THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN REPAIR AND RECOVERY FROM ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

By

DR ANDREW RICHARD LISLE MEDFORD

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DOCTOR OF MEDICINE (MD) to
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DECLARATION

I hereby declare, in accordance with University of Edinburgh Higher Professional Degree Regulations that I, Dr Andrew RL Medford, the candidate, am a graduate (MBChB 1995) of the University of Edinburgh and have been engaged in the practice of Medicine for at least one year since graduation. This thesis deals with a subject of study in the MBChB curriculum that also arises from contemporary medical practice.

I also hereby declare this thesis has been composed by myself, Dr Andrew RL Medford, the candidate; that the work discussed here is my own and that any technical advice, expertise or contribution from another party has been acknowledged in this thesis; that I, the candidate, have not submitted this thesis for candidature for any other degree, postgraduate diploma or professional qualification.

Some of the work in this thesis has been published in peer reviewed scientific journals. This is included in Appendix 1 and acknowledged in the text.

I hereby confirm the above statements to be true in accordance with University of Edinburgh Higher Professional Degree Regulations.

Andrew RL Medford (Dr)

ABSTRACT

Acute Respiratory Distress Syndrome (ARDS) is the most extreme form of acute lung injury and continues to have a significant morbidity and mortality. Unfortunately, the mechanisms involved in the recovery and repair of the lung following ARDS remain poorly understood. An understanding of these is pivotal to improving outcome from acute lung injury. Several observational studies have suggested a potential relationship between Vascular Endothelial Growth Factor (VEGF) in the lung and the development/outcome of ARDS. In this thesis, three potential mechanisms underlying these observations have been explored:

1. What is the anatomical distribution of VEGF receptor and isoform expression in normal and ARDS lung? How does this change at early and later time points following acute lung injury?

2. Are human type 2 alveolar epithelial (ATII) cells a source of and target for VEGF? How does exposure to a pro-inflammatory milieu modify their expression of VEGF isoforms and receptors?

3. Is there a relationship between a functional VEGF polymorphism and susceptibility to developing and severity of ARDS?

I have demonstrated VEGF receptor expression on both sides of the alveolar-capillary membrane with upregulation in later ARDS. All three principal isoforms ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) are expressed in normal human lung with uniform downregulation of all three in early ARDS, which normalises with increasing time following injury. I have not found any evidence of VEGF isoform switching.

I have also demonstrated human ATII cells are both a significant cellular source of and a target for VEGF (via VEGF receptor expression) confirming autocrine VEGF activity in the lung. VEGF is an ATII cell survival factor. ATII cells differentially respond to pro-inflammatory stimuli by increasing VEGF isoform but not receptor expression, which may serve as a regulatory control mechanism.

Finally, I have demonstrated the VEGF 936 T allele increases susceptibility to and the severity of lung injury. The T allele is associated with an increase in plasma VEGF level in ARDS patients but intra-alveolar levels are unaffected.

DEDICATION

I would like to dedicate this thesis to my parents, Valerie and Richard Medford. I thank them for their enduring personal support that has allowed me to complete this work.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ALI	Acute lung injury
ALP	Alkaline phosphatase
AM	Alveolar macrophage
AN	Alveolar neutrophil
APACHE II	Acute Physiological and Chronic Health Evaluation II score
APACHE III	Acute Physiological and Chronic Health Evaluation III score
APII	APACHE II score
APIII	APACHE III score
AQP3	Aquaporin 3
AQP5	Aquaporin 5
ARDS	Acute respiratory distress syndrome
ATI	Alveolar type 1 epithelial cell
ATII	Alveolar type 2 epithelial cell
ATP	Adenosine triphosphate
B_2M	Beta-2-microglobulin
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BNP	Brain natriuretic peptide
BrdU	Bromodeoxyuridine
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CT scan	Computed tomographic scan
CXR	Chest radiograph
DAB	Diaminobenzidine tetrahydrochloride
dNTP	Deoxyribonucleoside triphosphate
DMSO	Dimethylsulphoxide
EDTA	Ethylenediamine-tetraacetic acid
ELF	Epithelial lining fluid

ELISA	Enzyme linked immunoabsorbent assay
eNOS	Endothelial nitric oxide synthase
FFPE	Formalin-fixed paraffin-embedded
FiO_2	Fractional inspired oxygen concentration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hanks balanced salt solution
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factor 1
HIF-2α	Hypoxia-inducible factor 2 alpha
H_2O_2	Hydrogen peroxide
HRE	Hypoxia response element
HUVECs	Human umbilical vein endothelial cells
ICU	Intensive Care Unit
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-1Ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
KCl	Potassium chloride
KGF	Keratinocyte growth factor
KH_2PO_4	Potassium di-hydrogen phosphate
LIS	Murray Lung Injury Score
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MIF	Macrophage inhibitory factor
MODS	Multiple organ Dysfunction Score

NaCl	Sodium chloride
Na_2HPO_4	Di-sodium hydrogen phosphate
NCS	Newborn calf serum
NO	Nitric oxide
NRP	Neuropilin
NRP-1	Neuropilin receptor 1
NRP-2	Neuropilin receptor 2
PAWP	Pulmonary artery wedge pressure
PBEF	Pre-B-cell colony-enhancing factor
PBM	Peripheral blood monocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEEP	Positive end expiratory pressure (cm H_20)
PKB	Protein kinase B
PKC	Protein kinase C
PlGF	Placenta growth factor
PaO_2:FiO_2	ratio of arterial oxygen tension (mmHg)/fractional inspired oxygen concentration
rpm	revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPS II	Simplified Acute Physiology Score II
SDS	Sodium docesulphate
sflt	Soluble VEGFR1
SOFA	Sepsis-related Organ Failure Assessment
SP-B	Surfactant protein B
SP-C	Surfactant protein C
TBE	Tris-borate ethylenediamine-tetraacetic acid
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor alpha
mTNF	Membranous tumour necrosis factor alpha
sTNF	Soluble tumour necrosis factor alpha

VC	Ventilated control
VEGF	Vascular endothelial growth factor
VEGFR1	VEGF receptor 1 (also known as *flt-1*)
VEGFR2	VEGF receptor 2 (also known as KDR or *flk-1*)
VEGFR3	VEGF receptor 3 (also known as *flt-4*)
vHL	von Hippel-Lindau
VILI	Ventilator-induced lung injury
VPF	Vascular permeability factor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

TABLE OF CONTENTS

CHAPTER 1:
INTRODUCTION AND BACKGROUND

This chapter will give an overview of acute respiratory distress syndrome (ARDS) with particular emphasis on its pathology and pathophysiology, demonstrating the importance of the alveolar-capillary membrane epithelium and also review the biology of vascular endothelial growth factor (VEGF).

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1.1 ACUTE RESPIRATORY DISTRESS SYNDROME

1.1.1 Historical overview

Ashbaugh and colleagues first described acute respiratory distress syndrome (ARDS) as a clinical syndrome in 1967[1]. Twelve patients had acute respiratory distress, cyanosis refractory to oxygen therapy, reduced lung compliance and diffuse chest radiograph infiltrates (see Figures 1.1, 1.2).

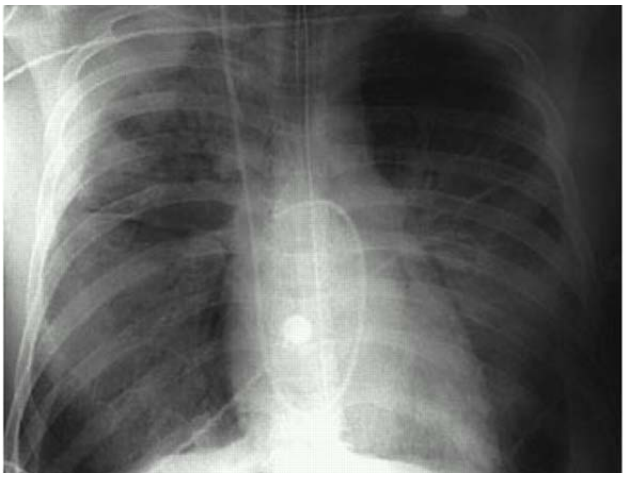


Figure 1.1: CXR of patient with early ARDS with typical bilateral infiltrates but normal heart size. Source: Ware LB et al.[2]

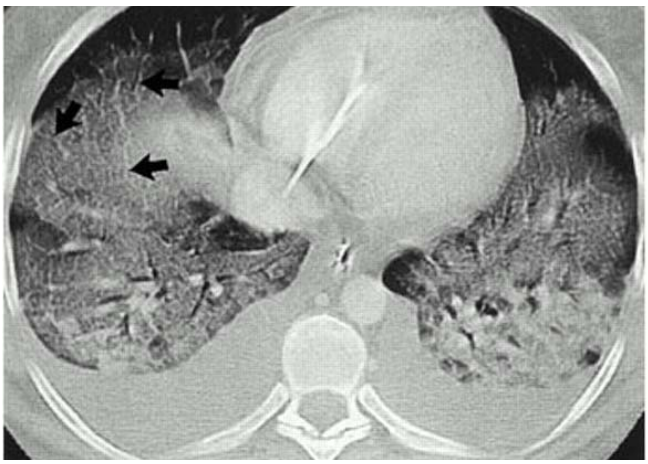


Figure 1.2: Computed tomographic (CT) scan of thorax in early ARDS showing typical bilateral infiltrates more prominent in dependent posterior zones, sparing the anterior zones. Source: Ware LB et al.[2]

These striking but uniform clinical, physiological, radiological and pathological findings distinguished them from 272 other ventilated intensive care unit (ICU) patients treated in Colorado General and Denver General Hospitals. The syndrome was initially called "Adult Respiratory Distress Syndrome" by Petty and Ashbaugh in 1971 as the pathological findings of the seven who died were almost identical to those in the infant respiratory distress syndrome with alveolar atelectasis, capillary engorgement and formation of hyaline membranes (see Figure 1.3)[3].

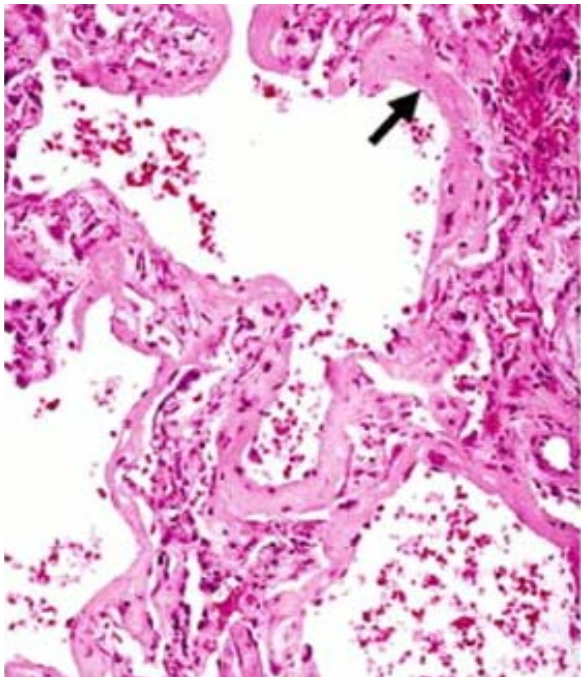


Figure 1.3: Histology of early ARDS showing early diffuse alveolar damage with neutrophilic alveolitis and intra-alveolar red cells with hyaline membranes (arrow). Source: Ware LB et al.[2]

The syndrome was subsequently renamed "Acute Respiratory Distress Syndrome" (ARDS) in recognition of the fact the same syndrome was also occurring in children (indeed, one of the original cohort in 1967 was aged 11 years old).

The syndrome is not new and a form of progressive pulmonary collapse was known to military surgeons during the First World War with pulmonary oedema noted following gunshot wounds to the head[4]. During the Second World War, later lung injury following trauma was described as "traumatic wet lung" which was observed following thoracic trauma, blast injuries, abdominal trauma and multiple fractures of long bones[5]. A pathological state "congestive atelectasis" was described in 1950 by Moon with airless, heavy lungs congested with debris and hyaline membrane formation lining the alveolar spaces at post mortem (reviewed in Petty et al[6]). The clinical and physiological features were not described at this time however.

In summary, it is evident that the pathophysiological complex we recognise as ARDS has many potential causes and has been known by other terms for a long time.

1.1.2 Definitions

ARDS has proved difficult to define because of its many causes and occurrence in a variety of clinical settings. The initial 1971 definition summarised the clinical features of ARDS well but lacked specific criteria to identify patients systematically. Most definitions since have been operational and rely upon three features of ARDS as shown in Table 1.1 below[6].

Criterion	Features
Oxygenation	Hypoxaemia and respiratory distress
CXR appearances	Diffuse alveolar infiltrates (see Figures 1.1, 1.2)
Compliance	Reduced respiratory system compliance

Table 1.1: Key features of ARDS

However problems remained: severity of hypoxaemia varied from one series to another; type and magnitude of infiltrates were seldom specified and compliance measurement required ventilation and was not readily reproducible.

In 1988, an expanded definition (the Murray Lung Injury Score, see section 2.1.3.1 and Table 2.4) was proposed that quantified the physiological respiratory impairment using a four-point lung injury scoring system based on four criteria shown in Table 1.2 below[7].

Criterion	Measurement
Respiratory support	Level of positive end-expiratory pressure (PEEP)
Oxygenation	Ratio of partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO_2:FiO_2)
Compliance	The static lung compliance
CXR appearances	The degree of CXR infiltrates

Table 1.2: Key features of Murray Lung Injury Score

This definition also included the presence or absence of non-pulmonary organ dysfunction and specified the clinical cause of lung injury. However, this scoring was not shown to be predictive of outcome in the first 72 hours and lacked specific criteria to exclude cardiogenic pulmonary oedema, a major differential[8]. Measurements of pulmonary artery wedge pressure (PAWP), have varied from less than 12 mmHg to less than 18 mmHg using such a definition[9 10].

1.1.3 1994 North American-European Consensus Statement

In 1994, the North American-European consensus conference, unified the definitions of acute lung injury (ALI) and ARDS, its most extreme form[11]. This definition was an attempt to standardise patient selection and allow direct comparisons of research into basic mechanisms and treatments for ARDS. ARDS (or ALI) was defined as shown in Table 1.3 below.

Criterion	Features
Time course	An acute process persisting for days or weeks
CXR appearances	Bilateral CXR infiltrates consistent with pulmonary oedema (see Figures 1.1, 1.2)
Clinical specificity	No clinical evidence of heart failure or absence of left atrial hypertension (verified by PAWP < 18 mmHg if necessary)
Oxygenation	PaO_2:FiO_2 < 200 mmHg for ARDS, PaO_2:FiO_2 < 300 mmHg for ALI

Table 1.3: 1994 North American Consensus Conference definition

The advantages of this definition are that it is simple to apply in the clinical setting, it recognises that there is a spectrum of lung injury severity and also attempts to exclude cardiogenic pulmonary oedema and chronic lung disease.

This definition is not without its limitations however and more appropriate definitions are awaited. The distinction between ALI and ARDS on the basis of worsened gas exchange does not reliably correlate with the underlying pathology. The degree of hypoxaemia does not predict survival. It takes no account of the amount of PEEP applied, which may profoundly affect oxygenation or lung compliance. For example, similar levels of oxygenation (and PaO_2:FiO_2) may be achieved with significantly different levels of PEEP that is not discriminated by this definition. Furthermore, it does not specify the cause of ARDS or take into consideration the different pathophysiology according to the site of the original injury, it does not consider the presence of multiorgan dysfunction (which does not always occur in ARDS, for example in chlorine inhalation or massive air embolism) and the radiographic findings are not specific and some data suggests that they are not applied consistently by observers[12].

It should be noted the definition does not demand the automatic measurement of PAWP but only as an aid to diagnosis, as PAWP can be commonly elevated over 18 mmHg in ARDS patients particularly if volume overloaded or with high intrathoracic pressures. A high PAWP does not exclude acute lung injury as such elevations may due to concomitant left ventricular dysfunction which occurs in up to 20% of ARDS patients[13]. If lung infiltrates and hypoxaemia fail to improve after normalisation of the PAWP, then there is likely to be an element of acute lung injury. However, myocardial ischaemia may lead to transient left ventricular dysfunction that may be missed if the PAWP is measured after resolution of the ischaemia so timing of the PAWP measurement is critical.

Measurement of brain natriuretic peptide (BNP) can be helpful if clinical evaluation does not exclude the possibility of cardiogenic pulmonary oedema. Plasma BNP < 100pg/ml (< 200mg/ml if glomerular filtration rate < 60ml/min) makes heart failure unlikely[12]. High levels do not always imply heart failure in critically ill patients as confirmed in an observational study showing high BNP levels did not discriminate between those with severe sepsis and those with acute heart failure[14].

Echocardiography may be helpful in discriminating (if clinical evaluation and plasma BNP are not helpful) particularly if there is demonstration of significant reduced left ventricular ejection fraction or severe aortic/mitral valve dysfunction to suggest heart failure. However, it may be difficult to exclude heart failure secondary to diastolic dysfunction, volume overload from acute renal failure and also milder left ventricular dysfunction secondary to ARDS (as described above in 20% of cases).

Other diagnostic techniques may help in the future. Increased microvascular permeability can be demonstrated at an early stage using gallium scintigraphy in ARDS to measure a gallium pulmonary leak index[15 16]. The practical value of this remains to be determined. Diffuse alveolar damage (see section 1.1.7.3, Chapter 1) can be detected early on BALF from cytological analysis[17]. Although BAL is usually well tolerated in ARDS, this technique may be limited by the lack of specificity of diffuse alveolar damage for ARDS, as it is well described in pulmonary fibrosis[18].

In summary, the 1994 definition could potentially be refined by including the level of PEEP, plasma BNP measurement (< 100pg/ml), echocardiography (absence of significant aortic or mitral valve dysfunction with reasonable ejection fraction) and pulmonary artery catheterisation take in context as necessary. This refinement should also incorporate the site of injury and aetiology, the presence of multiple organ dysfunction and a more specific definition of radiographic findings. Perhaps in the future, measurements of protein leak to document increased pulmonary vascular permeability or early bronchoscopic detection of diffuse alveolar damage may also accelerate the diagnosis and improve the specificity of this definition.

1.1.4 Epidemiology

The lack of a consistent definition of ARDS until more recently, and the myriad of causes and clinical features has hindered an accurate estimation of the true incidence of ARDS. Early estimates in North America were of an incidence of 75 per 100,000[19]. More recent studies in the UK and USA have suggested much lower incidences (1.5 to 4.5 per 100,000) but these were carried out before the 1994 consensus definition[20 21]. The first study using the 1994 consensus definition suggested a higher incidence of 13.5 per 100,000 in a Scandinavian population[22]. Experience of investigators screening in the ARDS Network Trial suggests the original estimate of 75 per 100,000 may be accurate[23]. Ongoing prospective studies using the 1994 consensus definition will clarify this issue.

The number of patients at risk of ARDS is unknown. The proportion of these that develop ARDS varies with the aetiology from 1.7% following cardiopulmonary bypass to 35.6% following aspiration in one study[9]. Prevalence studies in the ICU setting have revealed a 16-18% prevalence of ARDS in the critically ill[22 24]. Demographics suggest an acute inciting event triggers lung injury with a time course of usually less than 24 hours to injury following onset of the event[25].

1.1.5 Predisposing factors

The risk factors associated with the development of ARDS are either those causing direct injury to the lung (pneumonia, aspiration etc.) or those causing indirect injury in the setting of a systemic process via blood-borne systemic inflammatory mediators (sepsis, pancreatitis etc). Table 1.4 displays a list of predisposing factors associated with ARDS. Sepsis is the most associated factor (40% of cases) with development of ARDS and the presence of multiple predisposing factors substantially increases the risk[9 26].

Direct	Remote
Pneumonia	Sepsis
Aspiration	Pancreatitis
Drowning	Trauma/multiple fractures
Fat and amniotic fluid embolism	Burns
Lung contusion	Massive blood transfusion
Smoke and toxic gas inhalation	Leukoagglutin reaction (transfusion-related)
Alveolar haemorrhage	Diabetic ketoacidosis
Reperfusion (post embolectomy)	Bone marrow transplantation
Unilateral lung reimplantation	Drug overdose: aspirin, cocaine, opioids etc

Table 1.4: Abbreviated list of conditions associated with ARDS.

1.1.6 Outcome and prognosis

Most studies until recently have reported a mortality rate of 40-60%[27-30]. Death occurs mainly due to sepsis or multiorgan dysfunction and not respiratory failure[28]. However, the recent therapeutic success of low tidal volume ventilation in the ARDS Network Trial indicates some deaths are directly related to iatrogenic ventilator-induced lung injury[23]. Some data suggest mortality is falling. One North American study found a 36% mortality in 1993 compared to 53-68% in 1983-7[30]. A second study from the UK revealed a reduction from 66% mortality in 1990-3 to 34% in 1994-7[31]. Possible reasons for this include improved treatment of sepsis, better ventilation strategies and improved supportive care.

Deaths continue to occur months after discharge. 28 day mortality reflects the acute disease and treatment but does not represent long-term survival very well. 60 day mortality (as used in the ARDS Network Trial) is thought by many to be the optimal endpoint[23]. Number of ventilator-free days may also be a more useful endpoint in addition to 60 day mortality. Pulmonary function in most survivors returns to normal or near-normal levels and most reach their maximal recovery at 6 months[32 33]. Mild restriction, a reduction in diffusing capacity and expiratory flow, bronchial hyper-reactivity and air trapping can occur. A more recent study suggests many ARDS survivors have a persistent extrapulmonary functional disability 12 months after discharge mainly due to muscle wasting and weakness with minimal lung function changes[33].

