cast my 'net wider'. For each picture, the computer detected each cell and by altering the colour definitions for the image detection software, I could instruct the program to either detect blue cells, brown cells, or all cells visible.

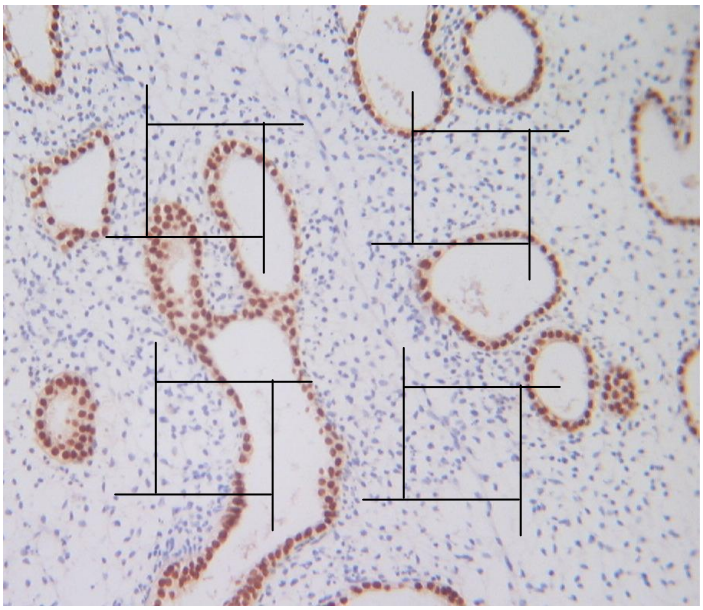


Figure 7.1: Performing a cell count manually within the 4 grids of known surface area enable a calculation of cell density that can be assumed for the tissue. With low microscopic magnification, this can be seen to be technically difficult due to high cell density.

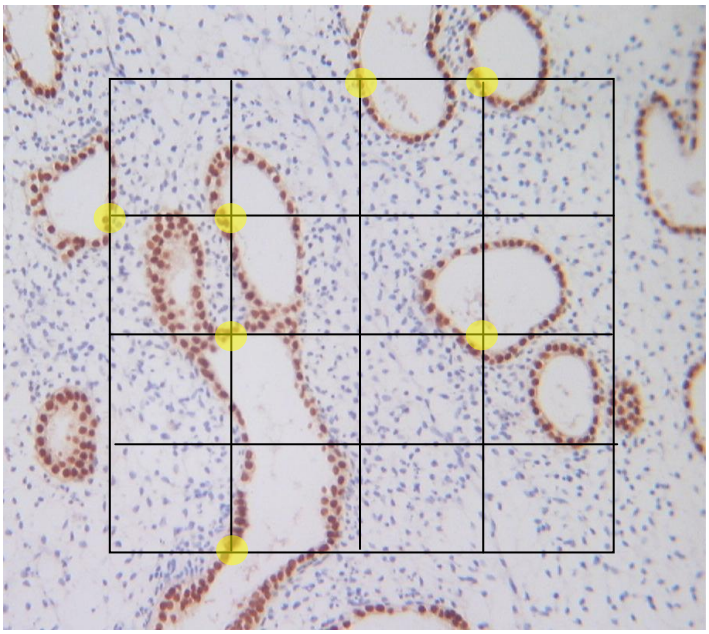


Figure 7.2: In this grid, counting the 25 intersections (5x5), I determine that 18 lie in the parenchyma and 7 lay in the airway compartment. Airway intersections are denoted with a yellow circle for clarity.

Structure proportional assessments

In determining which percentage of explant tissue was lumen vs. mesenchyme I chose to analyse the entire section of the tissue as much of this work was carried out at low power magnification.

In classical stereology, the process that would be utilised for manual work would be to overlay a grid over each picture. Each intersection could then be observed and recorded whether it occupied an airway structure (epithelium or lumen) or mesenchyme. The numbers of each could then be divided by the total quantity to give a percentage for both. An example of this is given in figure 5.2. Here there are 25 points of intersection on the grid of which 18 lay in parenchymal tissue, whilst 7 are overlaying airway structures. By this I can infer that 72% (18/25x100) of the tissue is parenchyma. This could then be repeated on multiple section fields to give a more representative average.