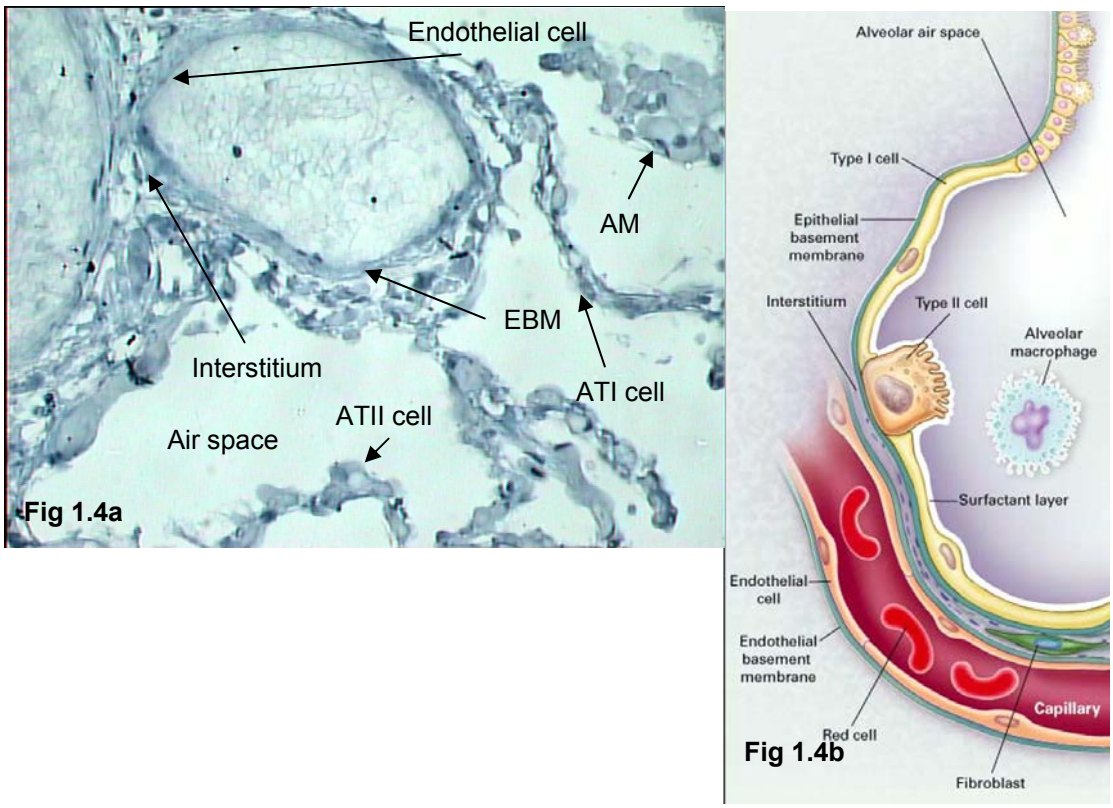
Prognostic factors predicting death at the time of diagnosis include chronic liver disease, non-pulmonary organ dysfunction, sepsis and age[8 22 34 35]. Oxygenation (P_aO_2/F_iO_2) is not a prognostic factor at onset of ARDS but 24-48 hours later predicts outcome[8 22 23 34 36 37].

To put ARDS mortality in context, it has been estimated from a mortality of 40% there are just under 16,500 deaths associated with ARDS annually in USA compared to 17,518 for emphysema and 16,516 HIV-related deaths[2]. Therefore, acute lung injury has significant implications for population health.

1.1.7 Pathogenesis and pathology

1.1.7.1 Alveolar epithelial cells – normal structure and function

In order to understand the pivotal importance of the alveolar epithelium in both pathogenesis and recovery from ARDS, it would be advantageous to briefly review the anatomy and physiology. The alveolar epithelium is composed of type I (ATI) and type II (ATII) cells (see Figures 1.4 a-b).



Figures 1.4a (x 40) and b: The normal alveolus, alveolar epithelial cells and alveolar-capillary membrane. Abbrevations: AM = alveolar macrophage, EBM = endothelial basement membrane. Source (fig 1.4b): Ware LB et al.[2]

ATI cells cover 93% of the alveolar surface, reflecting their ability to stretch into flattened cells with very little depth but with a large surface area to facilitate gas exchange[38]. ATI cells contain few organelles and also function in regulating water transport[39].

In contrast, ATII cells (see Figures 1.4 a-b) are cuboidal with microvilli and comprise only 7% of the alveolar surface[40]. They enable transepithelial transport of fluid and electrolytes and are the progenitor cells to ATI cells responsible for regeneration of ATI cells following lung injury[41]. Surfactant synthesis and secretion is a unique feature of ATII cells. This is essential to prevent airway collapse. They also have functions in coagulation/fibrinolysis and host defence[42]. Culture of ATII cells is complicated by the fact that they differentiate to an ATI cell phenotype after a few days[38 40 43]. Phenotypically (methods discussed in Chapter 2, see Figures 2.1, 2.2 and 2.3), they can be distinguished from ATI cells by their cuboidal shape; expression of alkaline phosphatase (ALP), surfactant protein C (SP-C) or aquaporin-3 (AQP3); absence of HTI_{56} or aquaporin-5 (AQP5), both markers mostly selective for ATI cells; and the presence of lamellar bodies at electron microscopy (see Figures 1.5, 1.6)[40 41 43-45]. AQP5 is expressed in other cells in the respiratory tract; airway smooth muscle and bronchial epithelium but not ATII cells[45 46].

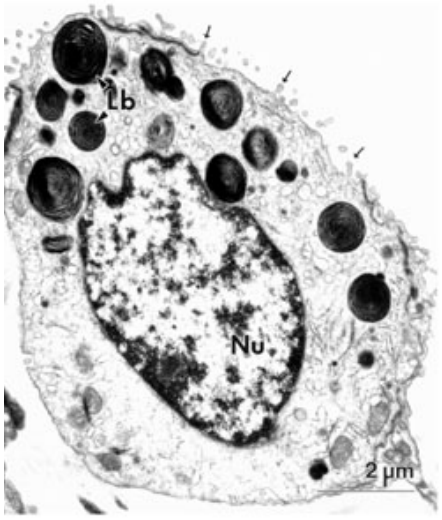


Figure 1.5: Transmission EM from ATII cell showing lamellar bodies (lb) and apical microvilli (arrows). Nu = nucleus. Source: Fehrenbach H et al.[42]

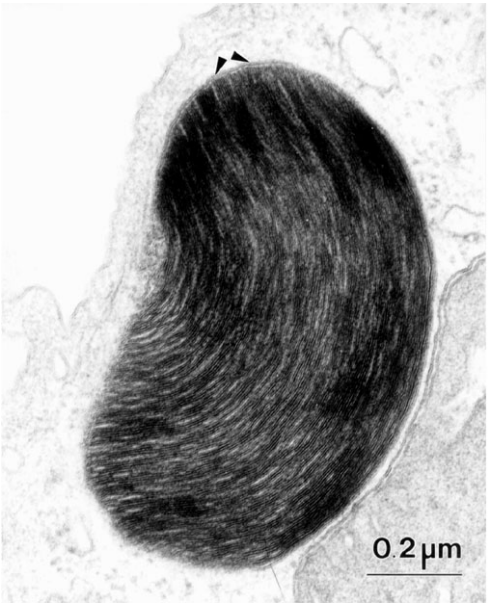


Figure 1.6: Transmission EM at higher power from ATII cell lamellar body showing densely packed phospholipid membranes bound by single limiting membrane. Source: Fehrenbach H et al.[42]

1.1.7.2 The lung as a target organ

The lung has certain unique features making it more susceptible as a target organ to the development of acute lung injury[47]. It is a highly vascular organ and the alveolar-capillary membrane (see Figure 1.4 a-b) is optimally designed to maximise gas exchange allowing extensive exposure to the outside environment. There are a myriad of intra-alveolar inflammatory cells including neutrophils, macrophages, epithelium and endothelium. Intrapulmonary inflammation and endothelial injury are key events in the development of ARDS.

It is important to be aware of the unique features of the alveolar-capillary bed that make it so susceptible to injury. Specifically, the alveolar-capillary bed is the main site of leucocyte migration in response to inflammation[48]. The lung architecture is also unique with oxygenated blood transported in the veins, a large neutrophil:pulmonary capillary size ratio (6-8μm versus 2-15μm) and close apposition of alveoli to the vascular bed; the distance for neutrophil migration is < 1μm from blood to air space. The alveolar surface area is estimated to be $100m^2$. Specifically, there is a complex network of short capillaries where the route from arteriole to venule crosses often greater than 8 alveolar walls containing more than 50 capillary segments. Compared to other vascular beds, there are many more lymphocytes and monocytes with about 50 times more neutrophils. The transit time for neutrophils through this bed is much longer than for red blood cells (6-26 seconds versus 1-4 seconds). The difference in transit times accounts for the increase in neutrophil concentration.

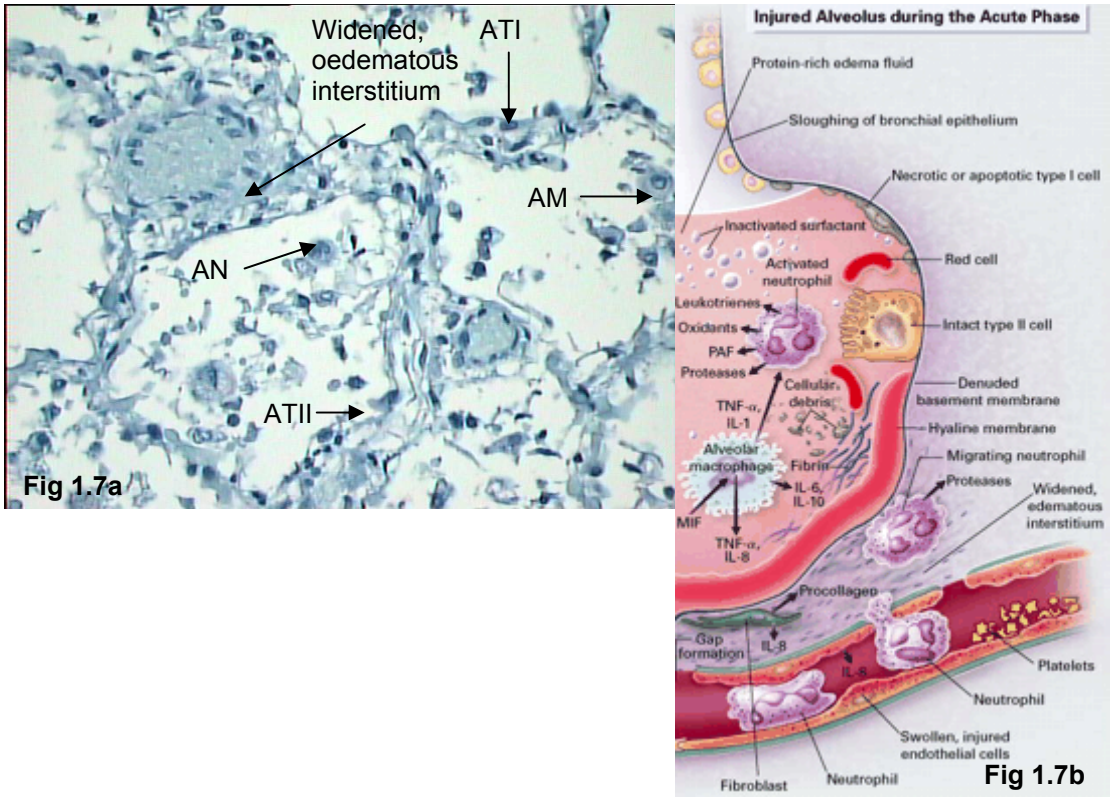
Other unique features to the lung are the different site of neutrophil migration ie) capillaries and not post-capillary venules as in the systemic vascular beds. Therefore, there is no need for tethering mechanisms in alveolar capillaries that are required to capture neutrophils across systemic post-capillary venules. The classical tethering, rolling and arrest of the neutrophil on inflamed endothelium is not applicable to the lung. Due to the high neutrophil:pulmonary capillary size ratio, about 50% of capillary segments require neutrophils to change their shape from spherical to "sausage-like" to pass. Changes in mechanical properties of neutrophils on activation during inflammation are pivotal in completely interrupting neutrophil transit. Other unique features of neutrophil migration in the lung remain to be explained. For example, 20% of neutrophil migration occurs through direct penetration of the endothelial cytoplasm.

Migration of neutrophils into the alveolar space can occur via β2-integrin dependent or independent mechanisms. Neutrophil-mediated proteolysis is not required for neutrophil migration in the lung. Although adhesion molecules are integral to neutrophil trafficking in the lung, physical factors are also important. Electron microscopy confirms fibrin strands at inflammatory sites emerging into the alveolar space, providing an adhesive substrate for migrating neutrophils and an "explosion" of neutrophils into the alveolus by a "pressure" mechanism from accumulating interstitial exudate.

1.1.7.3 Pathology of evolving ARDS

The pathology of ARDS progresses with time although pathological studies are largely derived from post mortem material that may not be entirely representative.

In the acute stage (see Figures 1.3, 1.7 a-b), (up to day 7 following onset of injury), there is an influx of protein-rich oedema fluid, neutrophils and other inflammatory cells and fibrin into the air spaces due to increased permeability of the alveolar-capillary barrier[49]. However, collagen deposition has also been shown to begin at an early stage (within 24 hours) in ARDS indicating fibroproliferation may occur earlier than previously thought and acute/chronic stages may be synchronous[50 51].



Figures 1.7a (x 40) and b: The injured alveolus in the acute phase of lung injury. Abbrevations: as in Figure 1.4a, also AN = alveolar neutrophils. Source (fig 1.7b): Ware LB et al.[2]

There may also be features of the initiating event eg) fat emboli, gastric aspiration. At the microscopic level, there is evidence of ATI cell injury with exposure of the underlying basement membrane[52]. ATII cells seem more resistant to injury[49]. Hyaline membrane formation (diffuse alveolar damage) occurs over the injured ATI cells (Figure 1.8) 48-72 hours after the insult described as diffuse alveolar damage[53] [54].

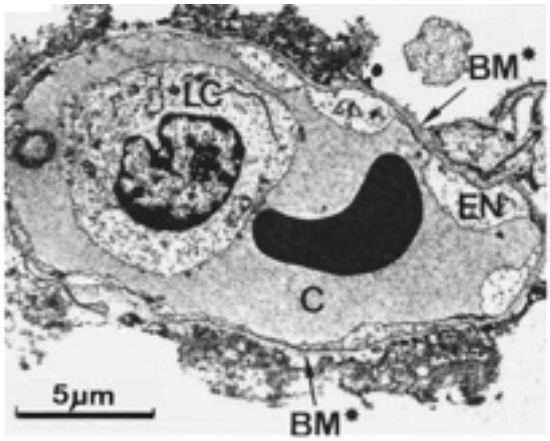
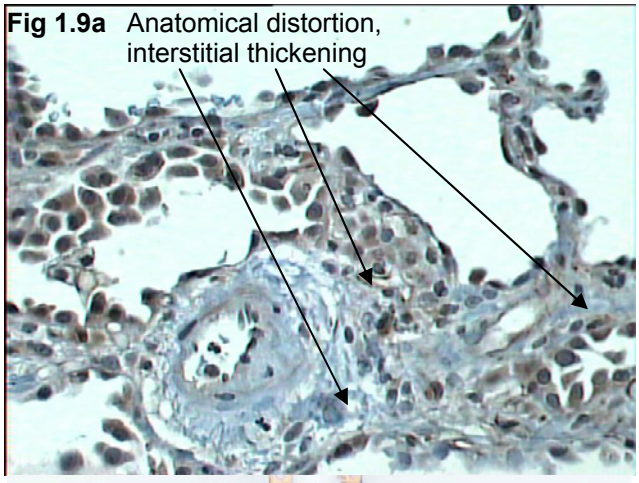
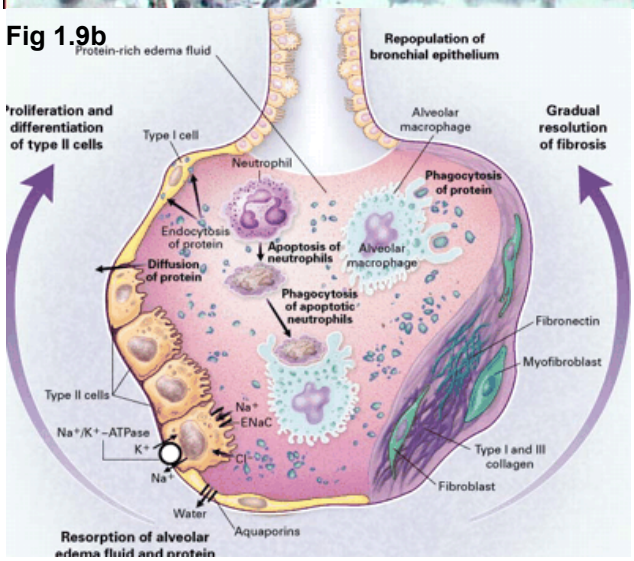


Figure 1.8: EM showing endothelial and epithelial injury. Swelling of the endothelium (EN) and intravascular neutrophil (LC) in capillary (C). Loss of alveolar epithelial cells with hyaline membrane deposition on basement membrane (BM*). Source: Ware LB et al.[2]

The chronic stage (after day 7) is variable. There may be rapid alveolar fluid clearance and epithelial repair followed by clinical recovery but in other cases oedema persists and intra-alveolar fibroproliferation occurs although this may often have started earlier as mentioned above (Figures 1.9 a-b, 1.10)[49-51].





Figures 1.9 a (x 40) and b: Histology of late stage of lung injury (fig 1.9a) and mechanisms important in either complete resolution or persistent fibrosis (fig 1.9b). NB: Staining on fig 1.9a DAB (brown) is for VEGF. Source (fig 1.9b): Ware LB et al.[2]

An organised exudate forms as myofibroblasts migrate from the injured epithelium into the fibrin-rich exudate. New blood vessels also fill the alveolar space[55]. Fibroproliferation (Figure 1.10) can progress to fibrosis distorting the lung architecture and impairing gas exchange (Figures 1.11a and b)[53].

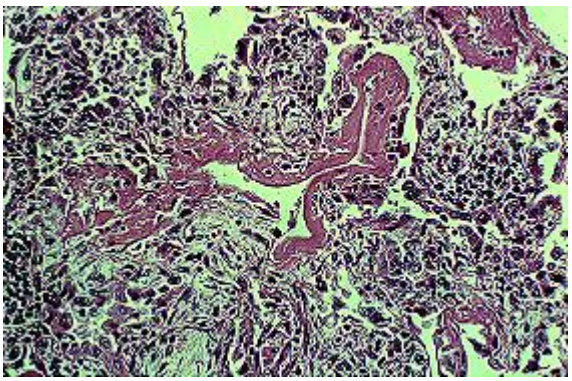


Figure 1.10: Collagen deposition in extracellular matrix of alveolar compartment, fibrosing alveolitis stage of ARDS. Source: Dr Nassif Ibrahim (see Acknowledgements).

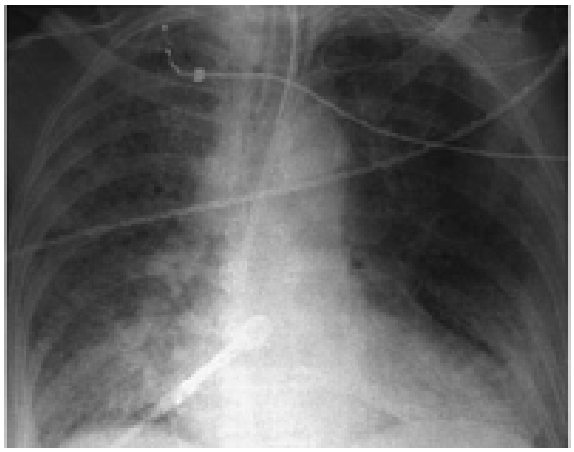


Figure 1.11a: Reticular opacities on CXR suggesting development of fibrosing alveolitis after day 7. Source: Ware LB et al.[2]

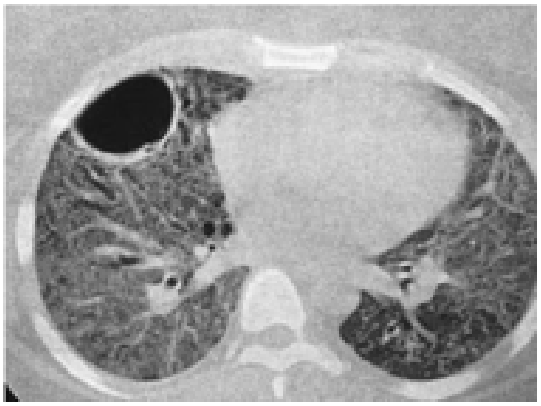


Figure 1.11b: Reticular opacities on CT with ground-glass shadowing during same phase as in Figure 1.11a. Source: Ware LB et al.[2]

Intrapulmonary levels of procollagen III peptide (a collagen precursor) are elevated very early after the onset of injury and is associated with increased risk of death[56 57]. Therefore, alveolar fluid clearance and regeneration of normal alveolar epithelium are critical to recovery from ARDS.

1.1.7.4 Endothelial and epithelial injury

Two separate barriers form the alveolar-capillary barrier, the microvascular endothelium and the alveolar epithelium (see Figure 1.4).

Increased vascular permeability and endothelial injury are established as being important in the development of pulmonary oedema in ARDS. Indeed, it has been suggested by some authors that ARDS is the pulmonary manifestation of a generalised endothelial injury ultimately leading to multi-organ failure[58]. Increased systemic levels of neutrophil elastase and L-selectin (involved in neutrophil activation and tissue migration) have been linked with the development of both ARDS and multi-organ failure[59 60].

The importance of epithelial injury has been recognised more recently although it has been documented in pathological studies over 25 years ago[61-63]. The degree of alveolar epithelial injury is an important predictor of outcome[64]. Plasma and ELF levels of KL-6 (an epithelial expressed glycoprotein) have been shown to be elevated in patients with ARDS and may also have prognostic value[65 66]. Indeed, non-survivors have higher plasma and ELF levels up to day 10 and day 2 respectively[65].

Alveolar epithelial injury can have serious consequences. It can result in increased permeability as the epithelial barrier is much less permeable than the endothelial barrier in normal circumstances and impaired removal of fluid from the alveolar space occurs[62 64 67 68]. It can also cause reduced surfactant turnover and production (also described in acute lung injury) and septic shock in patients with bacterial pneumonia[69-71]. Perhaps most importantly, it can result in translocation of biochemical mediators of fibrosis and lead to fibrosis from disorganised or insufficient repair especially if the injury is severe[56 72].