Again, I chose not to use the sampling approach. Having the facilities to examine the entire field was expected to yield more representative data than extrapolating data from limited areas. I used the Qwin image analysis package to manually draw on the screen all airways, inclusive of epithelium. These areas were automatically filled by the computer providing an airway map. A second digital image was produced encircling the tissue to provide denominator, and thus a proportion of the tissue that was airway could be calculated. The additional benefits of the image analysis package are that the individual characteristics of the airways can also be calculated; thus I was able to obtain the average cross sectional diameter of the airway and the number of airways per unit area of tissue.

Once again, the use of manual labelling would have proved time consuming, thus I utilised the abilities of the Qwin package to detect pixels of particular colour settings as determined by the user. I was thus able to automatically detect the brown pixels of the epithelium in cytokeratin stained tissue, then manually fine-edit these images to mirror the underlying airways (Figure 5.3). This facility made the ability to sample the individual bodies represented by vasculature or actin bodies feasible. Under normal circumstances these bodies are too numerous, irregular and small to accurately be able to draw around to enable the computer to calculate the area covered and average size of each.

Distance

The measurement of a distance manually in an image is fairly simple to do, even in the absence of a computer. For the photo or image of interest, another image is taken at exactly the same microscope magnification settings. The slide being photographed is a graticule which provides a microscopic ruler to act as a calibrator. Thus any distances measured can be converted using this technique.

Measurements of perimeters are far more difficult however and use of stereology for this purpose is beyond the scope of this thesis. Fortunately, my software rendered these techniques obsolete, thus any object detected could automatically have its perimeter calculated.

More challenging however was the calculation of average distances of one group of objects from another. I wished to calculate the distance from my blood vessels to the nearest airway lumen. As set out in chapter 3, I devised my own ‘macro’ program to

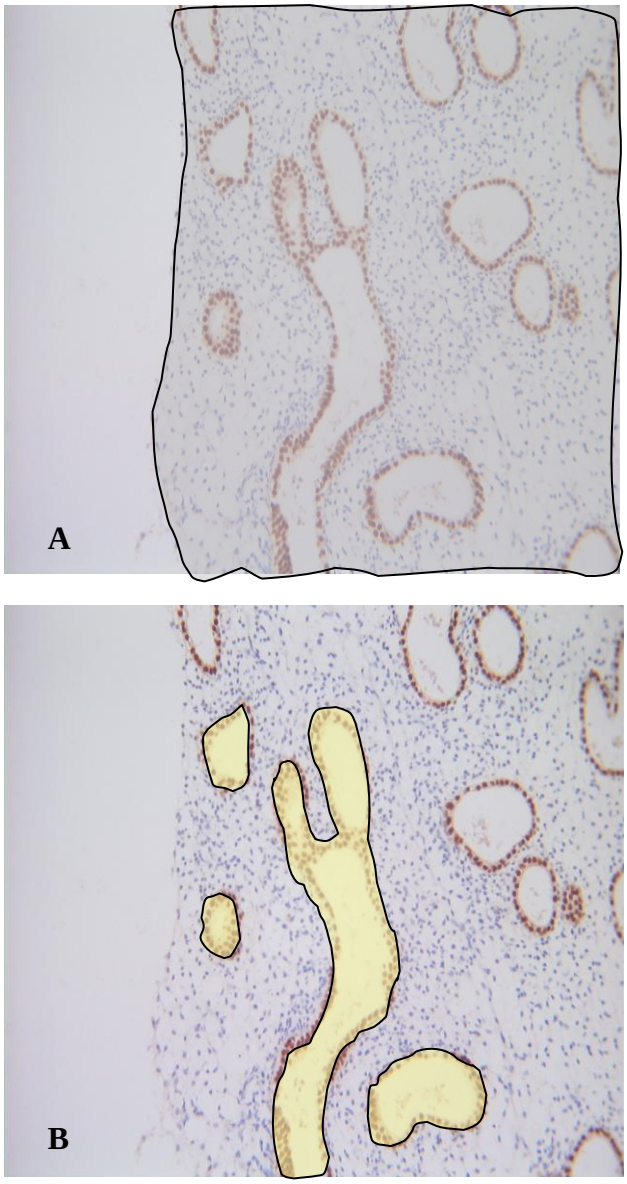


Figure 7.3: In calculating percentage of tissue characteristics, two binary overlays are generated. The first (A) denotes the total area of tissue within the visual field. The second (B) denotes the individual airways- four such airways have been filled for clarity.

enable this.