1.1.7.5 Neutrophil-dependent lung injury

Neutrophils accumulate early in the course of ARDS in lung specimens and predominate in BALF from ARDS patients[52 61 63]. Activation of neutrophils leads to generation of cytotoxic substances harmful to the alveolar epithelium including reactive oxygen species, proteolytic enzymes, eicosanoids and cationic peptides. In addition, other substances released may enhance the inflammatory response in the lung such as growth factors, cytokines and chemokines[2 73].

However, neutrophilic damage to the lung is not the only mechanism for development of ARDS as this has been described in patients with neutropaenia[74]. Moreover, granulocyte colony-stimulating factor (given to augment neutrophil numbers) in severe pneumonia patients does not increase the incidence or severity of lung injury[75]. Hence, neutrophil-independent mechanisms are also important in the development role of lung injury and neutrophils also have an important in host defence.

1.1.7.6 Cytokines

A complex pro-inflammatory cytokine cascade initiates and amplifies the inflammatory response to lung injury[76]. Inflammatory cells, alveolar epithelium and fibroblasts are all potential sources of such cytokines. Extrapulmonary factors can also regulate intrapulmonary cytokine production in ARDS such as macrophage inhibitory factor (MIF) increasing interleukin-8 (IL-8) and tumour necrosis factor alpha (TNF-α)[77]. The balance between pro- and anti-inflammatory mediators is also important as demonstrated in ARDS alveolar macrophages compared to "at risk"[78]. Several endogenous inhibitors have been described including interleukin-1-receptor antagonist (IL-1Ra), IL-8 autoantibodies, soluble tumour necrosis factor alpha (sTNF) and interleukin-10 (IL-10)[63 79].

1.1.7.7 Ventilator-induced lung injury

Mechanical ventilation of the lung can cause lung injury by two principal mechanisms and hence play a role in the pathogenesis of ARDS. Firstly, patients are subjected to high inspired oxygen concentrations which is toxic itself[80]. In rats, death occurs within 60-72 hours when exposed to greater than 95% oxygen[81].

Secondly, ventilation at high volumes and pressures can cause physical lung injury leading to increased oedema in the injured lung[82-84]. This is corroborated by the observed reduction in mortality (9% absolute reduction) with low tidal volume ventilation strategies in the ARDS Network Trial[23]. Mechanisms may involve alveolar over-distension and cyclical opening and closing of alveoli during ventilation[85-87]. Both mechanisms can lead to pro-inflammatory cytokine release and, moreover, a protective ventilation strategy can reduce both pulmonary and systemic cytokine responses[88 89]. A recent study highlights the importance of the pulmonary epithelium as a source of pro-inflammatory cytokines in this context[90].

1.1.7.8 Other mechanisms of injury

Acute lung injury involves a complex process with multiple pro- and anti-inflammatory pathways. Abnormalities of production, composition and function of surfactant may contribute to alveolar collapse and gas exchange abnormalities[71 91].

Pulmonary blood flow abnormalities and injury to the pulmonary vasculature related to micro- and macro-thrombosis are well described in ARDS[56 92-94]. A recent trial (PROWESS) has shown the benefits of activated protein C in severe sepsis syndrome, of potential relevance to acute lung injury[95].

1.1.8 Resolution

Alveolar oedema is resolved (Figure 1.9) by active transport of sodium and chloride into the interstitium[96]. Removal of oedema is associated with improved oxygenation, shorter ventilation and increased survival. Water follows passively via water channel proteins (aquaporins) predominantly expressed on AE cells[39 96]. Soluble protein is removed primarily by diffusion between alveolar epithelial cells. Insoluble protein is removed by endocytosis and transcytosis by alveolar epithelial cells and by phagocytosis by macrophages[97].

The ATII cell is required to proliferate and differentiate into ATI cells to reconstitute the alveolar epithelial barrier[42]. This covers the denuded basement membrane restoring the normal alveolar architecture increasing the fluid-transport capacity of the alveolar epithelium. Keratinocyte (KGF) and hepatocyte growth factors (HGF) and epithelial growth factors are known to be involved in this process.

The mechanisms underlying resolution remain poorly understood. Apoptosis may be important in neutrophil clearance in the injured lung. High intrapulmonary levels of apoptotic markers are present and broncho-alveolar lavage fluid (BAL) can cause epithelial cell apoptosis[98 99].

1.2 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

1.2.1 Background and biological actions

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a 34–46 kDa disulfide-linked dimeric glycoprotein and was first isolated from tumour cells[100 101]. VEGF and VPF were discovered from separate sources but were subsequently found to be identical by cDNA cloning and protein sequencing. It will be referred to as VEGF hereafter[102]. It is expressed by a variety of cell types including tumour cells, epithelial cells, macrophages, smooth muscle cells, neutrophils and platelets[103-106].

The key functions of VEGF are on the vascular bed. It is a potent endothelial cell mitogen *in vitro* and a potent angiogenic factor which has been demonstrated in a variety of *in vivo* models[100 107]. Embryos lacking a single VEGF allele have a lethal phenotype due to abnormal vascular development and lung architecture emphasising it is perhaps the most critical regulator of vascular development[108]. VEGF also increases microvascular permeability 50,000 times more potently than histamine[109]. It increases hydraulic conductivity in isolated microvessels mediated by calcium influx[110]. Permeability is increased via structural alterations in paracellular "tight" junctions and transcellular "caveolae"[111 112]. VEGF can induce fenestrations in some non-fenestrated vascular beds[113].

VEGF has other effects on the vascular bed. It is chemotactic for vascular endothelial cells and monocytes[114-116]. Studies also suggest it may act as a survival factor for vascular endothelium via its anti-apoptotic action and up-regulation of anti-apoptotic proteins Bcl-2 and A1[117 118]. It also has complex effects on coagulation including activation of procoagulant tissue factor[116 119].

VEGF has also been shown to be mitogenic on other cell types including human foetal pulmonary epithelial cells and neuronal cells[103 120 121]. Other studies have suggested an induction of collagen synthesis in human mesangial cells and osteoclast-mediated bone resorption and neurotrophic effects on neuronal cells[120 122].

Because of the importance of VEGF to angiogenesis and maintenance of the vascular bed, it is unsurprising that clinical trials of anti-VEGF therapy are in progress in solid tumours, and VEGF therapy in ischaemic vascular disease[123-127].

1.2.2 Why investigate VEGF in ARDS?

From the background on ARDS, it is clear that an increase (either functional or physical) in microvascular permeability (one of the main biological functions of VEGF) is one of the cardinal features of ARDS. Secondly, angiogenesis (another core function of VEGF) is a recognised histological feature in the lung following recovery from lung injury as it is in other forms of wound repair. Thirdly, high intrapulmonary concentrations of VEGF (approximately 10ng/ml) have been detected in normal human subjects, more than 500 times the levels in normal plasma although the reasons for this remain unclear at present as significant oedema and angiogenesis do not occur in healthy lungs[128]. Finally, it is evident that VEGF receptors are abundant in the vascular bed and VEGF itself is abundant in the lung and many of the mediators implicated in ARDS cause VEGF release.

These data have led to the hypothesis that VEGF has an important role in acute lung injury.

1.2.3 VEGF receptors

1.2.3.1 VEGF receptors 1 and 2 (VEGFR1, VEGFR2)

VEGF binds two related tyrosine receptor kinases, VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2). They have seven immunoglobulin-like domains with specific functions, a single transmembrane region and a consensus tyrosine kinase sequence interrupted by a kinase-insert domain (Figure 1.12)[129-131].

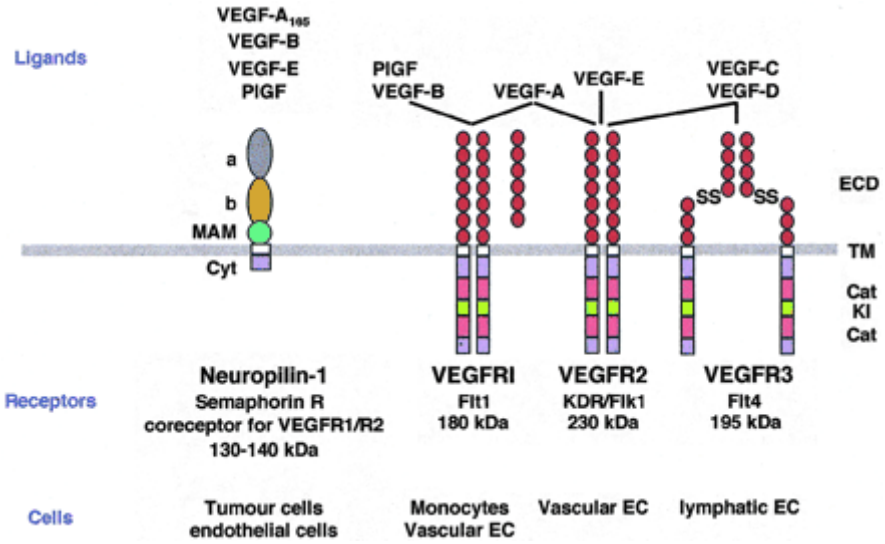


Figure 1.12: structure of VEGF receptors, main cellular sites and ligands. Source: Zachary I[132]. Cyt = cytoplasmic domain, PlGF = placental growth factor, ECD = extracellular domain. TM = transmembrane hydrophobic membrane-spanning domain. Red ovals denote Ig-like loops. Large cytoplasmic domain consists of catalytic domain (CAT) and non-catalytic kinase insert (KI).

VEGFR1 and VEGFR2 (the main receptors) were initially thought to be largely confined to vascular endothelium[133 134]. However, they have subsequently been detected elsewhere including activated macrophages, ATII cells and neutrophils[103 104 135-137]. VEGF is therefore capable of acting on both (epithelial and endothelial) sides of the alveolar-capillary membrane compatible with a significant biological function in the airspace in addition to its well-known effects on the vascular bed.

1.2.3.1.1 VEGFR1

Accumulating evidence supports a more inhibitory function for VEGFR1. VEGFR1 activation induces monocyte chemotaxis but not proliferation in cells lacking VEGFR2[114 138 139]. VEGFR1 knockout mice die between days 8.5 and 9.5 *in utero* from excessive proliferation of angioblasts supporting a negative regulatory role on VEGF by VEGFR1 at least during early development[140 141]. In addition, targeted deletion of the tyrosine kinase domain (but not the VEGF binding domain) on VEGFR1 does not cause death or obvious vascular defects[139]. Other studies have not confirmed a decoy function but suggested additional roles in haematopoiesis and release of tissue-specific growth factors eg) in the liver[142 143].

1.2.3.1.2 VEGFR2

Current evidence is consistent with VEGFR2 being the main signalling receptor for VEGF bioactivity ie) angiogenesis, proliferation and permeability[144 145]. Activation of VEGFR2 causes proliferation in cells lacking VEGFR1. VEGFR2 knockout mice fail to develop blood islands or organised blood vessels resulting in early death[146]. VEGFR2 also has a pro-survival function with anti-apoptotic effects on human umbilical vein endothelial cells (HUVECs)[147].

1.2.3.2 Other VEGF receptors

There are other important receptors activated by members of VEGF or its superfamily (see section 1.2.5). VEGF receptor 3 (VEGFR3) is not a receptor for VEGF itself but for VEGF-C and VEGF-D (see section 1.2.5) and is principally localised to lymphatic endothelium in adults[148]. These will not be discussed further in this thesis.

1.2.3.2.1 Neuropilin receptors (NRP-1, NRP-2)

Neuropilin receptors (NRP-1, NRP-2) are isoform-specific VEGF-binding sites of different size and affinity to VEGFR1 and VEGFR2[149 150]. They are expressed by endothelial cells in many adult tissues but lack the intracellular component containing tyrosine kinase activity and are regarded as VEGF co-receptors, being unable to signal themselves without the involvement of VEGFR2. They are known to bind semaphorins and are involved in neuronal guidance.

Studies are consistent with NRP-1 acting as a co-factor, augmenting the effects of VEGF (see Figure 1.12). It is isoform-specific, recognising exon 7 of VEGF (binding $VEGF_{165}$ but not $VEGF_{121}$, see section 1.2.4 later) and increases the effect of $VEGF_{165}$ by enhancing its binding to VEGFR2. This might partially explain the greater mitogenic potency of $VEGF_{165}$ compared to the $VEGF_{121}$ isoform. Studies also support a role for NRP-1 in vasculogenesis and angiogenesis. NRP-1 knockout and overexpressing mice both die prematurely from vascular defects[151 152].

NRP-2 also exhibits isoform-specificity and can form complexes with VEGFR1 in endothelial cells *in vitro*[153 154]. Unlike NRP-1, NRP-2 knockout mice have no vascular defects but some neuronal adverse effects consistent with a less important role in vascular development[155].

1.2.3.2.2 Soluble receptors

Soluble, truncated inhibitory forms of some of the receptors exist functioning as natural inhibitors. Isoforms of NRP-1 and NRP-2 exist generated by alternate splicing[156]. Soluble VEGFR1 (*sflt*) contains only the first 6 immunoglobulin-like domains and can also dimerise with VEGFR2 as well as bind soluble VEGF as efficiently as VEGFR1 reducing unbound VEGF levels[157]. Variation in soluble receptors may be one cause of regulation of free VEGF levels. One recent clinical study described a significant increase in intrapulmonary *sflt*, which was postulated to account for the apparent fall in free intrapulmonary VEGF levels in ARDS[158].

1.2.4 VEGF isoforms

The VEGF gene has been localised to chromosome 6 (6p21.3) with eight exons separated by seven introns[159] (see Figure 1.13).

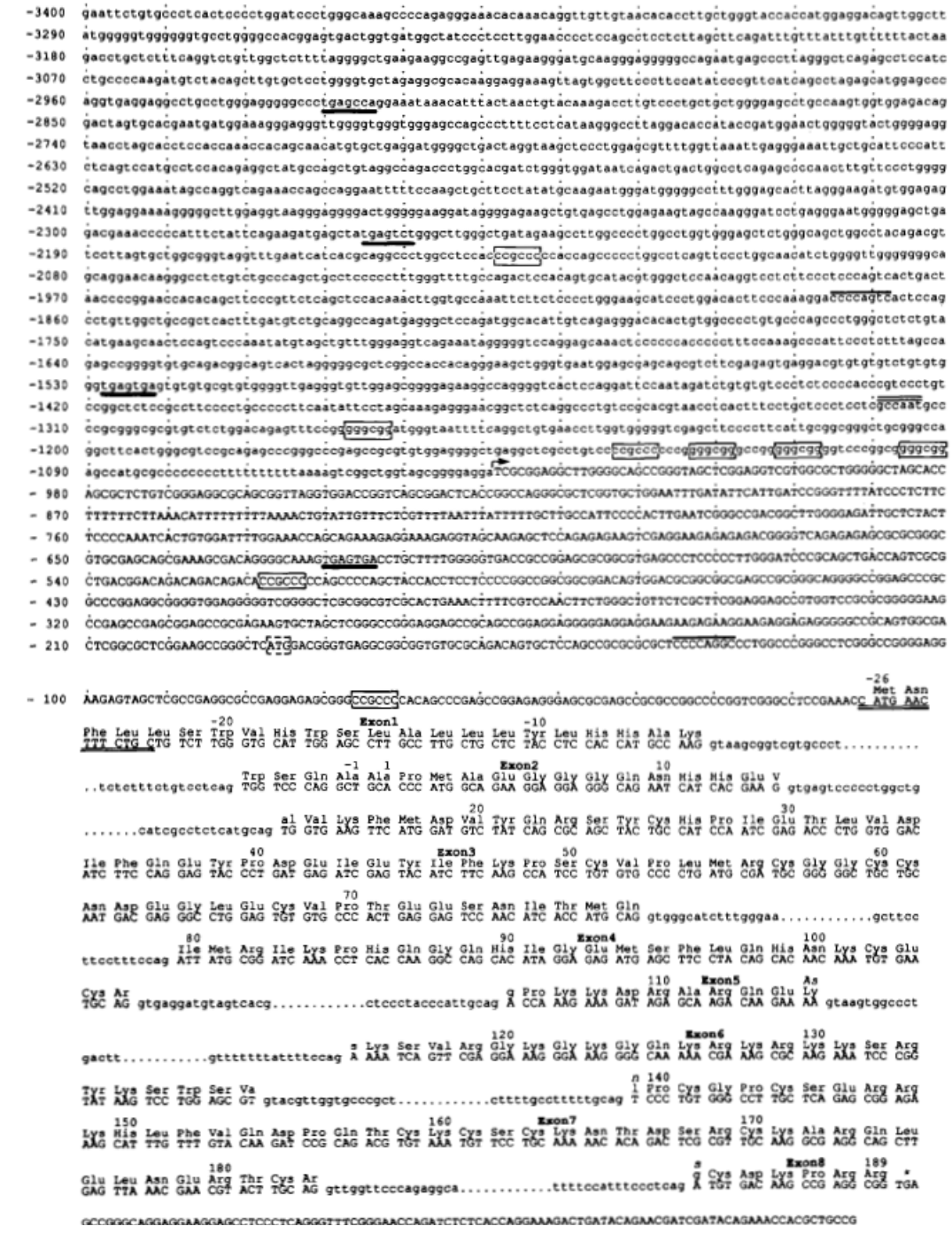


Figure 1.13: Partial nucleotide sequence of human VEGF gene. Upper case letters are exon nucleotides and lower case are intron or 5'-UTR nucleotides. Amino acids are numbered from alanine residue denoting amino terminus of protein. Nucleotides are numbered from translation start site. Boxes represent areas closely matching transcriptional control consensus sequences. Source: Tischer E et al[160].

Exon 1 encodes the signal sequence, exon 2 the N terminus, and exon 3 the dimerisation domain. VEGF dimerisation is necessary for biological activity[161]. Exon 3 encodes the VEGFR1 binding domain, exon 4 the VEGFR2 binding domain and the terminal part of exon 7 encodes the NRP binding domain (illustrated in Figure 1.14). N-glycosylation occurs in exon 3 (Aspartic acid, 74) which is a post-translational effect required for effective VEGF protein secretion but has no effect on VEGF function[162 163].

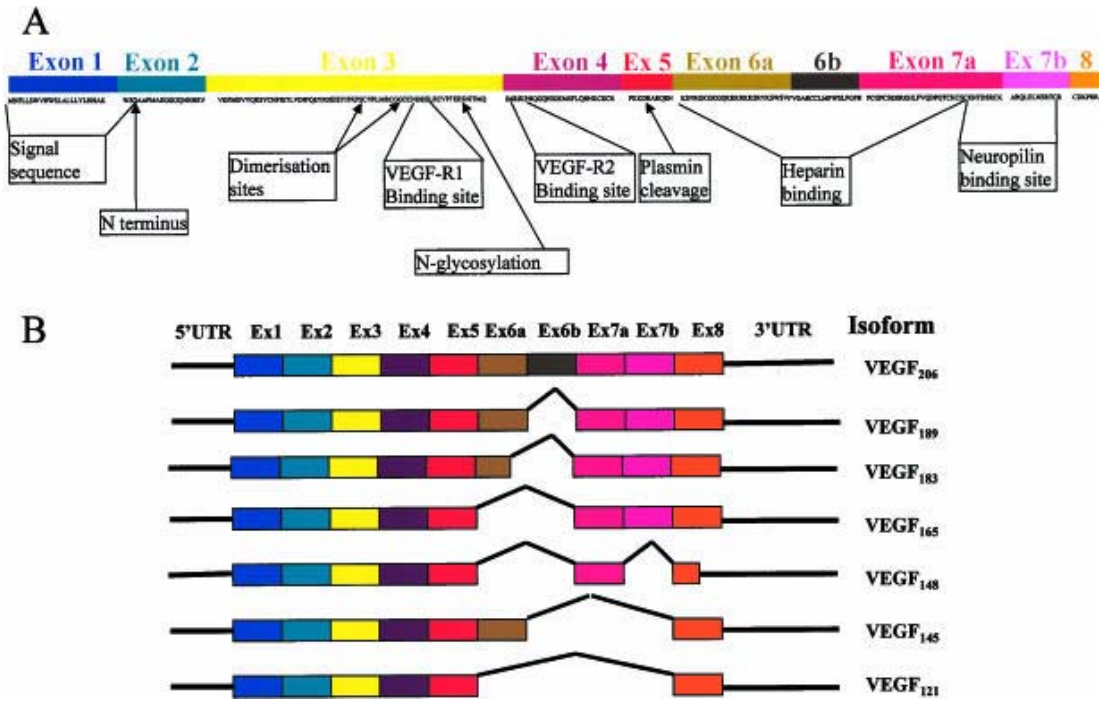


Figure 1.14 A: Exon structure of VEGF mRNA. Amino acid sequence corresponding to mRNA sequence given underneath. Functional domains shown. B: Schematic of VEGF isoform protein structure with different properties arising from differential presence of the heparin-binding functional domain (exon 6 and 7). NB: Promoter region in 5'-UTR and VEGF 936 C/T polymorphism (see Chapter 5) in 3'-UTR region. Source: Bates DO et al.[164]

Alternative splicing of the VEGF transcript from exons 5 to 8 leads to the generation of several different isoforms with variable diffusibilities depending on their length (Figure 1.14): $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$[160 165]. Exon 6 (not present in $VEGF_{121}$ and $VEGF_{165}$) and exon 7 provide heparin-binding affinity; exon 8 (present in all active isoforms) is necessary for the stimulation of mitosis. A plasmin cleavage site is present near the end of exon 5 resulting in a 110 amino acid peptide ($VEGF_{110}$) when VEGF is cleaved by plasmin, which loses the ability to stimulate mitosis. Therefore, the mitotic ability resides in the carboxyl terminus of VEGF. There are four cysteine bonds in the $VEGF_{165}$ and $VEGF_{189}$ that provide the VEGF dimers with their characteristic shape.

1.2.4.1 Main VEGF isoforms

The longer isoforms are highly basic and remain virtually completely cell-associated whereas $VEGF_{121}$ (lacking both exons 6 and 7) is freely diffusible[160 166]. $VEGF_{165}$ (lacking exon 6 but not 7) possesses intermediary properties being largely soluble but a distinct fraction remains cell-associated[167]. It is the predominant isoform and most biologically active in the physiological state[168].