Overall, the use of computer assisted image analysis together with a rigorous relatively unbiased methodology to sampling has enabled us to perform quantitative analysis on a large number of specimens and on relatively large areas of tissue from each section. Rather than having to provide a descriptive interpretation of the abnormal morphology seen, I was able to generate numerical results and thus determine whether my experiments proved statistically significant or not.

However, a thorough understanding of the tools available need to be attained before usage, as when incorrectly applied the results may not be valid. As with all other data analysis systems, the expression ‘Garbage In, Garbage Out’ is particularly applicable to image analysis.

Chapter Six:

Conclusions

The delivery of human infants prematurely into the extrauterine environment exposes these vulnerable patients to relatively high oxygen tensions when compared to *in utero* conditions. It is believed that these high oxygen tensions contribute to the development of Chronic Lung Disease of Prematurity (CLD, also known as Bronchopulmonary Dysplasia).

The work carried out in this thesis aimed to investigate the processes involved in the development of CLD, and the role that oxygen plays in the underlying processes that are associated with this pathological diagnosis.

I hypothesised that high oxygen tensions can lead to remodelling of the developing lung by causing specific and distinct effects in the epithelial and mesenchyme compartments. I was particularly interested in the effect of oxygen on the vascular and airway smooth muscle within the mesenchyme. I was also interested in determining the role that proliferation and apoptotic cell death had in mediating these changes.

To be able to carry out this work, I developed or utilised a number of methodologies, some specific to this thesis.

Much of the work was carried out on human fetal lung. In adapting the methods of McCray[137], my group developed a culture methodology that enabled maintenance of lung from fetuses from the pseudoglandular stage of lung development for 4 days after dissection into small pieces, and submersion into a collagen based semisolid culture medium. By exposure of these explants to high (95%) and normal (21%) oxygen concentrations for up to 72 hours after establishment of culture, I was able to

harvest these explants and then proceed with analysis. The aim with these experiments was to analyse the acute effects of oxygen on fetal lung development.

Histological processing of the lungs was carried out by myself with staining of the tissue by immunohistochemistry for a number of proteins including cytokeratin (epithelium), CD31 (endothelium), Ki67 (proliferating cells), and activated caspase-3 (apoptosis). For each, multiple experiments were carried out to determine optimal tissue preparation and optimal antibody concentrations and exposure times.

Finally, analysis of these tissues was carried out by the development of original morphometric image processing techniques for which I employed specialist image analysis software.

Technical difficulties were encountered at all stages of the experimental development, requiring development in expertise in culture methodology, experimentation with harvesting techniques, and attempts at numerous 'recipes' for immunohistochemistry for each antigen of interest.

After a comprehensive review of the literature regarding the pathology and pathogenesis of CLD, I recognised that oxygen could act on lung via a number of mechanisms. This could be by the regulation of intracellular processes resulting in increased or decreased expression of growth factors such as VEGF and KGF, each with a distinct effect of cell growth and function. Alternatively, oxygen could be acutely toxic to lung either via the generation of Reactive Oxygen Species or via upregulation of transcription factors such as NFκB which induce an inflammatory response in the host.

My initial work concentrated on the development of the fetal explant model, and the characterisation of the changes attributable to oxygen as opposed to the effects of culture.

I found that culture in hyperoxic conditions (95% oxygen content) produced visible structural differences when compared to explants maintained in normoxic conditions (21% oxygen). Hyperoxic tissue demonstrated relative dilatation of airways with a median cross sectional area of 14000μm^2 (IQR:10500-22000) compared to 5800μm^2 (IQR:3300-8800) for normoxia cultured controls. Additionally there was an increase in the proportion of tissue occupied by airway lumen after 72 hours culture under hyperoxic conditions [43% (IQR: 24-48%)] when compared to normoxia [15%, (IQR: 12-23%) , and thinning of epithelium in dilated airways with a mean epithelial thickness [6.4μm (IQR: 6-7)] relative to the corresponding normoxic tissue [8.7μm (IQR: 8-10)].

These changes have been previously described in other explant models, however these were not in response to the hyperoxic environment I utilised. I discussed in Chapter 2 that these changes are seen in experiments utilising serum free culture media and have suggested an acceleration of lung development as a physiological response to prepare the lung for the extrauterine oxygen rich environment. The changes thus represent movement of pseudoglandular lung into the saccular phase and have previously been supported by demonstration of flattening of epithelial cells and expression of surfactant proteins at a critical stage of lung development.