As already discussed, the carboxyl-terminal heparin-binding domain (111-165) is thought to be critical for mitogenic potency, and $VEGF_{121}$ is not as mitogenic as $VEGF_{165}$[169]. $VEGF_{189}$ seems to be less active than $VEGF_{165}$ or $VEGF_{121}$ *in vivo* in a model of glioblastoma clones overexpressing the VEGF isoforms implanted into mouse brains[170]. The $VEGF_{189}$-overexpressing clones resulted in negligible haemorrhage compared to the other two isoforms and less angiogenesis[170]. The heparin-binding isoforms appear to have a more significant role in vascular development as mice expressing only the murine equivalent of $VEGF_{121}$ ($VEGF_{120}$) die shortly after delivery because of circulatory problems (with impaired lung microvascular development and airspace maturation) whereas those expressing the longer isoforms live for 2 weeks[171-173]. In addition to their variable diffusibilities and functional effects, there is evidence of variability in VEGF receptor activation; for example, NRP-1 is activated by $VEGF_{165}$ but not $VEGF_{121}$[174].

1.2.4.2 Rarer VEGF isoforms

More recent studies have identified other rarer $VEGF_{145}$, $VEGF_{148}$ and $VEGF_{183}$ isoforms (see Figure 1.14) in cell lines, human glomerular tissue and human Müller cells[175-177]. $VEGF_{145}$ is unusual in binding very well to the extracellular matrix and seems to be largely confined to reproductive tissues[175 178 179]. As discussed, plasmin, can also cleave the cell-associated isoforms to $VEGF_{110}$ [157 168]. Both $VEGF_{110}$ and $VEGF_{148}$ lack exon 8 explaining their reduced potency compared to $VEGF_{165}$.

1.2.4.3 Novel inhibitory isoform ($VEGF_{165}b$)

Since this research was started a novel inhibitory isoform (see Figure 1.15) has been identified, $VEGF_{165}b$[180 181].

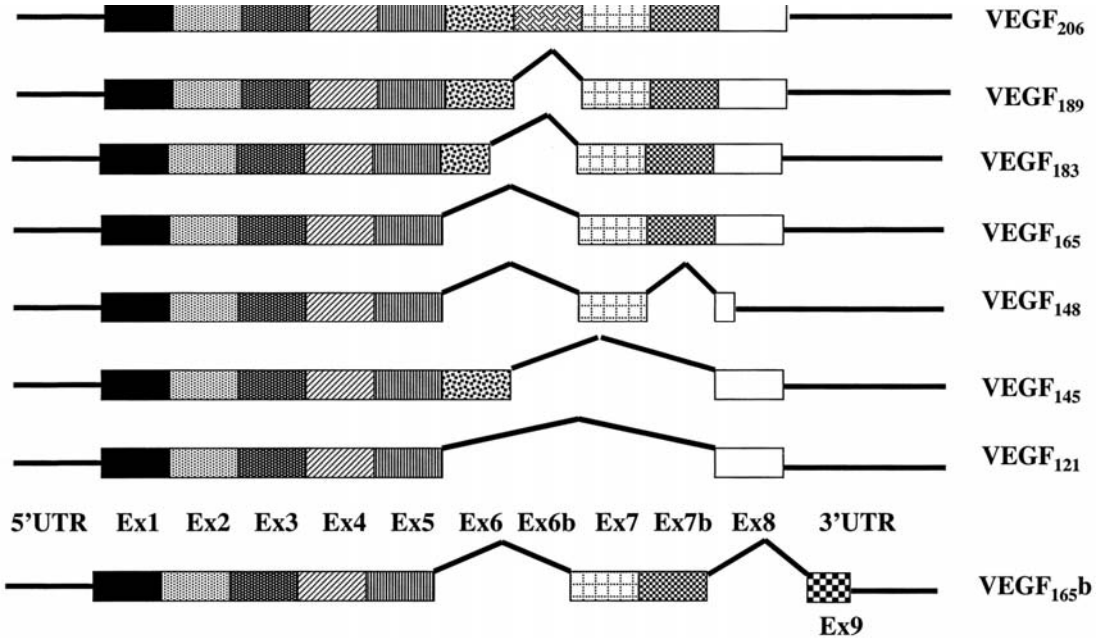


Figure 1.15: Exon structure of $VEGF_{165}b$ mRNA (bottom of figure) as compared to "normal" VEGF isoform structure. Source: Bates DO et al.[180]

Conditioned medium containing this isoform significantly inhibits $VEGF_{165}$-mediated vascular endothelial proliferation, migration and vasodilatation when co-incubated with $VEGF_{165}$ raising the possibility of an anti-angiogenic isoform. The transcript is identical to $VEGF_{165}$ except for the replacement of exon 8 with exon 9 containing an alternative 6 amino acids (Figure 1.16).

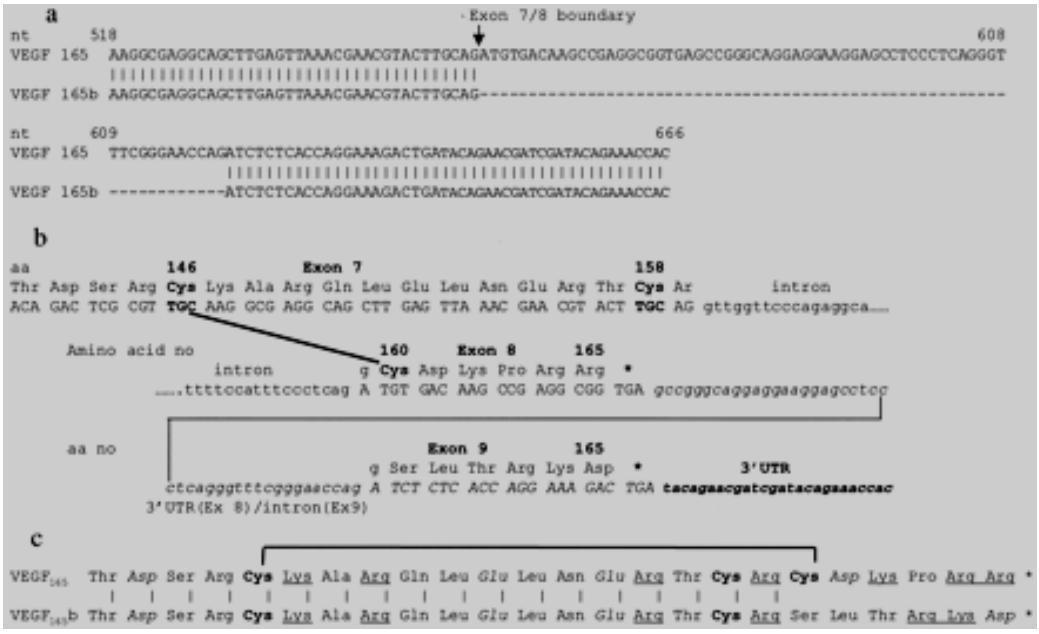


Figure 1.16: A: Nucleotide sequence of $VEGF_{165}$ and $VEGF_{165}b$ cDNA. 66 base pairs downstream of exon 7 missing from $VEGF_{165}b$. B: exon structure of carboxyl terminal end of $VEGF_{165}$ and $VEGF_{165}b$. The 3'UTR sequence of exon 8 contains an intronic sequence for exon 9. C: Predicted amino acid sequence of $VEGF_{165}$ compared with $VEGF_{165}b$. Italics denote acidic and underline basic residues. Source: Bates DO et al.[180]

In summary, the relative proportion of isoforms may have profound functional effects on VEGF biology. Therefore, the regulation of isoform switching may be critical in determining its functional importance in the lung.

The loss of two positively-charged terminal amino acids and the loss of Cys_{160} residue (resulting in loss of a disulphide bond with Cys_{146}, see Figure 1.16 and hence lack of proximity to the carboxyl terminus to the VEGFR2 binding site in exon 4) leads to a significant change in folding and tertiary structure thought to affect VEGFR2 signalling (thought to be the main signalling receptor[162 182]).

1.2.5 VEGF superfamily

A superfamily of VEGF-related proteins exists with similar structure and function: VEGF-B, -C, -D, -E and placental growth factor (PlGF) (see Figure 1.17)[168]. This thesis will focus on the role of VEGF-A (termed VEGF throughout this thesis). Isoforms are described for the other superfamily members (see Figure 1.17), but their genes are located on different chromosomes (see Table 1.5).

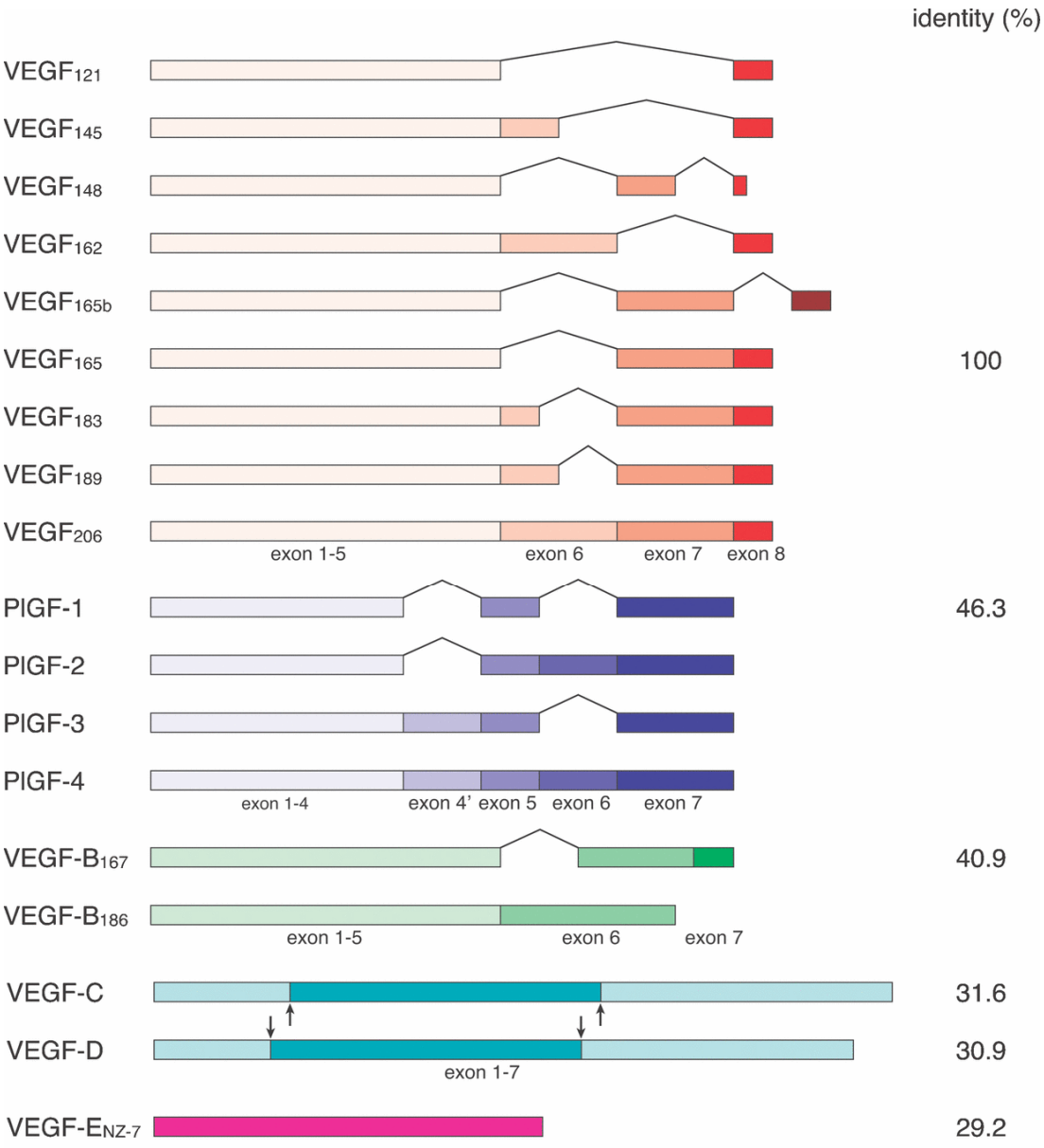


Figure 1.17: Comparison of structure of VEGF superfamily. Percentages on right indicate amino acid homology with $VEGF_{165}$. PlGF = placental growth factor. Arrows denote areas of proteolytic cleavage for VEGF-C and VEGF-D. Source: Takahashi H et al[183].

Specific antibodies to the VEGF superfamily members (with the exception of $VEGF_{165}b$, discussed in section 6.2.1, Chapter 6) are available. These antibodies are specific in their binding (with the exception of VEGF-A antibody which will detect $VEGF_{165}b$ in addition to the three VEGF isoforms, as discussed above). The detailed methodology of the antibodies used is discussed in section 2.3.3, Chapter 2 but the essential differences between the other superfamily antibodies are summarised in Table 1.5.

Antibody	% AA homology with $VEGF_{165}$	Binding area	Detects	Datasheet (Santa Cruz code)
VEGF-A	100	6p21.2: (N)	$VEGF_{121,165,189}$ isoforms	http://datasheets.scbt.com/sc-152.pdf (sc-152)
VEGF-B	41	11q13: AA 1-70 (N)	VEGF-B isoforms	http://datasheets.scbt.com/sc-13083.pdf (sc-13083)
VEGF-C	32	4q34: AA 203-410 (C)	VEGF-C isoforms	http://datasheets.scbt.com/sc-20714.pdf (sc-20714)
VEGF-D	31	Xp22.1: AA 211-354 (C)	VEGF-D isoforms	http://datasheets.scbt.com/sc-13085.pdf (sc-13085)
PIGF	46	14q24.3: AA 51-100	PIGF isoforms	http://datasheets.scbt.com/sc-20714.pdf (sc-20714)

Table 1.5: Specific characteristics of VEGF superfamily antibodies (no specific VEGF-E antibody available). All are raised in rabbit species and polyclonal. AA = amino acid, N = N terminus, C = C terminus.

VEGF-C is a specific ligand for VEGFR3, but can also activate VEGFR2 with an estimated mitogenic potency 100 times less than VEGF itself. Its primary role is thought to be in lymphangiogenesis[184]. Indeed, it is expressed largely near VEGFR3 on lymphatic endothelium after development[185] [186]. VEGF-D transcripts are expressed in lung as well as other organs and it is thought to have similar roles in lymphatic vessel development and also vascular development having many structural similarities to VEGF-C[187]. VEGF-E has similar structure to $VEGF_{121}$ but superior mitogenic activity almost equal to $VEGF_{165}$[188]. It activates only VEGFR2. PlGF binds to VEGF receptor 1 (VEGFR1) and has significant homology with VEGF, although with weaker mitogenic activity than VEGF itself[189] [190]. It can be mitogenic on human umbilical vein endothelial cells in the form of a dimer with VEGF (HUVECs)[191].

In addition to the VEGF superfamily, there are possibly a further family of tissue-specific VEGFs (most recently, the endocrine gland derived VEGF (Eg-VEGF)[192]. It has identical biological function to VEGF although structurally not related but pronounced target-cell selectivity. In addition, there are numerous other growth factors with angiogenic (basic fibroblast growth factor and the angiopoeitin family) beyond the scope of this thesis[193] [194].

In summary, VEGF is only one of many members with similar (but in general less potent) functional properties in a superfamily. The functions of the other members of the VEGF superfamily are poorly understood but this demonstrates the further complexity of VEGF biology.

1.2.6 Regulation of VEGF expression

Any change in VEGF receptor expression, soluble receptors or alternate splicing to change the relative isoform expression would theoretically alter VEGF bioactivity. However, there are some important regulators of VEGF expression independent of such changes of relevance to the normal and injured lung that will be discussed further.

1.2.6.1 Regulation of VEGF expression by hypoxia

Oxygen is a key regulator of VEGF bioactivity, hypoxia being a potent stimulus to VEGF expression. In ARDS, tissue hypoxia is well described and high flow oxygen is often used therefore there may be a relationship between these factors and VEGF bioactivity[2]. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric protein regulated by removal of the HIF-1 alpha subunit through ubiquination and proteosomal destruction in normoxic conditions[195]. Hypoxia inhibits HIF-1 ubiquination and allows binding of HIF-1 to a hypoxia response element (HRE) upstream of the VEGF gene in promoter, enhancer and intronic sequences increasing VEGF transcription[196 197]. Hypoxia also increases VEGF mRNA stability[198 199].

Oxygen also regulates VEGF receptor expression. Hypoxia upregulates VEGFR1 and VEGFR2 expression *in vivo*[200]. VEGFR1 has a HIF-1 consensus sequence in its promoter region, whereas VEGFR2 does not and is thought to be upregulated by post-transcriptional paracrine mechanisms[201 202].

1.2.6.2 Other mechanisms of regulation

Aside from oxygen-related regulatory mechanisms, other oxygen-independent mechanisms exist. In ARDS, intrapulmonary increases in pro-inflammatory cytokines such as TNF-α and LPS are well-described[2]. Both of these cytokines can upregulate VEGF expression although these findings were described outside the air space in mononuclear and endothelial cells[203 204]. More recent data has described similar findings for LPS in an alveolar epithelial cell line, A549 cells[205]. TNF-α may also converge with the VEGF signalling pathway (discussed in section 1.2.7).

Importantly, VEGF can regulate its own activity by direct regulation of its receptors, at least in the vascular bed. VEGF activation of VEGFR2 increases VEGFR2 gene expression and cellular levels and can also upregulate VEGFR1 and *sflt* expression in endothelial cells[206 207].

Mechanical stretch may be important, particularly relevant in the context of mechanical ventilation and ventilator-induced lung injury[208]. Pulmonary ischaemia in the presence of normoxia in ventilated ferret lungs is associated with an increase in HIF-1 alpha mRNA but not protein[209].

Other mechanisms exist but are not discussed in further detail and they are not relevant to the lung eg) tumour suppressor gene inactivation which is relevant to non-necrotic vascular tumours[210].

1.2.7 VEGF signalling

The complexity of VEGF signalling pathways is illustrated in Figure 1.18. VEGF appears to be able to stimulate almost all known signalling pathways in endothelial cells in culture. 46 different signalling molecules have been identified as being activated by VEGF in endothelial cells in one review including phospholipases C and A, thromboxane A2, protein kinases B and C PKB, PKC), PI3 kinase, nitric oxide (NO) and mitogen-activated protein kinase (MAPK)[211]. This does not take account of other signalling molecules in target cells other than endothelium.

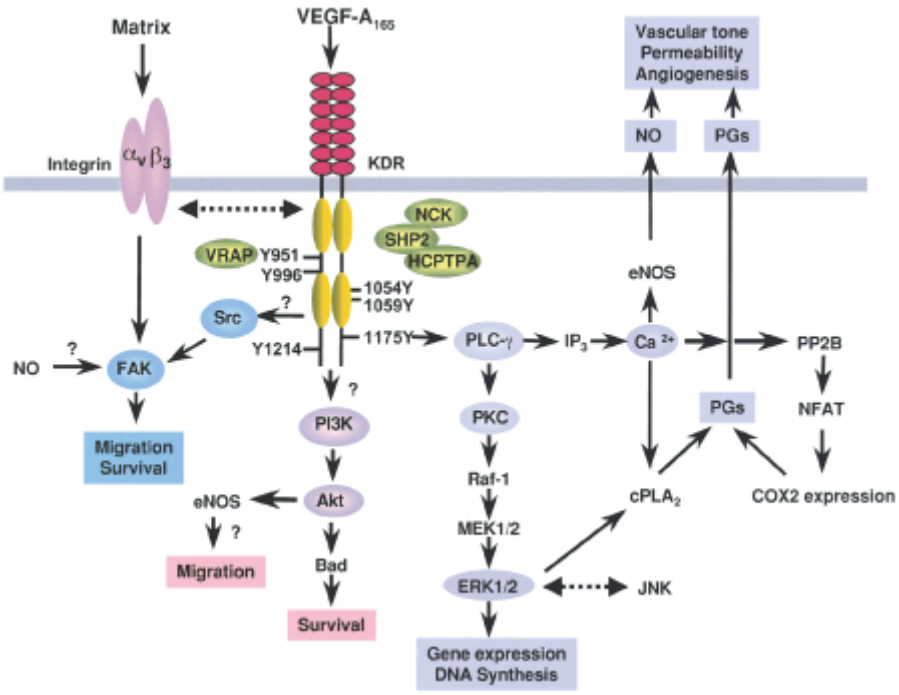


Figure 1.18: Schematic representation of VEGF signalling included illustrating its complexity. VEGFR2 (KDR) is the main signalling receptor. Source: Zachary I et al.[212]

There is growing evidence that the signalling pathways for VEGF induced permeability and angiogenesis diverge. Inhibition of p38 MAPK enhances VEGF-mediated angiogenesis but inhibits VEGF-mediated permeability on endothelial cells *in vitro*[213 214]. Furthermore, PKC inhibition prevents VEGF-induced proliferation and angiogenesis *in vitro* but induces permeability *in vivo* in endothelial cells and mice[215].

In endothelial cells, mTNF seems necessary for VEGF-mediated permeability but not angiogenesis *in vitro* and *in vivo*[214]. Upregulated mTNF leads to continuous p38 MAPK activation in endothelial cells and selective inhibition of mTNF (rather than sTNF) and/or p38 MAPK inhibits VEGF-mediated permeability *in vitro*. mTNF and VEGF signalling pathways may converge at the level of p38 MAPK, with mTNF having a permissive role in VEGF-mediated permeability (Figure 1.19). Indeed, TNF-α knockout mice do not have abnormal vasculature unlike their VEGF counterparts[216]. Inhibition of p38 MAPK is also associated with prevention of endothelial cell apoptosis *in vitro* following VEGFR2 activation suggesting MAPK may be implicated in other VEGF functions[217].

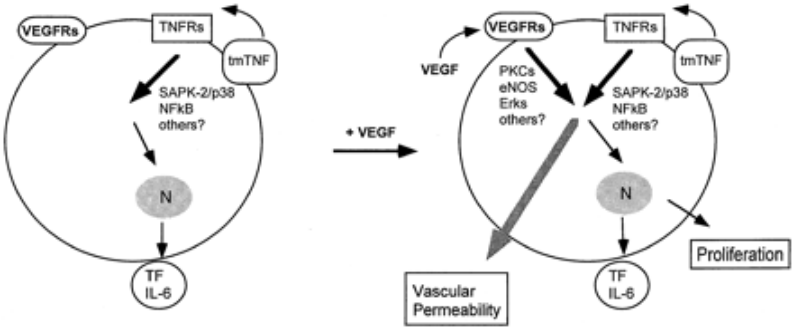


Figure 1.19: Potential convergence of continuous TNF and VEGF signalling pathways leading to vascular permeability. Source: Clauss M et al.[214]

1.2.8 VEGF Polymorphisms

Significant interindividual variations in plasma VEGF level and gene expression have been reported[105 218 219]. Several functional VEGF polymorphisms have been described recently. Given its possible role in ARDS, such polymorphisms (although there are other explanations) are attractive candidates to influence individual susceptibility (which is known to be highly variable) to developing ARDS from a given "insult" eg) an individual will have an estimated 40-60% risk of developing acute lung injury following Gram negative sepsis[9]. VEGF is among those genes with a potential role in ARDS that is polymorphic[220-222].

1.2.8.1 VEGF +936 C/T polymorphism

A CT substitution at position 936 distal to the start of translation in the 3'-untranslated region of the VEGF gene on chromosome 6 is associated with reduced plasma levels in both heterozygotes and homozygotes[220]. This substitution results in altered binding of the transcription factor activator protein 4 (AP-4) although whether the abolishment of the AP-4 binding site is of any relevance to the reduction in VEGF protein expression remains unclear at present[220]. AP-4 is known to enhance expression of some viral genes by binding to their enhancer sites e.g. SV-40[223]. However, the relation between this polymorphism and intrapulmonary inflammatory cell and epithelial cell production of VEGF is unknown at present. Another group failed to find a significant difference in serum level with genotype for the same polymorphism however this was a non-Caucasian population[224].

In other lung diseases, no association has been found between the same polymorphism and susceptibility to COPD although it has been implicated with altered susceptibility to sarcoidosis[225 226]. There are a growing number of studies confirming associations with extrapulmonary diseases, either inflammatory or neoplastic but these are not discussed further here[227-231].

1.2.8.2 Other functional VEGF polymorphisms

Other functional VEGF polymorphisms currently under investigation in this context include –634 G/C in the 5'UTR (associated with reduced VEGF production from peripheral blood monocytes (PBMs) stimulated with lipopolysaccharide[222]). Conversely, the same Japanese group found significantly higher serum levels with the same polymorphism[224]. The –1154 G/A and –2578 C/A polymorphisms in the promoter region are associated with higher VEGF production from stimulated PBMs[232]. Individuals with the A allele also have an 18 nucleotide insertion at –2549 Individuals without the insertion have an almost doubling of transcriptional activity[233]. Such polymorphisms have been extensively studied in the context of extrapulmonary disease and are not considered further here as they have yet to be investigated in lung disease[232-236].

1.2.9 VEGF and the lung

A variety of *in vitro*, animal and human studies demonstrate the functional importance and abundance of VEGF in the normal lung. Studies in ALI need to consider both sides of the alveolar-capillary membrane. Isolated cellular studies looking at epithelial or microvascular endothelial cells give additional insight to animal models and clinical studies as discussed below.

1.2.9.1 VEGF and the alveolar space

In vitro studies have demonstrated an abundance of VEGF in lung tissue, especially in alveolar epithelium including A549 cell-line and primary human cultured type II pneumocytes[205 237-239]. Indeed, the highest levels of VEGF mRNA are found in animal and human lung suggesting that alveolar epithelium is the predominant source[205 237]. Data from animal and human studies confirm these findings. Although the embryonic role of VEGF is well recognised, adult murine lungs contain a significant amount of VEGF mRNA transcript[240]. In normal human lung air spaces, VEGF is compartmentalized (measured in fluid obtained at bronchoalveolar lavage) to 500 times higher than those in the plasma[128].

Human foetal airway epithelial (ATII) cells are known to express VEGF receptor 2 (VEGFR2), the main functioning VEGF receptor, which would facilitate an autocrine role in the air space for VEGF in addition to its well-known paracrine effects on the vascular bed[103 241]. Such an autocrine role has been described on other specialised epithelial cells outside the lung, in the kidney[106].

In summary, high levels of VEGF exist in normal human lung despite the lack of angiogenesis, oedema or excess microvascular permeability occurring in the physiological state. Receptors are expressed in the airspace compatible with a biological (but as yet unclarified) role for VEGF in the uninjured state.

1.2.9.1.1 Studies suggesting pathological role for VEGF in alveolar space

Theories that VEGF is solely pathological in the lung causing excess vascular permeability do not account for these findings. Nevertheless, a wealth of data is consistent with a pathological role of VEGF in the lung. A549 cells (although not entirely typical of primary human alveolar epithelial cells) release VEGF in response to pro-inflammatory stimuli potentially involved in ARDS, including LPS, neutrophil elastase and KGF[205]. In one LPS-induced murine model of lung injury, intrapulmonary levels of VEGF increased following injury for 96 hours mirroring an increase in bronchoalveolar lavage fluid protein and neutrophils, with significant VEGF localisation to lung epithelium[242]. In an acid-induced murine model of lung injury, high tidal (injurious) volume ventilation strategies increase lung VEGFR2 (the main signalling receptor for VEGF bioactivity) protein expression consistent with a possible role in ventilator-induced lung injury[243]. On the vascular side of the alveolar-capillary membrane, clinical studies confirm an elevation in plasma VEGF in early ARDS (the first 24 hours) with normalisation of levels in recovery (after day 4) in survivors but no non-survivors. A significant expression from peripheral blood monocytes *in vitro* was noted with a 48% reduction in albumin flux across human pulmonary endothelial cell monolayers using the soluble VEGFR1, *sflt*[244]. Such data are observational and do not imply causation. Significantly, a protective ventilatory strategy failed to reduce VEGF expression in the murine acid model, suggesting the VEGF response may be secondary to more critical events[243].

More compelling data come from animal models focussed on the alveolar side of the ACM where VEGF over-expression appears to be directly harmful. Adenoviral delivery of $VEGF_{165}$ to the murine lung leads to non-cardiogenic pulmonary oedema and increased pulmonary capillary permeability on the basis of histology, lung weight wet/dry ratios, elevated albumin permeability and Evans blue dye assay although viral-induced damage to the alveolar-capillary membrane may have contributed[245]**.** Overexpression of $VEGF_{164}$ in neonatal transgenic mice respiratory epithelial cells leads to pulmonary haemorrhage, endothelial destruction and alveolar remodelling in an emphysema-like phenotype[246]. The emphysema-like changes may be secondary to the vascular disruption, as surfactant protein B (SP-B) production was not affected[246].

These data are consistent with a possible pathological role for VEGF in lung injury. However, many of the data are observational which may indicate secondary responses to more critical events. In addition, the overexpression models may have involved damage to the alveolar-capillary membrane exposing the underlying endothelium to the higher physiological levels of VEGF in the air space leading to excessive oedema. Moreover, these data do not account for the consistent findings of abundant VEGF in the normal lung. In this regard, a growing body of evidence is consistent with a protective role for VEGF in the lung.

1.2.9.1.2 Studies suggesting protective role for VEGF in alveolar space

To date, observational studies in humans of lung injury consistently show a reduction in intrapulmonary VEGF levels in the early stages of ARDS[247 248]. This is consistent with a hyperoxic lung injury model in rabbits, where alveolar epithelial expression of VEGF was reduced[135]. Several other investigators have found similar reductions in intrapulmonary VEGF levels in other forms of lung injury including high altitude pulmonary oedema in adults, bronchopulmonary dysplasia, persistent pulmonary hypertension of the newborn, idiopathic pulmonary fibrosis, smokers or even in the early stages after lung transplantation[248-253]. Recovery of intrapulmonary VEGF levels to pre-injury levels has been noted following recovery from both acute lung injury in humans and the hyperoxic rabbit model and high altitude pulmonary oedema as well as later post transplant[247-249 253].

Considerable evidence suggests VEGF acts as an alveolar epithelial mitogen and stimulant. Exogenous VEGF acts as a growth factor on human foetal and neonatal murine pulmonary epithelial cells and is capable of restoring A549 cell proliferation after exposure of the cells to acid injury[103 254 255]. HIF-2α–deficient foetal mice (with consequent fatal respiratory distress syndrome in neonatal mice due to impaired surfactant production by type 2 pneumocytes) have lower intrapulmonary VEGF levels and expression on type 2 pneumocytes[254]. Intrauterine or postnatal intratracheal delivery to the neonatal mice of $VEGF_{165}$ protected against developing respiratory distress syndrome and increased surfactant production[254].

Further intervention studies interrupting VEGF signalling in some way lend further support to a protective role in the lung on the alveolar side. Chronic VEGFR2 blockade (given subcutaneously) in rats leads to alveolar apoptosis and emphysema[256]. In addition, lung-targeted ablation of the VEGF gene by adenoviral delivery in the adult mouse leads to a persistent emphysema phenotype for at least 8 weeks. Interestingly no inflammation or proliferation but increased apoptosis is seen in these lungs[257]. VEGF partially mediates the protective effects of IL-13 in a murine hyperoxic model of lung injury with transgenic overexpressing IL-13 mice[258]. The beneficial effect of IL-13 is significantly abrogated by VEGF blockade and IL-13 selectively upregulates the murine $VEGF_{164}$ isoform in normoxia but the other $VEGF_{120}$ and $VEGF_{188}$ isoforms in hyperoxia. The exact mechanisms of the VEGF-mediated protection are not fully understood.

These data suggest possible survival function for VEGF in the alveolar space. VEGF may have a pneumotrophic function and be an autocrine epithelial growth factor in the lung. It is already known to be a survival factor for the vascular bed via induction of anti-apoptotic proteins but to date this has not been demonstrated in alveolar epithelium[118].

1.2.9.1.3 Conflicting data and possible reasons

As stated previously, some of the data are conflicting and other data does not conclusively corroborate the above hypothesis. In one cellular study, $VEGF_{164}$ failed to show a proliferative effect on rodent primary ATII cells. This may be related to the doses used, the species differences, the methodology of thymidine and bromodeoxyuridine (BrdU) incorporation and it should be noted a rise in SP-B transcription was detected[259].

VEGFR2 is the main signalling receptor for VEGF but little evidence is available to evaluate its role in acute lung injury[241]. Abadie et al.[260] using homogenates from early and late human necropsy or surgical open biopsy ARDS lung tissue demonstrated no difference in VEGFR2 expression compared to controls. However, ATII cell proliferation and VEGFR2 expression were noted in injured tissue and these data do not exclude a protective role for VEGF.

Abadie et al.[260] in the same study using the same tissue failed to find any change in lung tissue homogenate VEGF levels following the early phase of injury in lung homogenates on immunohistochemistry but this may be due to different time points being used. In another observational clinical study, Maitre et al.[249] failed to find a difference in serum VEGF levels in ARDS in contrast to the elevation of plasma VEGF levels noted by Thickett et al[244]. This is surprising as serum VEGF levels would be expected to be at least two times higher than plasma levels due to *ex vivo* platelet and neutrophil release[105]. However, methodology might again account for this. ELISA antibodies and control group severity were different (higher index of oxygenation in the latter study control group) to other observational clinical studies.

In summary, the current data appear conflicting but it is speculated there is a common underlying mechanism. Whilst it is possible the changes in VEGF level during injury and recovery may reflect changes secondary to repair and regeneration of the injured alveolar epithelium, time points of the animal models and clinical studies differed as did the degree of endothelial injury in the over expression animal models which might partially account for the apparently conflicting data. Changes in splice variant and soluble receptor expression may also contribute although published data are limited. This leads to more specific hypothesis; that in ARDS, alveolar-capillary membrane damage may contribute to oedema resulting from exposure to the underlying endothelium to higher (usually intrapulmonary) concentrations of VEGF but that paradoxically, VEGF acts as an alveolar epithelial mitogen facilitating resolution of oedema following recovery from acute lung injury once the alveolar-capillary membrane is restored.

1.2.9.2 Effects on pulmonary vasculature

Pulmonary hypertension occurs in ARDS as well as endothelial injury and increased microvascular permeability[2]. Studies looking at the effect of VEGF on the endothelial side of the alveolar-capillary membrane are limited. VEGF is known to reduce trans-endothelial resistance of bovine lung microvascular endothelial cells for less than 60 minutes at concentrations less than 10ng/ml and stimulate endothelial cell chemotaxis maximally at 10ng/ml[261]. Most published studies have looked at the vasculature overall and potential development of pulmonary hypertension rather than at microvascular level.

VEGF may indeed have protective effects on the pulmonary vasculature[262 263]. In an ovine model of chronic intrauterine pulmonary hypertension, whole lung VEGF protein expression is down-regulated, with reduced VEGF expression in the airway epithelium, vascular endothelial and smooth muscle cells on immunohistochemistry[264]. Indeed, transgenic mice overexpressing TNF-alpha (lung or systemic) display features of pulmonary hypertension associated with reduced VEGF and VEGFR2 mRNA expression[265].

Other data conflicts. VEGF–overexpressing transgenic mice develop an abnormal vasculature and lethal phenotype indicating the need for tight regulation of this molecule[266]. Hypoxia in rats and guinea pigs is associated with elevated VEGF mRNA and protein in pulmonary arteries as well as pulmonary hypertension[267 268]. In rats, mean pulmonary artery pressure correlated with the degree of hypoxia, vascular remodelling and VEGF levels in the pulmonary arteries[268].

The above studies are observational and do not imply causation. Interventional studies favour a protective role against pulmonary hypertension. Administration of VEGFR tyrosine kinase inhibitor in newborn rats leads to pulmonary hypertension and abnormal lung structure[269]. Administration of a specific $VEGF_{165}$ inhibitor leads to more significant features of pulmonary hypertension histologically and haemodynamically associated with decreased endothelial nitric oxide synthase (eNOS) expression[264]. Furthermore, adenovirally-delivered $VEGF_{165}$ protects against hypoxic pulmonary hypertension in rats possibly via increased endothelial nitric oxide (NO) production[270]. Treatment of newborn rats with a VEGFR2 inhibitor decreases arterial density and vascular growth as well as alveolarization[271].

In summary, interventional studies suggest that VEGF may have a role in preventing pulmonary hypertension. The apparent conflicting observational data may indicate that hypoxia is simply an over-riding regulatory factor to VEGF bioactivity even if pulmonary hypertension has developed.

1.2.10 VEGF in other lung disease

Further information as to the role of VEGF in the normal human lung comes from studies in other lung disease, which often (although not always) correlate with findings from studies in ALI.

As in ALI, reduced intrapulmonary (airway or BAL) VEGF levels seem to be associated with alveolar epithelial destruction and emphysema. As discussed previously, animal studies have shown VEGF receptor blockade leads to a model of non-inflammatory emphysema[256 272]. In human studies, sputum, BALF and whole lung tissue VEGF levels are reduced in emphysema although the question remains whether this is a secondary phenomenon related to alveolar epithelial damage or a primary event[273-276]. Similar findings have been noted for whole lung VEGFR2 levels[273]. *In situ* hybridisation suggests that the principal reduction in VEGF and VEGFR2 may be in alveolar epithelial cells[273]. Reduced BALF VEGF levels also occur in idiopathic pulmonary fibrosis (IPF), smokers with normal lung function and elderly normal subjects, where alveolar epithelial injury occurs[252 277].

As in ALI, recovery from other injuries is associated with recovery of intrapulmonary VEGF levels. BALF VEGF levels rise following successful recovery from lung transplantation[253]. Similar findings to ALI occur in the vascular bed with elevation of VEGF levels during the acute phase. Elevated serum VEGF levels occur in cystic fibrosis (CF) and decrease following treatment of CF exacerbations and extra thoracic sarcoidosis[277-279].

In contrast to ALI, intrapulmonary VEGF levels are elevated in inflammatory and neoplastic diseases[280 281]. Elevated pleural fluid VEGF levels occur in empyema, parapneumonic and malignant effusions[282]. Elevated sputum VEGF levels occur in chronic bronchitis and asthma with an inverse correlation with FEV_1 suggesting an association with severity of airway inflammation[275 283]. *In situ* hybridisation in human asthmatic biopsies shows upregulated VEGF, VEGFR1 and VEGFR2 mRNA expression on a variety of cell types[284]. Elevated BALF VEGF levels have also been described in acute eosinophilic pneumonia[285]. Elevated lung tissue VEGF levels correlate with poor differentiation of non-small cell lung cancer histologically[286].

1.2.11 Preliminary conclusions: injurious or protective?

In conclusion, the normal human lung contains significant amounts of the angiogenic factor VEGF, without significant angiogenesis. Published data conflicts. Many studies have suggested that VEGF may contribute to the development of non-cardiogenic pulmonary oedema. Other data has proposed a more protective role in the alveolar epithelium following injury. Differences in the degree of endothelial injury may explain the conflicting data. It is speculated that it functions as a pneumotrophic factor behaving as an autocrine factor facilitating repair following injury but that when it is disrupted, tissue damage occurs with oedema across the exposed endothelium and emphysema.

Therefore, VEGF in the lung may be a paradox: protecting and regenerating the epithelial surface yet contributing to the generation of pulmonary oedema across the underlying endothelium if disruption of the alveolar-capillary membrane occurs as in ARDS.

Anti-VEGF therapy is already under investigation for lung cancer, vascular disease, pulmonary hypertension and chronic inflammatory diseases[287]. In the long term, therapy modulating the VEGF system may be of more value with better understanding of its role in the lung, and the regulatory mechanisms influencing VEGF bioactivity including changes in splice variants, transcription factors, pro-inflammatory cytokines, and soluble receptors.

1.2.12 Thesis hypothesis

VEGF has an important role in recovery and repair in ARDS

1.2.12.1 Thesis aims

In order to investigate this hypothesis, I have considered the following questions:

1. Whole lung VEGF receptor and isoform expression:
   - What is the anatomical localisation of VEGF receptors in normal and injured human lung?
   - Is there a change in VEGF receptor expression in normal, early and later ARDS lung?
   - What is the VEGF isoform expression in normal and injured human lung?
   - Are there any changes in absolute or proportionate isoform expression?

2. Human ATII cells:
   - Do human ATII cells express significant amounts of VEGF and is it altered in response to LPS?
   - Is VEGF a mitogen for human ATII cells?
   - Is human ATII cell expression of VEGF isoforms and its receptors altered by pro-inflammatory cytokines?

3. VEGF +936 C/T polymorphism:
   - Does this polymorphism influence susceptibility to the development of or severity of ARDS?
   - What is the relationship between the polymorphic genotype and plasma and BAL VEGF levels in patients at risk or with ARDS?

CHAPTER 2:
MATERIALS AND METHODS

This chapter will describe the techniques used in this thesis and will directly refer to the results chapters 3 to 6. It will also describe the source and nature of the materials used as well as details surrounding classification and risk stratification of patients. Specific details regarding the generation of solutions, oligonucleotide sequences, PCR reaction conditions and ELISA standard curves are situated in the appendices for ease of reference.

The ATII cell extraction technique used in this chapter, (sections 2.3.4 -2.3.7)has been published in another study in Am J Resp Cell Mol Biol.

Armstrong L, Medford ARL, Uppington KM, Robertson J, Witherden IR, Tetley TD, Millar AB.

Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells.

Am J Resp Cell Mol Biol 2004 31(2): 241-5.

2.1 Subjects

2.1.1 Ethics and consent

The North Bristol NHS Trust Local Research Ethics Committee approved all studies including the use of anonymised archival lung tissue (see section 2.2.1). Patients were fully consented before lung resection. ITU patients had bronchoscopy as part of routine clinical practice. For normal and ventilated control patients, informed consent was obtained before venepuncture or bronchoscopy. For sedated ventilated patients, consent was given by the senior physician in charge of the ITU where it was felt that bronchoscopy was in the clinical interests of the patient as a means of detecting nosocomial infection, removal of secretions, mucus plugs or foreign bodies or to aid in other diagnoses.

Normal subjects of good health were recruited by internal and external advertisement in the University of Bristol campus. Exclusion criteria were: significant past medical history of systemic disease (especially chronic lung disease, cancer, vascular disease or chronic inflammatory disease), non-Caucasian ethnicity, a smoking history, age greater than 30 years and not an undergraduate. The rationale for these exclusion criteria comes from observational studies that have noted changes in intra-alveolar VEGF levels with smoking, lung disease, inflammatory diseases, cancer, and increasing age (discussed in sections 1.2.9.1.2 and 1.2.10, Chapter 1). In addition, in any genetic study reducing ethnic variation minimises a further source of confounding (discussed in section 5.4.2, Chapter 5).

It should be noted the 2004 Human Tissue Act does have implications for future research using archival tissue such as that described in sections 2.2.1 but at the time of full ethical approval and even after completion of the relevant experiments, the Act was not in place. Therefore, the anonymised archival lung tissue experiments were conducted with full ethical approval at the time (pre-2004) and did not contravene any such Act.

2.1.2 Definitions

2.1.2.1 ARDS and ALI

As described in section 1.1.3 and Table 1.3 in Chapter 1, ARDS and ALI were defined using the 1994 American-European Consensus Conference definition as summarised in Table 2.1[11].

Criterion	Features
Time course	An acute process persisting for days or weeks
CXR appearances	Bilateral CXR infiltrates consistent with pulmonary oedema
Clinical specificity	No clinical evidence of heart failure or absence of left atrial hypertension (verified by PAWP < 18 mmHg if necessary)
Oxygenation	PaO_2:FiO_2 < 200 mmHg for ARDS, PaO_2:FiO_2 < 300 mmHg for ALI

Table 2.1: 1994 North American Consensus Conference definition

2.1.2.2 "At risk"

As per previous studies in the literature, "at risk" patients (as quoted in Chapter 5) were strictly defined[98]. "At risk" patients were ventilated and had similar degrees of physiological disturbance but without acute lung injury, ie) no CXR infiltrates and PaO_2:FiO_2 > 300mmHg.

Patients with suspected sepsis were considered to be "at risk" of ARDS if they fulfilled the criteria shown in Table 2.2.