Although this acceleration of lung development may appear to be a rapid but normal maturation, this might represent a dysregulated lung development in response to

hyperoxia that has its closest equivalence in CLD. This implies a disordered, non-physiological developmental response. Alternatively, these findings could be related to the acute effects of oxygen toxicity whereby it is via tissue damage and increased vascular permeability that I note my observations, making this a model of acute lung injury in the preterm infant.

To assist determining which of these three processes is most likely to be occurring in response to hyperoxia, I proceeded to analyse the changes in the vascular compartment. The culture of human lung explants in hyperoxic conditions resulted in morphological changes in the vasculature characterised by pruning of the blood vessels and apposition of these vessels towards the airways. Hyperoxic culture samples had less vasculature in mesenchyme [4% (IQR: 2-5)] than paired normoxic samples [7% (IQR: 5-8)] and had reduced vessel to airway lumen distance [35μm (32-38μm) vs. 25μm (21-30μm) for normoxic and hyperoxic conditions respectively]. The apposition of vessels to airway most resembles the accelerated lung development seen with other aspects of the explant model i.e. saccular dilatation, epithelial thinning and surfactant expression. The pruning of blood vessels more resembled the loss of vasculature seen in both acute lung injury and CLD as a result of direct oxygen toxicity and loss of angiogenic factors such as VEGF. However aspects of this pruning produced an appearance of maturation of the vasculature rather than frank destruction. Overall therefore, I hypothesised in Chapter 3 that hyperoxia leads to removal of blood vessels due to pruning, representing a maldevelopmental maturation of lung as seen in CLD.

The underlying processes causing these changes within the explant model could be categorised into changes in cell differentiation or cell numbers. These cell numbers

are altered in response to the balance between proliferation and cell death, principally by apoptosis. I proceeded to analyse these two phenomena in Chapter 4.

Using Ki67 staining as a marker for active cell proliferation, I noted there was an overall decrease in proliferating cells during explant culture from baseline. The effects of normoxic culture were analysed according to cell type demonstrating that there was a significant decrease in proliferation in the mesenchyme compartment [12% (IQR: 9-13) on Day1, reducing to 4% (IQR: 2-5) for N72], but that epithelial proliferation was preserved [13% (IQR: 9-16) on Day 1 and 10% (IQR 4-16) for N72]. The addition of hyperoxic conditions to culture had no discernible effect on proliferation indices in either compartment when compared to normoxia [3% (IQR: 2-4) for mesenchyme and 13% (IQR: 10-16) for epithelium in H72 tissue].

Although hyperoxia did not appear to influence the rate of proliferation in these experiments, my discussion covered a number of points of interest.

1) Epithelium and mesenchyme appeared to have differing proliferative responses to culture. I reviewed the literature and noted previous work that demonstrated that epithelium and mesenchyme have potentially independent responses to culture; however these responses are inconsistent and appear to be dependant on secondary factors such as presence of serum, growth factors, maturity of the subject and nature of the model i.e. cell culture, explant culture or *in vivo* studies.

2) Oxygen related effects on proliferation may not follow a linear association. Of particular interest is was the observation by Pandya[10] that a bell-shaped response was noted when oxygen tension was linked to proliferation rates in

airway smooth muscle cells.

3) That compared to *in utero* conditions, both the normoxic and hyperoxic environments used (21% and 95% respectively) were in fact relatively hyperoxic. This may go some of the way to explain why I was able to demonstrate a suppression of proliferation in mesenchyme both in normoxia and hyperoxia branches of my experiments.

My work looking at the contribution of apoptosis to the changes seen within my hyperoxic lung culture model appeared to confirm that hyperoxia induced apoptosis limited to the mesenchyme was in part responsible for the regression of this compartment that I have noted. Within the mesenchyme, apoptotic indices were similar in Day 1 cultured lung and 72 hours normoxic cultured lung (Medians 3% (IQR:2-5.5%) vs. 4.3% (IQR:4-4.6%) respectively). Culture in hyperoxic conditions resulted in significantly higher apoptotic indices than both Day 1 tissue and normoxic 72 hours tissue, with a median of 11% (IQR: 7-15%). Epithelial cell apoptosis was relatively unaffected by culture in either normoxia or hyperoxia [Median apoptosis rate: 1% (IQR: 0.5-2.7) for Day 1, 4.8% (IQR: 3.9-5.6) for N72 and 2% (IQR: 1.3-2.9) for H72].