Primary criteria (≥2 of the following)		Secondary criteria (≥2 of the following)	
Temperature	> 39°C or < 36°C	**Systemic vascular resistance**	< 800 dyn·s/cm^5
White cell count	< 4,000/dl or > 14,000/dl	**Blood pressure**	unexplained hypotension (SBP < 90 mm Hg for > 60 minutes)
Blood culture	a positive blood culture	**Acid-base status**	Ongoing metabolic acidosis with anion gap > 20 mEq/l
Clinical suspicion	strongly suspected source of infection	**Inotropes**	inotrope use to achieve SBP > 90 mm Hg
		Platelet count	< 81,000/dl

Table 2.2: "At risk" definition in patients with sepsis

Patients requiring massive transfusion were considered to be "at risk" of ARDS if intubated or on mask CPAP requiring > 15 units in 24 hours. Patients with acute pancreatitis were considered to be "at risk" of ARDS if displaying similar physiological features to those with sepsis described above except without a suspected source of infection or positive blood culture. Patients with inhalational injuries were considered to be "at risk" of ARDS if intubated or on mask CPAP.

Some "at risk" patients had elevated lung injury scores but no CXR changes and were therefore not able to be included in the "acute lung injury" or ARDS group. Several studies have identified differing levels of circulating cytokines according to the site of the initiating insult. In this thesis, primary (direct) and secondary (remote) lung injury are classified as in Table 2.3.

Primary (direct)	Secondary (remote)
Community acquired pneumonia	Extrapulmonary sepsis (intra-abdominal, vertebral, intracranial, cellulitis, endocarditis, unexplained bacteraemia)
Hospital acquired pneumonia	Pancreatitis
Ventilator-associated pneumonia	Massive blood transfusion
Aspiration pneumonia	Diabetic ketoacidosis
Inhalational injury	

Table 2.3: Classification of primary and secondary lung injury

Several patients had both primary and secondary lung injury and were classified as mixed lung injury. A significant BAL culture was regarded as $\geq 10^5$ colony forming units per ml.

2.1.3 ITU severity scores

There has been a proliferation of scoring systems using various parameters to predict disease severity and outcome in ARDS[7 288-293]. These scores are helpful in allowing direct comparison of cohorts between different centres and different studies. Many attempts have been made to externally validate such prognostic models[294 295]. A number of inflammatory markers correlate with some of the scores (IL-6, IL-8). The Multiple organ Dysfunction Score (MODS) and the Sepsis-related Organ Failure Assessment (SOFA) score have also been used by some investigators[292 293]. These scores were not used in this thesis although some studies have demonstrated their prognostic value[292 296]. In this thesis, a variety of other scores were used to correlate with VEGF +936 genotype, as described next:

2.1.3.1 Murray Lung Injury Score

The Lung Injury score (LIS) was developed by Murray et al. and assesses severity of ARDS indicating the degree of pulmonary injury and oxygenation[7]. It has not been shown to predict outcome from ARDS unlike other scores[35 297]. This is perhaps not surprising as the predominant cause of death in ARDS patients is multi-organ failure and only 10% die with refractory hypoxaemia[28]. The scoring system is outlined in Table 2.1.

Value	CXR: consolidated quadrants	Hypoxaemia: PaO_2:FiO_2 ratio (mmHg)	PEEP: (when ventilated) (cm H_20)	Lung compliance: (if available) (ml/cm H_20)
0	None	>300	<5	>80
1	1	225-299	6-8	60-79
2	2	175-224	9-11	40-59
3	3	100-174	12-14	20-39
4	All 4	<100	>15	<19

Table 2.4: Scoring system for Murray Lung Injury Score

The final value is obtained by dividing the aggregate sum by the number of components used. In this thesis, pulmonary compliance measurements were not available. Murray et al. proposed that ARDS be defined as a LIS greater than 2.5; however, as discussed previously (Section 2.1.2) the 1994 American-European Consensus Conference definition was used to classify the cohorts for the polymorphism study as this is currently the internationally accepted definition in use by critical care investigators[11]. Therefore, the LIS values for some of the ARDS cohort are not all above 2.5. This exemplifies the fact that the 1994 definition is somewhat less stringent requiring only a threshold oxygenation index and bilateral CXR infiltration.

2.1.3.2 Acute Physiological and Chronic Health Evaluation (APACHE) scores

The APACHE score was introduced in 1981 assessing 34 different parameters making it complex and difficult to use[298]. Since then it has been refined and simplified to the APACHE II score which evaluates 12 physiological variables including the Glasgow Coma Scale allocating points for age and comorbidity[288]. It has been used to compare severity of disease between centres as both a research and audit tool[299]. In an effort to improve its predictive ability, it was then refined to the APACHE III score by re-evaluating the selection and weighting of the various physiological parameters[289]. The score is the sum of three groups of variables (physiology, age and chronic health). Neither the APACHE II or APACHE III scores can predict the risk of mortality with total accuracy as yet[300].

2.1.3.3 Simplified Acute Physiology Score II (SAPS II)

The SAPS II score includes 17 variables: 12 physiology variables, 3 underlying disease variables (acquired immunodeficiency syndrome, metastatic cancer and haematological malignancy), age and type of admission (scheduled surgical, unscheduled surgical and medical)[290]. It can predict mortality in ARDS although again has its limitations[35 290 295].

2.1.3.4 Severity scores and cytokine studies

One of the main problems in interpretation of ARDS trials from different centres is the heterogeneity of case mix. Aetiology of ARDS has been associated with risk of developing the syndrome and outcome as discussed in Chapter 1. Use of robust scoring systems is important to allow comparison.

Severity scores also allow association of inflammatory mediators with severity of physiological derangement. APACHE II score correlates with IL-6 and IL-10 levels in community acquired pneumonia[301]. Cytokine levels may therefore have additional prognostic value in ARDS patients.

2.1.4 Endpoints and other measures

Mortality is the ultimate disease severity score. As discussed in Chapter 1, 60 day mortality has been recommended as a useful additional endpoint to the traditional 28 day mortality as used in other trials[23]. For the ARDS cohort, the presence of systolic hypotension (< 100 mmHg) was noted.

2.2 Samples

2.2.1 Archival lung tissue

Archival anonymised formalin-fixed and paraffin embedded (FFPE) normal and ARDS lung tissue was obtained from Frenchay Pathology Department. The average time from death to post mortem was 6 hours and it is acknowledged that there would have been a finite risk of necrolysis and protein degradation. The cause of death in all cases of archival lung tissue was either remote (gut) or local (intrapulmonary) sepsis. In all cases, the post mortem had been performed for clinical reasons to further understand the cause and mechanism of death rather than by law. Issues surrounding selection bias and necrolysis are discussed in sections 3.4.2 (Chapter 3) and 6.2.1 (Chapter 6). ARDS lung tissue was designated "early ARDS" where onset was within 48 hours and "later ARDS" for onset after day seven.

2.2.2 Resected human lung tissue

Portions of "normal" human lungs were obtained from Thoracic Surgical Unit Bristol Royal Infirmary from cancer patients undergoing resection as a source of tissue for human ATII cell isolation and culture (see Sections 2.3.4 and 2.3.5).

2.2.3 Normal subjects and ventilated ITU patients

10ml whole blood samples were obtained in sterile EDTA tubes either by venepuncture for normal patients or *in situ* arterial line for ventilated patients.

Fibreoptic bronchoscopy was performed with an Olympus flexible non-video bronchoscope in ventilated "at risk" and ARDS patients in the presence of a senior anaesthetist. Suctioning of the endotracheal/tracheostomy tube was performed prior to BAL. The patient was prepared by pre-oxygenation with 100% oxygen for at least 2 minutes, with adequate sedation with/without paralysis at the discretion of the senior anaesthetist. Lignocaine spray was not used and continuous monitoring of oximetry, ECG and haemodynamics was performed. The bronchoscope was inserted into either an endotracheal or tracheostomy tube (minimum size 8) using a swivel catheter mount adapter. When passing the bronchoscope down into the lung, care was taken to avoid suctioning secretions in the tube or proximal airways to avoid contamination. The tip of the bronchoscope was wedged into the right middle lobe and 20ml sterile saline was injected, aspirated and discarded. A new trap (a chilled siliconised bottle to minimise cell loss due to adherence) was positioned and 3 x 60ml (180ml) aliquots of 0.9% sterile saline buffered with 8.4% sterile sodium bicarbonate were instilled to obtain broncho-alveolar fluid (BALF) – see section 2.3.1 for BALF processing details. On average, a 35% (65ml) BAL yield was obtained, (range 5 – 90%). Finally, another trap was positioned and a further 20ml sterile saline injected and aspirated for use as a clinical sample for suspected ventilator-associated pneumonia for the intensivist team with clinical responsibility. At this point, the bronchoscope was redirected and wedged into the subsegment thought to be implicated in possible disease as assessed radiographically. All bronchoscopy procedures were performed by the same bronchoscopist (Dr Andrew Medford).

Ideally, all ventilated ICU patients would have been sampled to be included in the analysis. In certain defined situations, this was not possible. No samples were taken in the case of refusal of consent. Blood or BALF samples were not taken for analysis in cases of serious transmissible disease. Other contraindications to bronchoscopy included unstable coronary syndromes, uncontrolled severe coagulopathies or uncontrolled bronchospasm. Logistical reasons included unavailability of bronchoscopy at the time of admission (weekends) or need for early extubation precluding bronchoscopy.

2.3 Techniques

2.3.1 Plasma and BALF extraction

Blood samples were centrifuged at 1300g for 10 minutes at 4^{o}C. Plasma was removed and stored at -70^{o}C for later analysis. BALF was strained through a single layer of gauze to remove any mucus clumps. The BALF was centrifuged at 500g for 5 minutes, and the supernatant was stored at -70^{o}C for further analysis.

Concentrated human ARDS BALF (x 100) was obtained by concentrating down ARDS BALF using Microcon YM-3 Centrifugal Filter Units (Millipore, Hertfordshire, UK). This was performed by Dr Lynne Armstrong (acknowledgements) and this fluid was used in the ATII cell culture (section 2.3.5, Chapter 2) and proliferation (section 2.3.8, Chapter 2) experiments. Briefly, 500µl aliquots of human ARDS BALF were pipetted into the sample reservoir in the vial with centrifugation at 14000g at 25^{o}C for 100 minutes. The sample reservoir was then placed inverted and spun in invert spin mode at 1000g for 3 minutes. The sample reservoir was then removed leaving the vial with concentrate to be stored at -70^{o}C for further analysis. The degree of concentration was confirmed by measuring concentrate urea concentration and initial BALF urea concentration.

2.3.2 DNA extraction

DNA was extracted using a standard phenol-free high salt method[302]. After centrifugation described above, the buffy layer was carefully transferred to a 15ml centrifuge tube adding 8mls of red cell lysis buffer (Appendix) mixing well and left for 20 minutes. A further centrifugation at 1300g for 10 minutes at 4^{o}C was performed. The red cell lysate was carefully removed as near as possible to the white cell pellet, resuspending the pellet in 3 mls of nuclei lysis buffer (Appendix). 0.6mls of 5x proteinase K (Sigma) solution [10mg/ml proteinase K in 1% sodium docesulphate (SDS)] was added mixing well and incubating at 55^{o}C for 3 hours.

4mls of 4.5M ammonium acetate (see Appendix) was added shaking vigorously for 15 seconds. A further centrifugation was performed at 1300g for 25 minutes at 4^{o}C. The supernatant was carefully removed and 8mls of absolute ethanol (Fischer Chemicals) added inverting gently to facilitate DNA precipitation. Samples were stored for 24 hours at -20^{o}C to optimise precipitation. The precipitated DNA was removed carefully from the ethanol and redissolved in 0.3mls of TE buffer (Appendix). The samples were then stored for a further 24 hours at 4^{o}C to allow proper dissolving.

Optical density was measured at 260nm (DNA content) to determine the DNA concentration using a GeneQuant II (Pharmacia Biotec, UK):

DNA concentration (µg/ml): OD_{260nm} x 50

Purity was measured at 260/280nm with a value of greater than 1.5 considered to be of good purity (pure DNA has a ratio of 1.8). DNA samples were standardised to 200ng/ml using RNAse free water.

If DNA samples were not of sufficient purity, they were "cleaned" as follows. 500µl of the sample was mixed with an equal volume of phenol:chloroform:isoamyl ethanol (25:24:1) (Sigma) and vortexed followed by centrifugation for 20 minutes at 13000g at 4^{o}C. The aqueous layer was carefully removed and again mixed with an equal volume of phenol:chloroform:isoamyl ethanol (25:24:1). The mixture was then vortexed and centrifuged for 20 minutes at 13000g at 4^{o}C. The aqueous layer was carefully removed and 1ml absolute ethanol and 50µl of 3M sodium acetate pH 6.0 (Sigma) added. The sample was stored for 24 hours at -20^{o}C to facilitate DNA precipitation. The precipitated DNA was carefully removed and washed in 1ml 70% ethanol. A final centrifugation for 20 minutes at 13000g at 5^{o}C was performed before carefully removing the ethanol and redissolving the pellet in 100µl of RNAse free water and measuring the DNA concentration as described above. This always resulted in improvement of the 260/280nm ratio to above 1.5 although was inevitably associated with a fall in DNA concentration.

2.3.3 Immunohistochemistry

2.3.3.1 Single stain immunohistochemistry

Normal, early and late ARDS lung tissue sections were obtained. Normal lung tissue implied that there was no lung involvement in the cause of death. ARDS lung tissue was subdivided into "early" (within 48 hours of onset) and "later" (after day 7). Paraffinised 4µm sections were dewaxed in serial xylene (BDH Laboratory Supplies, Poole, UK), dehydrated in absolute ethanol (BDH Laboratory Supplies, Poole, UK) and soaked in running water for 5 minutes. Antigen retrieval was performed via pressure cooking for one minute 45 seconds in 0.01M tri-sodium citrate (BDH Laboratory Supplies, Poole, UK) buffer (pH 6). 0.1% saponin (Sigma-Aldrich, Dorset, UK) in phosphate buffered saline (PBS), pH 7.3 was used to permeabilise the membrane and was used as a wash buffer and antibody diluent.

Sections were outlined after further rinsing in water with a hydrophobic pen (DAKO). Endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) (Sigma) in absolute methanol (BDH Laboratory Supplies, Poole, UK) for 10 minutes. Sections were incubated in 2.5% horse blocking serum (Vectastain Universal Quick Kit, Vector Laboratories, Peterborough, UK) to block non-specific binding sites. Avidin and biotin binding sites were then blocked with specific blocking sera (Vector Laboratories, Peterborough, UK). After washing the optimal concentration of primary antibody was applied for 1 hour (see Table 2.2).

Antibody	Species	Santa Cruz code	Dilution (concentration)
VEGF	Rabbit	sc-152	1:500 (0.4µg/ml)
VEGFR1	Rabbit	sc-316	1:750 (0.27 µg/ml)
VEGFR2	Rabbit	sc-505	1:75 (2.67 µg/ml)
NRP-1	Rabbit	sc-5541	1:350 (0.57 µg/ml)
AQP3	Goat	sc-9885	1:500 (0.4 µg/ml)
Isotypic IgG	Rabbit	sc-2027	1:150 (2.67 µg/ml)
Isotypic IgG	Goat	sc-2028	1:150 (2.67 µg/ml)

Table 2.5: Primary antibodies used in immunohistochemistry (all polyclonal)

Rabbit polyclonal antibodies to VEGF, VEGFR1, VEGFR2 and NRP-1 (Santa Cruz) were used as primary antibodies. The optimal concentration of the primary antibody was pre-determined using a variety of concentrations and assessing the most specific staining. Isotypic rabbit or goat IgG (Santa Cruz) at the same concentration was used as a negative control depending on the species of the primary antibody. In addition, specific blocking peptides for VEGF, VEGFR1 and AQP3 (Santa Cruz) were co-incubated with these primary antibodies before immunohistochemistry to confirm specificity of staining (results were identical to using isotypic negative controls).

After washing, a pan-specific biotinylated secondary antibody was added for 10 minutes followed by streptavidin-peroxidase complex for 5 minutes after a further wash and diaminobenzidine tetrahydrochloride (DAB) substrate solution (Vector, SK-4100) for 10 minutes after washing yielding a brown colouration in areas of staining (Vectastain Universal Quick Kit, Vector Laboratories, Peterborough, UK). Sections were washed in distilled water and then counterstained in haematoxylin (Sigma) prior to serial dehydration and dewaxing in absolute ethanol and xylene before mounting with DPX mountant (BDH Laboratory Supplies, Poole, UK). Slides were examined by light microscopy (Zeiss-Axioskop).

Image capture and semi-quantitative densitometry were achieved using Histometrix 6 software version 1.4 (Kinetic Imaging) linked to a JVC TK-C1360B camera with a resolution of 470 TV lines. The user selects pixels representing immunopositivity and this threshold is memorised by the software. Anything within the selected pixel range can be accounted for and is expressed as a percentage of the pixels in the selected area. Histometrix therefore gives a composite intensity score per unit area derived from the staining intensity value divided by the staining cross-sectional area assessed. Densitometry was performed on all slides from the same procedure assigning the same random colouration as unit of intensity on each slide. Histometrix also allows random (computer-generated) selection of areas on the section. Five randomly chosen areas on each section for each patient (n = 4) were assessed giving twenty values. Densities on negative control sections were subtracted from positively stained section densities to control for background pixel intensities detected by Histometrix.

If dual staining was to be performed, slides were rinsed in distilled water following the DAB substrate solution stage. Then, a non-specific blocking solution was applied (as described earlier) followed, after a wash, by the second primary antibody for 1 hour (AQP3, see Table 2.1). After washing, a pan-specific biotinylated secondary antibody was added for 10 minutes followed by streptavidin-peroxidase complex for 5 minutes after a further wash (as described previously). Instead of DAB substrate Vector VIP substrate solution was applied (Vector, SK-4600) for 2 minutes yielding a purple colouration. Dual stained slides were counterstained after rinsing with methyl green (Vector, H-3402) according to manufacturer's instructions. Briefly, this involved incubating slides to 60^{o}C in a slide warmer for 5 minutes and rinsing in distilled water before dehydration and dewaxing (as described earlier). This methodology was adopted following a process of elimination of others as described below.

2.3.3.2 Unsuccessful dual staining immunohistochemistry

Initial investigations were undertaken using goat polyclonal SP-C primary antibodies (Santa Cruz, sc-7705). This stained many other cells as well as ATII cells, especially alveolar macrophages. It became clear that AQP3 antibodies were more specific, because of relatively less non-specific alveolar macrophage immunostaining. A goat polyclonal AQP5 antibody (Santa Cruz, sc-9890) was considered as a negative marker for ATII cells and a positive marker for ATI cells but was not used because of non-specific staining on alveolar macrophages despite optimisation of blocking techniques. In terms of technique, using Vector Alkaline Phosphatase (Vector, SK-5100) (more diffuse staining than peroxidase and difficult to visualise red against brown DAB) and use of nickel with DAB substrate solution (more localised peroxidase technique but grey colouration difficult to visualise against brown DAB) were suboptimal. Because of the anatomical proximity of VEGF and AQP-3 expression in ATII cells, this limits differentiation by dual staining techniques. Limited attempts at immunofluorescence were halted by persistent non-specific staining of alveolar macrophages despite optimisation of blocking protocols.

2.3.4 ATII cell extraction from resected human lung specimens

ATII cells were obtained from resected lungs using the established method of Witherden and Tetley[43 303]. Briefly, the lung segment was inflated with DCCM (React Scientific, Troon) and then perfused with 0.15M sterile saline massaging the tissue to remove alveolar macrophages and monocytes. The tissue was digested in 0.25% trypsin in Hanks balanced salt solution (HBSS) (Sigma) twice at 37^{o}C for 15 minutes (10mls per 5cm^{3} of tissue) and then chopped into 1-2mm^{3} pieces in the presence of newborn calf serum (NCS) (Invitrogen, Paisley). 250 μg/ml DNAse I in 7 ml HBSS (Sigma) was added to the suspension. After vigorous shaking for 5 minutes, the suspension was filtered through a large gauge (~500μm) and then a 40μm mesh (Fahrenheit, Milton Keynes). The filtered suspension was then centrifuged at 300g for 10 minutes at 4^{o}C, the cell suspension was resuspended in 15 ml HBSS/15 ml DCCM containing 100 μg/ml DNAse I. Serial adherence steps were performed at 37^{o}C for 2 hours putting the cell suspensions in a T-175 and then a T-75 flask after centrifugations at 300g for 10 minutes at 4^{o}C, resuspending each time in 30mls of complete ATII culture medium. This led to purification due to adherence of contaminating alveolar macrophages and fibroblasts. Serial cytospins and cell counts were performed using a cytocentrifuge (Shandon cytospin 3) and Neubauer haemocytometer (see Appendix)**.** The non-adherent cells were centrifuged at 300g for 10 minutes at 4^{o}C. 1 x 10^{6} ATII cells/ml (in complete ATII culture medium) were added to collagen-coated (Vitrogen 100, Cohesion Technologies, Palo Alto, USA) 24 well plates (Corning). The correct number was determined from cell counts (see Appendix) and differential staining for alkaline phosphatase (see section 2.3.6).

2.3.5 ATII cell culture

1 x 10^6 ATII cells were cultured in 1 ml complete ATII culture medium (NB: for the proliferation experiments only 100µl of 2 x 10^4 ATII cells/ml of complete ATII culture medium were cultured in 96 well plates until 30% confluence was attained before proceeding with mediator incubation and MTS reagents – see section 2.3.8). After 24 hours of adherence, any non-adherent cells and medium were removed and fresh complete medium added. The cells were then incubated at 37^{o}C and after 16 hours the medium was removed and the cells washed with HBSS. Fresh complete medium was then added and the cells incubated for a further 24 hours to establish confluent monolayers with ATII morphology. Cells were phenotyped (see below) before culturing and adding pro-inflammatory cytokines for 4 hours at day 3 and extracting RNA (see section 2.3.6).

ATII cells were then incubated with a variety of different mediators (made up in complete medium) as shown in Table 2.6. Importantly, the same batch of LPS (*Escherichia Coli* 0111:B4, L4391, Sigma-Aldrich) was used in all LPS experiments.