I reviewed the literature to confirm the role that oxygen has via a number of mechanisms in inducing apoptosis. I then demonstrated the role that apoptosis plays in a number of biological scenarios. These scenarios include acute lung injury, as a prelude to chronic lung injury, and are also recognised within normal and accelerated lung development after delivery.

I suggested in the discussion of Chapter 4 that apoptosis induced during acute lung

injury is reactive, whereas the apoptosis seen in accelerated lung development is adaptive, enabling the preterm infant to more effectively exchange gas via loss of mesenchyme. Both are these processes contribute to the development of CLD where even in the absence of obvious acute lung injury, I note the development of maldeveloped lungs in extremely preterm infants. I believe my explant model of hyperoxia may represent aspects of both acute lung injury and acceleration, particularly by reference to the findings in the vascular compartment in Chapter 3.

I have therefore shown from my work, that high concentrations of oxygen can prove detrimental to the development of the lung. Both epithelium and mesenchyme respond differently to hyperoxia. A spectrum of responses exist which span 'Acute Lung Injury' through to lung maldevelopment. This latter maldevelopment may arise via adaptive processes which may in the short term serve to assist respiration in the preterm infants, and is primarily an apoptotic response in the mesenchyme. These various interactions are summarised in Figure 6.1.

The work carried out provides a basis for future experiments utilising the explant culture model with hyperoxic exposure. The strength of the model is that by exposure of the lung to differing oxygen tensions, an array of mechanistic investigations can shed light on the effect of hyperoxia on epithelium and mesenchyme. Members of my laboratory have already characterised the responses of the lung explants in altered expression of VEGF, implying a hyperoxia mediated suppression of this angiogenic factor. With the characterisation of the effects of hyperoxia on the explants, we can also now investigate the potential for pharmacological interventions to ameliorate these changes. Specifically, we are now pre-treating the explants with dexamethasone to determine the effects of this commonly used therapy on developing fetal lung.

Although these experiments attempt to provide a model with which to investigate the effects of oxygen on lung development in the premature infant both via toxic and accelerative processes, we need to be mindful that in the preterm infant, oxygen is not a factor that acts in isolation. As reported in the Introduction to this thesis, there are a concert of factors influencing the responses of lung to oxygen and which each may have a detrimental effect on the lung independently[221 222]. Exposure of the lung to inflammation as a response to infection in the uterus or after delivery can cause acute lung injury or chronically, an accelerated maldevelopment resulting in Chronic Lung Disease[57]. Iatrogenic factors such as the use of assisted ventilation also produce acute and chronic lung changes via the inflammation that results from atalectotrauma and volutrauma[223 224]. Despite the use of these modalities for three decades, we are little further in finding alternatives to these damaging treatment modalities.

In summary, I hypothesise that these acute injuries and accelerated attempts at lung development combine to produce some of the varying clinical pictures of CLD. Although these changes provide ability in the short term for the preterm infant to adapt to extra-uterine environment, the cost over the longer term is the disabling disease that characterises these survivors of extreme prematurity.

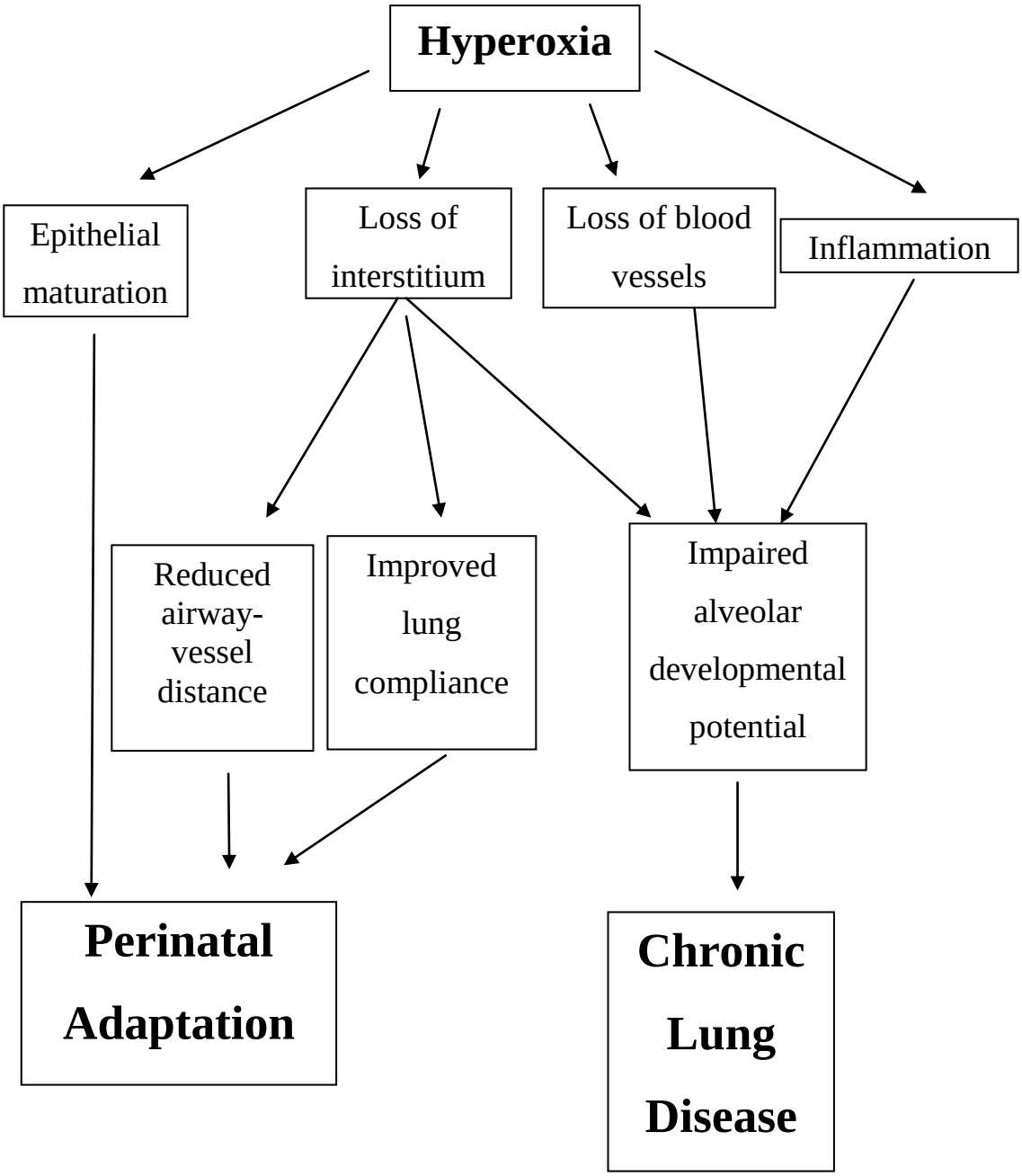


Figure 6.1: Scheme showing potential effects of hyperoxia on lung development. Initial responses of epithelium maturation and regression of interstitium by hyperoxia induced apoptosis may assist gaseous exchange by reducing distance between airway and vasculature and by increasing lung compliance. The trade off however is that reduced interstitium and loss of vasculature acutely and with time lead to reduced potential for alveolar formation and both impair gaseous exchange chronically.

Appendices:

Appendix 1

Routine to calculate the properties of FLOCM tissue vasculature.

Input variables for filename and number of slides

```
PauseText   ( "input file name" )

Input   ( FILLY$ )

PauseText   ( "Input number of pictures" )

Input   ( NO )

Open output files

Open File   ( C:\Porus\MD\FLOCM\cd31\cdfield.Q5D, channel #1 )

Open File   ( C:\Porus\MD\FLOCM\cd31\tissuefield.Q5D, channel #2 )

Open File   ( C:\Porus\MD\FLOCM\cd31\airwaydistance.Q5D, channel #3 )

Loop commence

For   ( X = 1 to NO, step 1 )

  Grey Util   ( Clear All )

  Image frame   ( x 0,  y 0,  Width 1024,  Height 1024 )

  Measure frame   ( x 256,  y 256,  Width 512,  Height 512 )

  Open file

  FILENAME$ = "c:\porus\md\flocm\cd31\"
```

```
X$ = STR$(X)
If  ( X<10 )
  ACQFILE$ = FILENAME$+FILLY$+"\dscf000"+X$+".jpg"
Else
  ACQFILE$ = FILENAME$+FILLY$+"\dscf00"+X$+".jpg"
Endif
ACQOUTPUT = 0
Read image  ( from file ACQFILE$ into ACQOUTPUT, import calibration )
Analyse image
Detect vessels into binary 0 and adjust with edit, final skeleton binary 1
PauseText  ( "Detect vessels" )
Colour Detect  [PAUSE]  ( RGB: 15-216, 0-212, 0-120, from Colour0 into Binary0 )
Binary Amend  ( Open from Binary0 to Binary0, cycles 1, operator Disc, edge erode off )
Binary Amend  ( Close from Binary0 to Binary0, cycles 2, operator Disc, edge erode off )
Binary Edit  [PAUSE]  ( Delete from Binary0 to Binary0, nib Fill, width 2 )
Binary Amend  ( Skeleton from Binary0 to Binary1, cycles 5, operator Disc, edge erode off )
Binary Amend  ( Prune from Binary1 to Binary1, cycles 5, operator Disc, edge erode off )
Draw airways into binary 3, with tissue as binary 4
```