Incubation	Concentration and source
BAL fluid	concentrated BAL fluid from ARDS patients (50 µl in 1ml complete medium)
LPS	10µg/ml LPS (Sigma)
TNFα	10ng/ml TNFα (Peprotech)
IL-1β	1ng/ml IL-1β (Peprotech)
$VEGF_{165}$	0.1, 1 and 10ng/ml human $VEGF_{165}$ (R&D)

Table 2.6: Incubations used for ATII cell culture experiments.

These cytokines were selected as being known to be associated with ARDS and/or sepsis and therefore providing information about ATII cell behaviour in an ARDS milieu. VEGF was included to assess its possible effect on ATII cells, in a possible autocrine role (see Chapter 1). The doses of cytokine are consistent with those used in the literature in other primary cell work. The highest dose of VEGF (10ng/ml) equates to the intrapulmonary measured levels noted in normal subjects, the middle dose (1ng/ml) represents typical intrapulmonary VEGF levels in early ARDS[128 247]. The lowest dose (0.1ng/ml) represents the extreme lower range of intrapulmonary levels in early ARDS[247].

2.3.6 ATII cell phenotyping

ATII cell cytospins were incubated in ALP stain (see Appendix) for 20 minutes at 37^{o}C. ATII cells were phenotyped by staining for alkaline phosphatase (ALP), see (Figure 2.1 below).

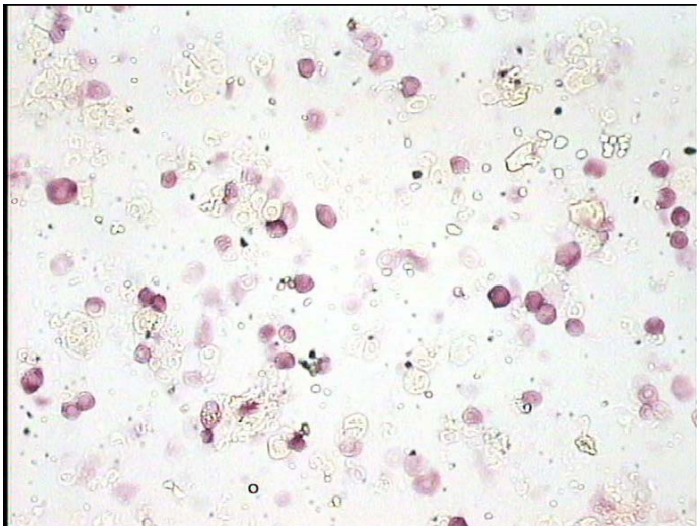


Figure 2.1: Alkaline phosphatase staining of ATII cytospin (magnification x 20)

RT-PCR (see Appendix for technical details) was also performed for surfactant protein C (SP-C) and aquaporin 3 (AQP3) as positive markers for ATII phenotype and AQP5 as a negative marker (ATI phenotype) as shown in Figure 2.2**.**

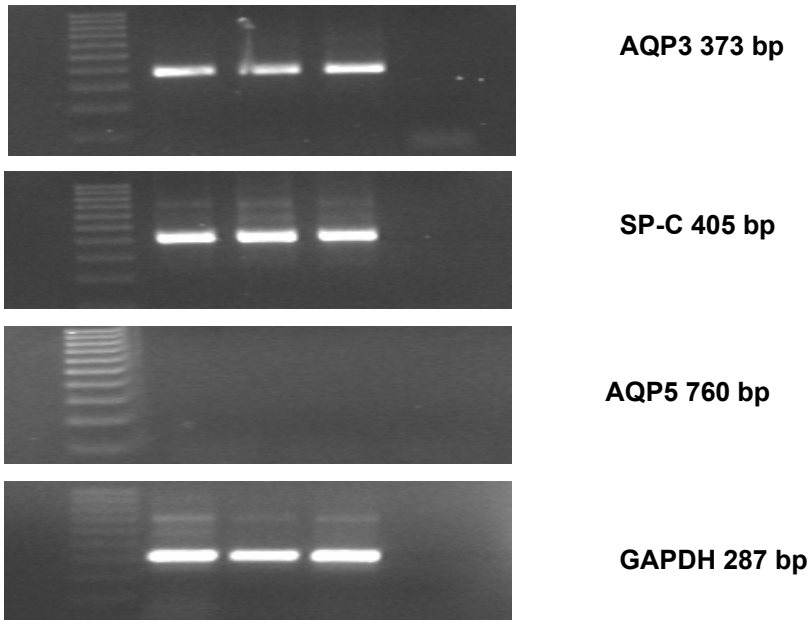


Figure 2.2: RT-PCR for ATII phenotyping

Electron microscopy (kindly performed by Dr Chris Neal, see Acknowledgements) also confirmed typical lamellar bodies (see Figures 2.3a and 2.3b below).

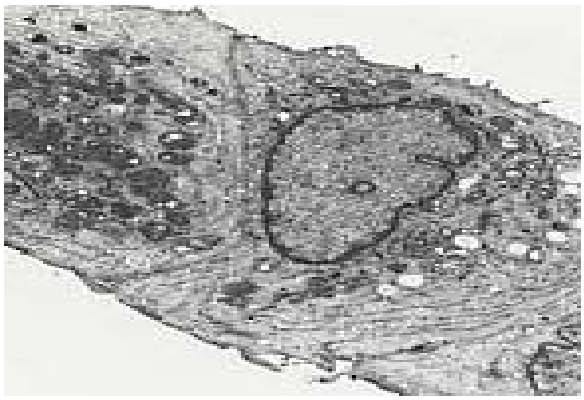


Figure 2.3a: electron microscopy of ATII cell confirming lamellar bodies

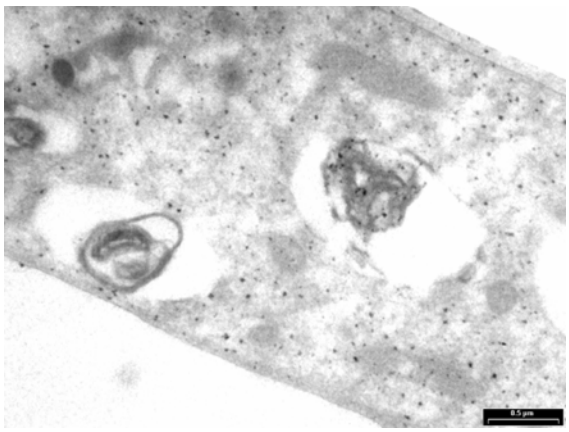


Figure 2.3b: Higher magnification of ATII cell lamellar bodies at electron microscopy

2.3.7 ATII cell RNA isolation

Total cellular RNA was extracted from ATII cells cultured at 1 x 10^6/ml using RNABee reagent (AMS Biotechnology, Abingdon, UK) according to manufacturer's instructions. Briefly, the supernatants were removed and the ATII cells were resuspended in 1ml RNABee reagent, scraping to ensure optimal cell removal. 200µl chilled chloroform was added with vigorous shaking for 15 seconds. Following centrifugation at 13000rpm for 15 minutes at 4^{o}C, the aqueous phase (containing the RNA) was carefully removed to another tube. An equal volume (usually about 400µl) of absolute isopropanol (Sigma) was added with 1µl of 20µg/µl glycogen (Roche) to aid the precipitation of RNA. Samples were stored for a minimum of 24 hours at -20^{o}C to aid RNA precipitation.

Samples were recovered from -20^{o}C storage and allowed to thaw on ice followed by centrifugation at 13000rpm for 15 minutes at 4^{o}C. The resulting supernatant was carefully removed so as not to disturb the RNA pellet. Rehydration and washing was performed using 1 ml 70% ethanol and vortexing. The supernatant was discarded and the pellet allowed to air dry for 5 minutes before being resuspended in 20µl RNAse free water. Optical density was measured at 260nm (RNA content) to determine the RNA concentration using a GeneQuant II (Pharmacia Biotec, UK):

RNA concentration (µg/ml): OD_{260nm} x 40

Purity was measured at 260/280nm with a value of > 1.7 considered to be of good purity (pure RNA has a ratio of 2.0). RNA samples were standardised to 200ng/ml using RNAse free water.

2.3.8 ATII cell proliferation

ATII cell proliferation was assessed using the CellTiter96®Aq$_{ueous}$ Non-Radioactive Cell Proliferation Assay (Promega) which is an accepted technique for indirectly assessing cell proliferation in culture[304]. A novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) is bioreduced by metabolically active cells in culture to a formazan product that can be detected and measured colorimetrically at 490nm (see Figure 2.4 below).

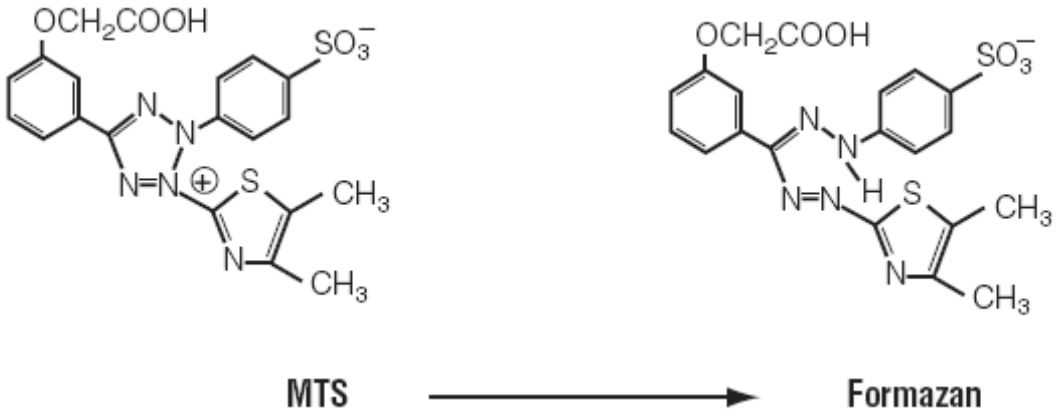


Figure 2.4: Structures of MTS tetrazolium salt and its formazan product. Source: Promega Corporation, Technical Bulletin No 169[305].

The absorbance at 490nm is directly proportional to the number of living cells in culture as shown in Figure 2.5.

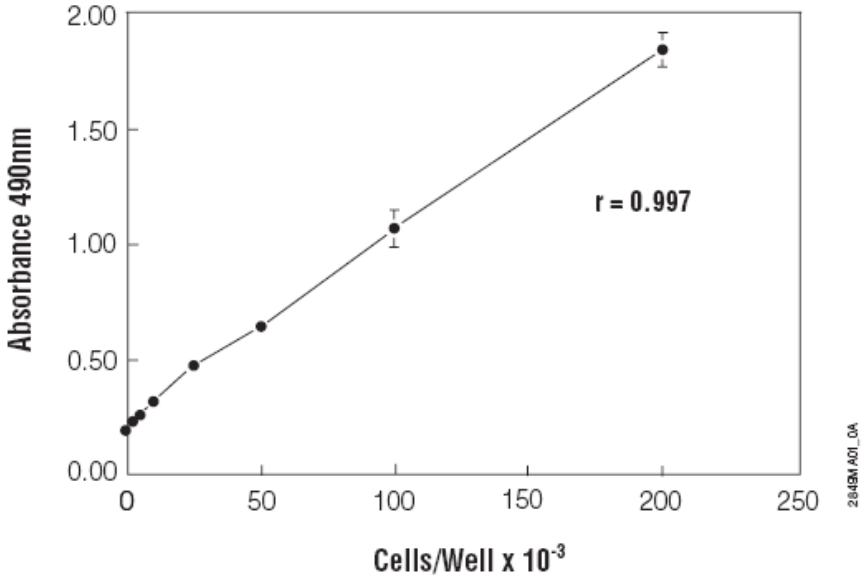


Figure 2.5: Effect of cell number on absorbance at 490nm using MTS assay in K562 cells (human chronic myelogenous leukaemia cells). Mean +/- SD of 4 replicates indicating linear response between cell number and absorbance at 490nm. Source: Promega Corporation, Technical Bulletin No 169[305].

This method has the advantage of speed, safety (no radioactivity or organic solvents) and stability. One theoretical problem is that an increase in absorbance although usually due to a proliferative response, may be related to an increase in cell metabolism without proliferation. The gold standard technique is [^{3}H]-Thymidine incorporation but technical data has shown that the MTS method performs very similarly in assessing B9 cell proliferation in response to IL-6 as shown in Figure 2.6 below.

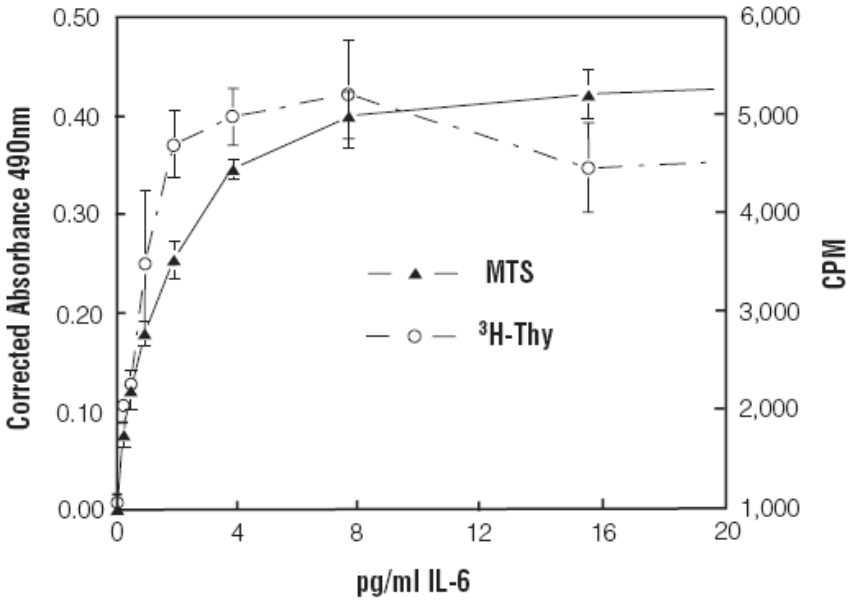


Figure 2.6: Proliferation of B9 cells in response to various concentrations of IL-6 measured using both MTS and [^{3}H]-Thymidine assays. Source: Promega Corporation, Technical Bulletin No 169[305].

100µl of 2 x 10^4/ml ATII cells were cultured in complete ATII medium in a collagen coated 96 well plates to achieve 30% confluence. They were then incubated in culture medium plus/minus a variety of mediators for 48 hours in triplicate as detailed in Table 2.7 below.

Incubation	Concentration and source
Control	complete medium only
sflt	10ng/ml *sflt* (R&D)
$VEGF_{165}$	0.1ng/ml $VEGF_{165}$ (R&D) 1ng/ml $VEGF_{165}$ 10ng/ml $VEGF_{165}$ 100ng/ml $VEGF_{165}$
$VEGF_{165}$ and *sflt*	10ng/ml $VEGF_{165}$ + 10ng/ml *sflt* 100ng/ml $VEGF_{165}$ + 10ng/ml *sflt*
BAL fluid	concentrated BAL fluid from ARDS patients (50µl in 1ml complete medium)
BAL fluid and *sflt*	concentrated BAL fluid from ARDS patients + 10ng/ml *sflt*
Positive control	50ng/ml KGF (Peprotech)

Table 2.7: Incubations used for ATII cell proliferation experiments

Next, 2mls of MTS solution were added to 100µl of phenazine methosulfate (PMS) solution. 20µl of this mixture was added to each well containing 100µl of 2 x 10^4/ml (previous experiments had indicated that 2 x 10^4/ml cells with 4 hours incubation with MTS reagents after 48 hours of mediators when at 30% confluence was probably the most linear response - see Figures 2.7a-c).

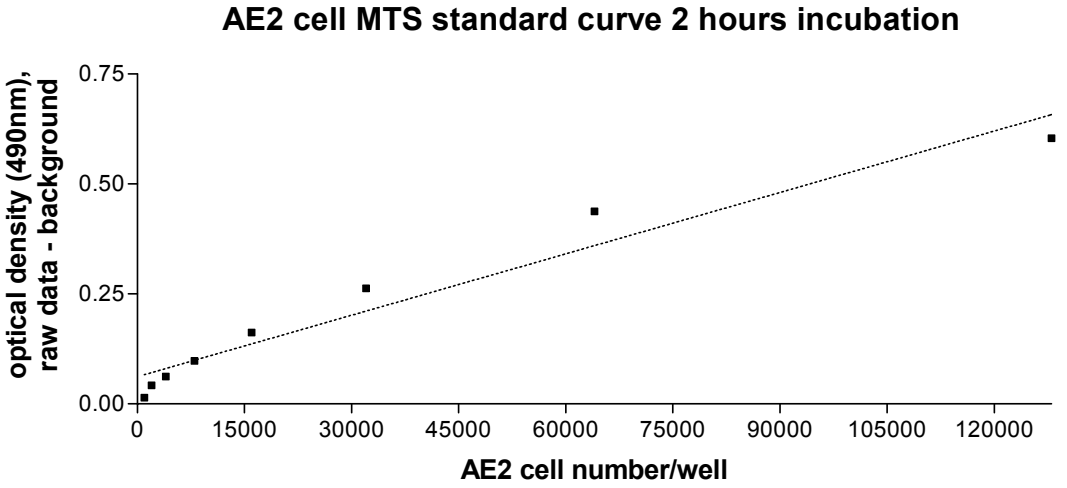


Figure 2.7a: Proliferation of ATII cells using MTS assay with 2 hour incubation and increasing cell number (linear regression, $r^2 = 0.95$, $p < 0.0001$). Dotted line denotes goodness of fit.

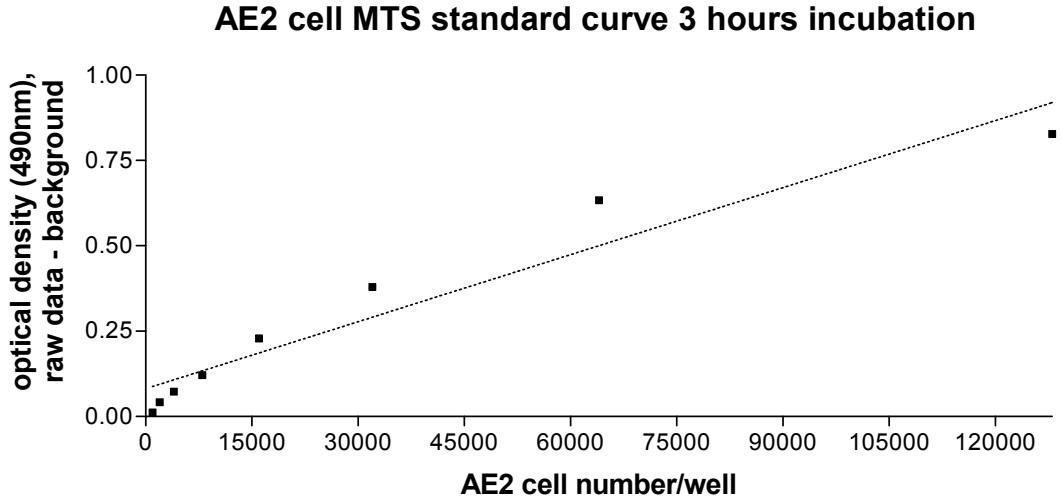


Figure 2.7b: As for Figure 2.7a but 3 hour incubation (linear regression, $r^2 = 0.93$, $p = 0.0001$).

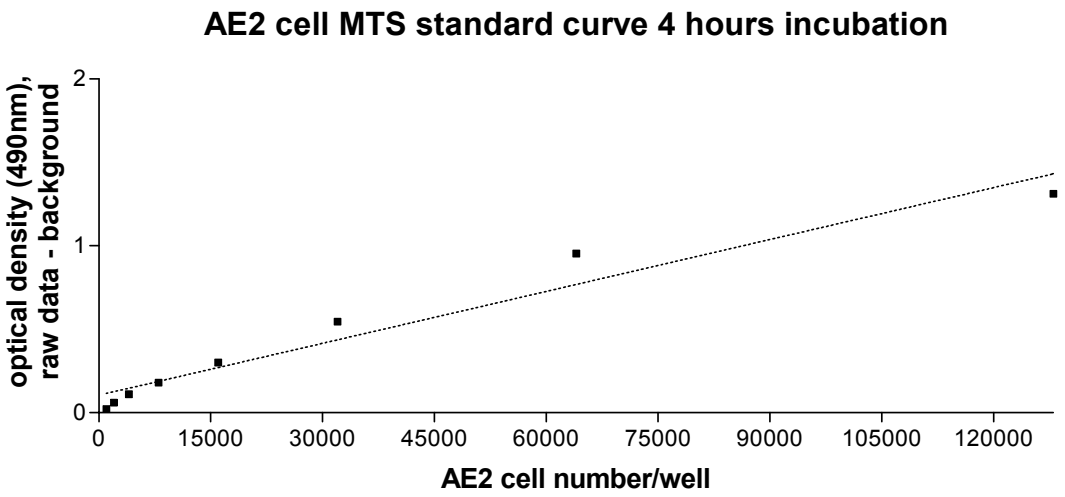


Figure 2.7c: As for Figure 2.7a but 4 hour incubation (linear regression, $r^2 = 0.95$, $p < 0.0001$).

As well as control wells, three background wells containing no medium were used to subtract background intensities. The plate was incubated for 4 hours at 37^{o}C in a humidified, 5% CO2 atmosphere before recording the absorbance at 490nm using an ELISA microplate reader (Biolinx 2.1 software connected to a MR7000 Dynatec ELISA microplate reader (Dynex technologies). Proliferation was calculated as a percentage of control proliferation with positive values indicating a net proliferative response versus control. Negative values indicate a net reduced response versus control. The MTS assay was interpreted as being validated in all experiments where the positive control resulted in an increase in proliferation above control. In any experiment, where the positive control failed to elicit a higher proliferative response than control, the results were deemed invalid and the experiment repeated.