PauseText ("Outline airway lumen")

Binary Edit [PAUSE] (Draw from Binary3 to Binary3, nib Fill, width 2)

Detect tissue into 2

Colour Detect [PAUSE] (RGB: 15-165, 0-172, 0-212, from Colour0 into Binary2)

Binary Amend (Close from Binary2 to Binary2, cycles 10, operator Disc, edge erode off)

Measure field (plane Binary2)

Selected parameters: Area

Measurements

Vessels-field

Measure field (plane Binary0)

Selected parameters: Area, Count

File Field Results (channel #1)

Vessels features-seperate files

Measure feature (plane Binary1, 32 ferets, minimum area: 4, grey image: Colour0)

Selected parameters: Area, Length, Breadth, Roundness, XCentroid,

YCentroid

OPENFILE$ = "C:\Porus\MD\FLOCM\cd31\cdfeat"+X$

CHAN = 5

Open File (OPENFILE$, channel #CHAN)

File Feature Results (channel #5)

Close File (channel #5)

Calculate grey readings

Binary Logical (copy Binary3, inverted to Binary4)

Binary to Grey (Distance from Binary4 to Colour1, operator Octagon)

Measure frame (x 276, y 276, Width 472, Height 472)

Measure Grey (plane Colour1, mask Binary1)

Selected parameters: Mean Red

File Grey Results (channel #3)

Measure frame (x 256, y 256, Width 512, Height 512)

Tissue-field

Binary Amend (Erode from Binary4 to Binary4, cycles 1, operator Disc, edge erode off)

Binary Logical (C = A AND B : C Binary4, A Binary4, B Binary2)

Measure field (plane Binary4)

Selected parameters: Area

File Field Results (channel #2)

Next (X)

Appendix 2

Routine to calculate contribution of apoptotic cells in tissue:

Open File (C:\Documents and Settings\Porus Bustani\My Documents\MD\babies\caspgest\cells.Q5D, channel #1)

Open File (C:\Documents and Settings\Porus Bustani\My Documents\MD\babies\caspgest\caspase.Q5D, channel #2)

For (X = 1 to 100, step 1)

Grey Util (Clear All)

Measure frame (x 31, y 61, Width 962, Height 838)

FILE$ = "c:\documents and settings\porus bustani\my documents\md\babies\caspgest\"

X$ = STR$(X)

If (X<10)

ACQFILE$ = FILE$+"\DSCF000"+X$+".jpg"

Else

ACQFILE$ = FILE$+"\DSCF00"+X$+".jpg"

Endif

ACQOUTPUT = 0

Read image (from file ACQFILE$ into ACQOUTPUT, import calibration)

Colour Detect [PAUSE] (RGB: 0-172, 0-167, 0-182, from Colour0 into Binary0)

Binary Amend (Open from Binary0 to Binary0, cycles 1, operator Octagon, edge

erode off)

Binary Segment (Ult Erode from Binary0 to Binary1, stepsize 1, max cycles 30, operator Disc)

Colour Detect (RGB: 0-209, 0-204, 0-110, from Colour0 into Binary2)

Measure field (plane Binary1)

Selected parameters: Count

File Field Results (channel #1)

Measure field (plane Binary2)

Selected parameters: Count

File Field Results (channel #2)

Measure field (plane Binary2)

Selected parameters: Count

Next (X)

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