2.3.9 Formalin-fixed paraffin-embedded (FFPE) RNA extraction

This method is a modified version of the Krafft technique[306]. 8 x 6µm sections were cut on a microtome (Jung-Biocut 2035, Westshore Technologies, Michigan, USA) from formalin-fixed paraffin-embedded (FFPE) blocks of archival normal, early (within 48 hours) and late ARDS (after day 7) lung tissue. Sections were dewaxed using repeated application of 800µl Histoclear II (National Diagnostics, Atlanta Georgia, USA), vortexing and centrifuging at 13,000 rpm for 5 minutes with 400µl absolute ethanol (Fisher Chemicals) washes discarding the supernatants. A final wash was performed with 800µl absolute ethanol.

Sections were then dried at 55^{o}C for 3 minutes before being digested for 6 hours in digest buffer (1mg/ml proteinase K (Sigma), 20mM Tris (Sigma), 20 mM EDTA (Sigma) buffer at pH 7.4, 1% SDS (Sigma) and RNAse free water – see Appendix) at the same temperature. After ice cooling, RNA was extracted using 500µl of phenol:chloroform:isoamyl ethanol (25:24:1) (Sigma) mixture removing the aqueous layer (containing the RNA) after vortexing and centrifugation (13,000 rpm at 4^{o}C for 2 minutes) repeating this extraction once.

RNA was precipitated adding 40µl of 3M sodium acetate (Sigma, see Appendix), 1µl of 20µg/µl glycogen (Roche) and 240µl absolute isopropanol (Fisher) mixing gently and leaving overnight at -20^{o}C. The RNA was then pelleted by centrifugation at 4^{o}C at 13,000 rpm for 10 minutes discarding the supernatant. A final wash and centrifugation (12,000 rpm for 5 minutes at 4^{o}C) in 80% ethanol was performed. RNA pellets were redissolved in 25µl RNAse free water by immersion at 55^{o}C in a water bath for 10 minute. Samples were then stored overnight at -80^{o}C before thawing and determining the RNA concentration as described previously (section 2.3.7).

2.3.10 Semi-quantitative RT-PCR

2.3.10.1 DNase treatment

Prior to RT-PCR, genomic DNA contamination (detected by using a no RT control on the original RNA) was removed from the RNA using RNAse-free DNase (Promega). Briefly, 8µl RNA, 1µl RQ1 RNase-free DNase and 1µl RQ1 DNase 10x reaction buffer (Promega) (see Appendix) was added, vortexed and incubated at 37^{o}C for 30 minutes before placing on ice. 1µl of RQ1 DNase stop solution (Promega) was added to terminate the reaction and then the samples were incubated at 65^{o}C for 10 minutes to inactivate the DNase. The treated RNA was checked for purity by having an absent no RT control again before being used for RT-PCR.

2.3.10.2 Optimisation of RT-PCR and theoretical problems

Before commencing RT-PCR the reaction conditions were optimised using temperature and magnesium gradients (see Figures 2.8a and b).

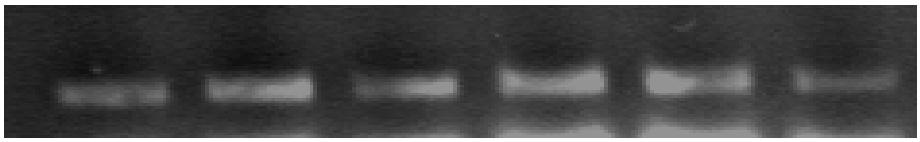


Figure 2.8a: Example of temperature gradient for $VEGF_{165}$ isoform RT-PCR.
Lanes correspond to increasing temperatures as follows:
1: 55^{o}C
2: 55.8^{o}C
3: 57.2^{o}C
4: 58^{o}C
5: 58.8^{o}C
6: 60^{o}C

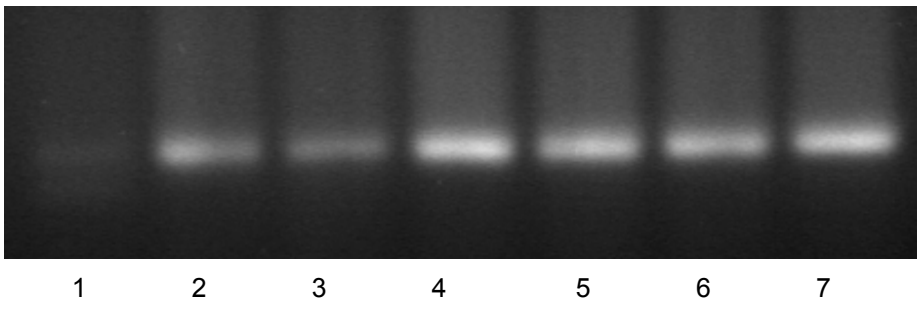


Figure 2.8b: Example of magnesium gradient for $VEGF_{165}$ isoform RT-PCR. Lanes correspond to increasing concentrations of magnesium chloride as follows (mM):

1: 0.5mM

2: 1mM

3: 1.5mM

4: 2mM

5: 2.5mM

6: 3mM

7: 3.5mM

Cycle gradients were used to demonstrate that the semiquantitative RT-PCR was detecting RNA in log phase and not plateau phase (see Figure 2.9).

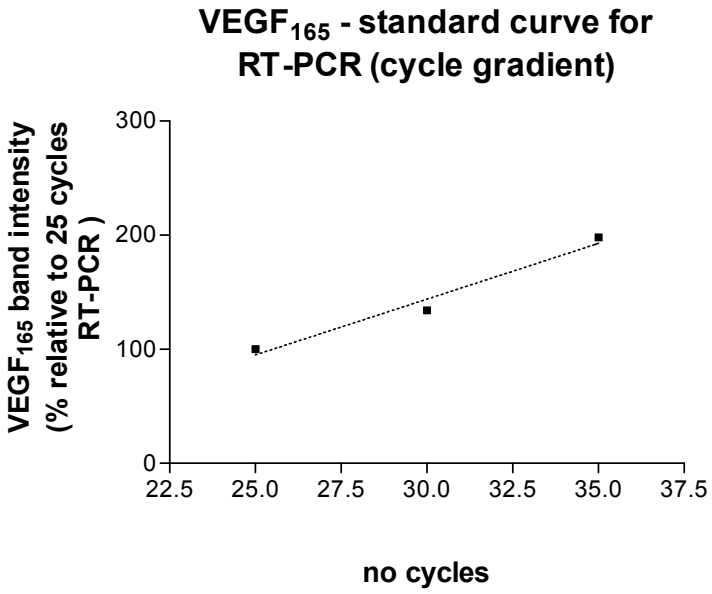


Figure 2.9: Example cycle gradient RT-PCR graph for $VEGF_{165}$ (linear regression r^2 = 0.97, p = 0.11). Dotted line denotes goodness of fit.

RNA concentration gradient (see Figures 2.10a and b) was used to demonstrate there was a linear response to RNA concentration although this technique is known not to be truly quantitative as compared to real-time PCR and subject to the problems of end-point PCR[307-309].

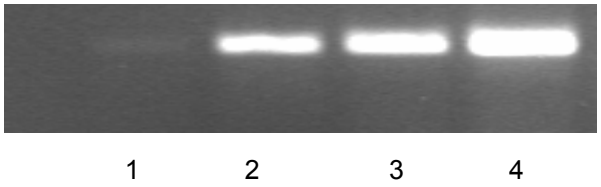


Fig 2.10a: Example gel from RNA concentration gradient for GAPDH. Lanes correspond to increasing amounts of RNA as follows:

1: 50ng

2: 100ng

3: 200ng

4: 400ng

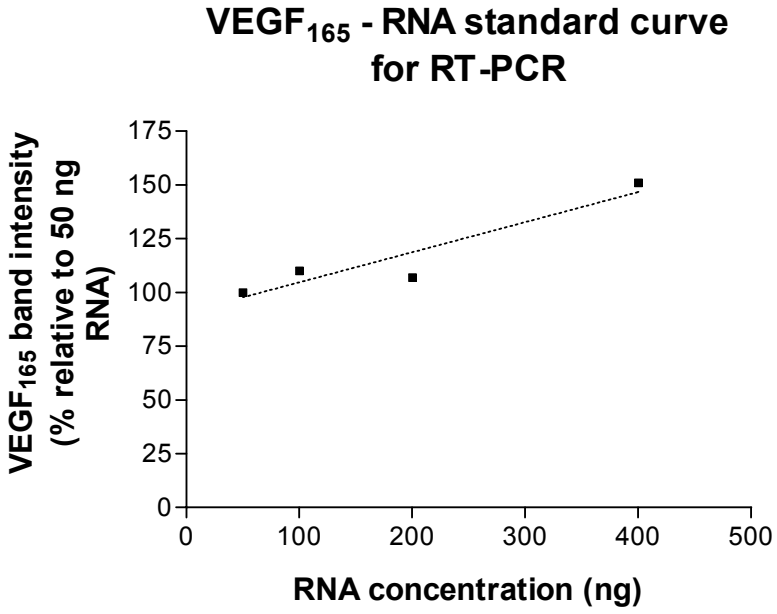


Fig 2.10b: Densitometric analysis of Figure 2.10a (linear regression, $r^2 = 0.88$, $p = 0.06$). Dotted line denotes goodness of fit.

Two-step RT-PCR at least offers the advantages of improved consistency, avoiding repetitive freeze-thawing of RNA samples and degradation, and increased sensitivity[309]. The preferred method to generate cDNA is using a Moloney Murine Leukemia Virus-based RT enzyme although oligo-dT is an established alternative. Theoretical problems with the oligo-dT method include variable primer efficiency due to sequence-dependent interactions and biased RT products due to RNA secondary structure and the length of the polyA tail[309].

All relative quantitative methods employ housekeeping genes. It is assumed the treatment or stimulation does not alter expression of it and that expression is constant although there is growing evidence for significant variation in GAPDH mRNA in asthmatic airways for example[310 311].

Other problems with RT-PCR include interassay variability (minimised using a mastermix here and measured at an average of 11.6%, see Figure 2.11), potential genomic DNA contamination (minimised using RNAse-free DNase treatment, see section 2.3.10.1) and post-PCR manipulations involving electrophoresis, imaging and densitometry (see section 2.3.11).

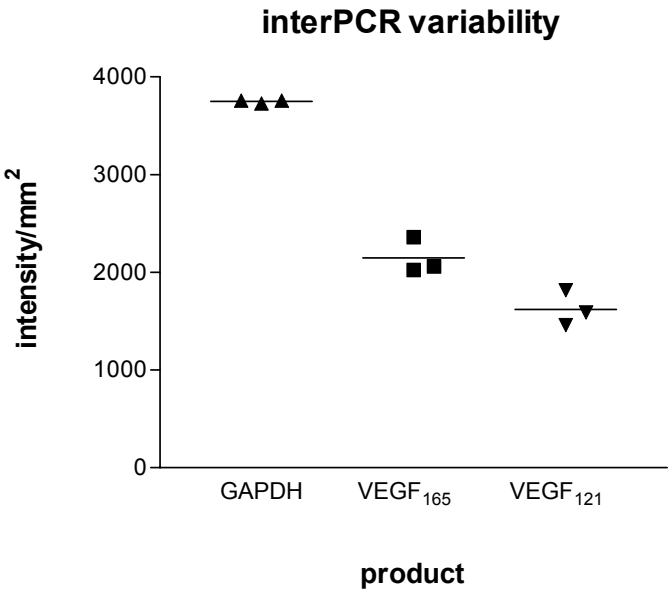


Figure 2.11: Example of triplicate repeat of ATII RT-PCR for $VEGF_{165}$, $VEGF_{121}$ and GAPDH.

2.3.10.3 cDNA preparation

cDNA synthesis was performed in a two-step reaction using 1µg of total RNA as a template. All reactions were carried out in a Peltier MTC-200 thermocycler. Reverse transcription was performed using a 20ul reaction [containing 5µl of 200ng/µl RNA, 0.4µl oligoDT primer (Promega UK Ltd, Southampton, UK) and 14.6µl of RNAse free water] at 65^{o}C for 10 minutes prior to ice quenching. The second stage was performed using a 15µl mix [4µl RT 5x buffer (Roche Diagnostic Ltd, Lewes, UK), 2µl 100mM DTT (Roche Diagnostic Ltd, Lewes, UK), 4µl 20mM dNTPs (Abgene, Epsom, UK) and 1µl expandRT (Roche Diagnostic Ltd, Lewes, UK)] added to the 20µl oligoDT mix at 42^{o}C for 1 hour.

2.3.10.4 cDNA polymerase chain reaction (PCR)

cDNA PCR was then performed using 1µl of cDNA to amplify for VEGF isoforms, VEGFR1, VEGFR2, NRP-1 and GAPDH or B_2M depending on the type of RNA. Amplifications were carried out in a 20µl reaction volume containing 13µl RNase free water, 1.2µl 25mM $MgCl_2$ (final concentration 1.5 mM) (Abgene), 0.4µl 25µM dNTPs (Abgene), 1µl of 20µM forward and reverse primers (Sigma), 2µl 10x reaction buffer (Abgene) and 0.4µl 5U/µl Taq DNA polymerase (Abgene).

Following denaturation at 94^{o}C for 3 minutes, the reactions were subjected to pre-determined optimal annealing temperatures for each molecule of interest (see Appendix for all temperatures, primer sequences, cycle number and product sizes). DNA extension was carried out at 72^{o}C for 30 seconds followed by denaturation at 94^{o}C for 30 seconds. Cycles were repeated a specific number of times for each molecule (see Appendix). There was a final temperature of 72^{o}C for 10 minutes to finalise any extension and the reaction was cooled to 4^{o}C before quantification.

All reactions were carried out in a Peltier MTC-200 thermocycler. A no cDNA sample was used as a negative control with a positive control cDNA known to express the product of interest (sequenced in the case of the VEGF isoform). A no RT control was used in addition to identify any genomic DNA contamination requiring DNase treatment (see section 2.3.10.1).

2.3.11 Electrophoresis, imaging and densitometry

Individual amplified products were visualised by running the samples on an agarose gel (Sigma) specific for each molecule (2% for VEGF isoforms, 1% for VEGFR1, VEGFR2, NRP-1 and AQP3 and SP-C, see Appendix) dissolved in 1x Tris-borate ethylenediamine-tetraacetic acid (TBE) containing 7.5µl 10mg/ml ethidium bromide (Sigma). Gel electrophoresis was performed using a 1x TBE running buffer (Appendix) at 80 volts on a BioRad PowerPac-300 for approximately 60 minutes. Samples were run against a relevant housekeeping gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] for all experiments except FFPE RT-PCR which used beta-2-microglobulin (B_2M)] and a 100 base pair (bp) ladder (Sigma) visualised by using a transilluminator (BioRad, Hertfordshire, UK) that allowed digital image capture. Semi-quantitative analysis and image capture was performed using densitometry for the amplified products using BioRad Geldoc software.

For FFPE RT-PCR of VEGF isoforms on whole lung, in addition to absolute densitometry values, proportionate mRNA expression was calculated for each isoform in normal, early ARDS and later ARDS lung. This was derived from the percentage densitometry value of the individual isoform divided by the sum of the densitometry values for all three isoforms.

2.3.12 Purification of DNA from agarose gels

(NB: All centrifugations described in the next 3 sections are 13000rpm for 1 minute unless otherwise stated).

Following, PCR the amplified product was carefully excised from the agarose gel with a clean, sharp scalpel and the DNA purified using the QIAquick gel extraction kit according to manufacturer's instructions (QIAGEN, West Sussex, UK). Briefly, the gel slice was weighed and 3 volumes of buffer QG added to 1 volume of gel. This was incubated at 50^{o}C for 10 minutes and vortexed until the gel had dissolved ensuring the pH was less than 7.5 (indicated by the resulting yellow colouration). 1 gel volume of absolute isopropanol was added. The mixture was transferred to a QIAquick spin column and centrifuged to bind DNA. The flow-through was discarded and 500µl of buffer QG added to remove any traces of agarose followed by a further centrifugation. A further 750µl of buffer PE was added to wash followed by centrifugation. After discarding the flow-through another centrifugation was performed. 50µl of buffer EB (10mM Tris-Cl, pH 8.5) was added to elute the DNA with a final centrifugation into a clean centrifuge tube. Samples were kept at -20^{o}C for subsequent cloning and sequencing.

2.3.13 Cloning of amplified RT-PCR products for sequencing

In order to sequence and confirm the amplified suspected $VEGF_{189}$ PCR product, the TA cloning kit (Invitrogen, Paisley, UK) was used according to manufacturer's instructions. Briefly, ligation of the excised DNA was performed by mixing 1µl of PCR product with 1µl of 10x ligation buffer (see Appendix), 2µl of pCR 2.1 vector (see Figure 2.8) (25ng/µl in 10mM Tris-HCl, 1mM EDTA pH 8.0), T4 DNA ligase (4.0 Weiss Units/µl) and 5µl of sterile water. The reaction was incubated at 14^{o}C overnight using a thermocycler (Peltier, PTC-200) and used for transformation into competent TOP10 *E.Coli* cells.

The following day, 2µl of the ligated reaction was carefully transferred into the *E.Coli* cells that had been slowly thawed on ice. Vials were placed on ice for 30 minutes. Transformation was achieved by heat shocking the competent cells in a water bath at 42°C for exactly 30 seconds for the uptake of the vector containing the PCR product and immediately placed on ice. The cells were incubated at 37°C for 1 hour at 225rpm in 250µl of SOC medium (see Appendix).

Transformed cells were spread on Luria-Bertani (LB) (see Appendix) agar plates containing 100µg/ml ampicillin (Sigma) and 40mg/ml X-gal (Sigma) and incubated for 18 hours at 37°C. Plates were transferred to 4°C for 2-3 hours to allow proper colour development. The transformed cells were selected by blue/white colour screening due to the presence of the *lacz* (encodes the first 146 amino acids of β-galactosidase) and the ampicillin resistance genes in the PCR2.1 vector (see Figure 2.12).

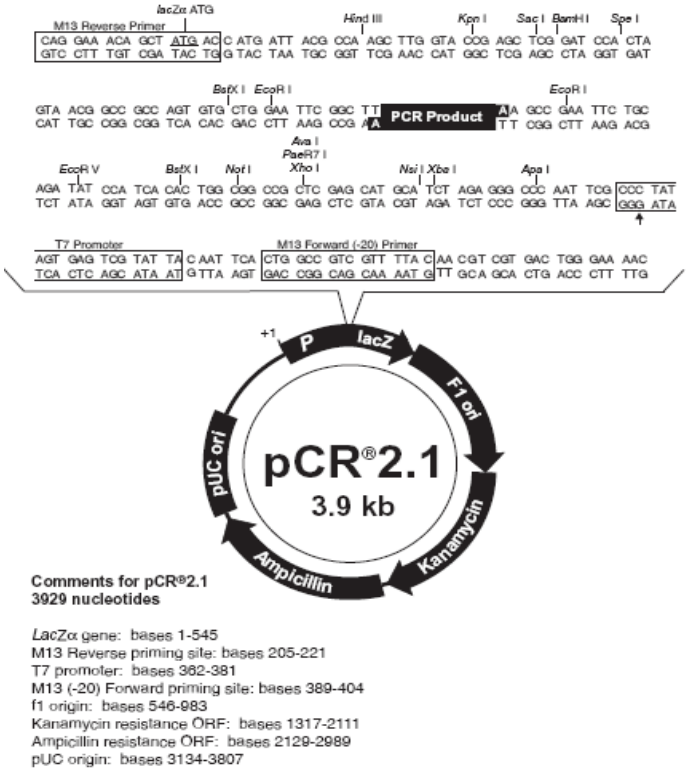


Figure 2.12: Structure of the linearized pCR 2.1 vector. Multiple cloning site shown with PCR product inserted by TA cloning. The arrow indicates the start of transcription for the T7 RNA polymerase. *EcoR1* sites flank the inserted PCR product on each side. Source: Invitrogen technical bulletin[312].

2.3.14 Plasmid purification of selected colonies

White colonies were grown overnight at 37^{o}C at 225rpm and plasmids were purified using the QIAprep Spin Miniprep Kit protocol according to manufacturer's instructions (QIAGEN). Briefly, bacterial cells were pelleted at 3000rpm for 10 minutes and resuspended in 250µl buffer P1 (containing RNAse A). 250µl buffer P2 was added gently inverting the samples to avoid shearing of DNA. 350µl buffer N3 was added repeating the inversion followed by centrifugation at 13000rpm for 10 minutes decanting the supernatant to a QIAprep column.

Samples present in the columns were centrifuged and the flow-through discarded. 500µl buffer PB was added to remove trace nuclease activity followed by centrifugation and discard of the flow-through. 750µl buffer PE was added to wash the DNA followed by another centrifugation to remove residual wash buffer. The DNA was eluted into a clean centrifuge tube after adding 50µl buffer EB.

2.3.15 Sequencing of amplified RT-PCR products

Sequencing was performed with the assistance of Mrs D Heinemann-Culpan (see Acknowledgements). The sequence of the presumed human $VEGF_{189}$ product was confirmed as human $VEGF_{189}$ (see Figure 2.13 below).

NTNCAGCTTGGNCGAGCTCGGATCCTAGTAACGGCCGCCAGTGTGCTGGAATTCGGCT
TTCACCGCCTCGGCTTGTCACATCTGCAAGTACGTTCGTTTAACTCAAGCTGCCTCGCC
TTGCAACGCGAGTCTGTGTTTTTGCAGGAACATTTACACGTCTGCAGATCTTGTACAAA
CAAATGCTTTCTCCGCTCTGAGCAAGGCCCACAGGGATTTTCTTGTCTTGCTCTATCTTT
CTTTGGTCTGCATTCACATTTGTTGTGCTGTAGGAAGCTCATCTCAAGCCGAATTCTGCA
GATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTA
TAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACC
CTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAAT
AGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAA
TGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCACGT
GACCGCTACACTTGCCAGCGCCCTACCGCCGCTCCTTTCGCTTTCTCCTTCCTTCTCGC
CACGTTCGCCGGCTTTCCCGTCAACTCTAATCGGGGGCTCCTTTAGGTTCGAATTAGTG
CTTTACGGCACTCGACCCNAAAAATTGATAAGGTGAGGGTCACGTANGGGCATCCCTG
ANAAAGGTTTCCCCTTTACTNGANCNGTCTTANNNGACCTGTCAATGGAAACCTACCAC
CGGCANTTTTGATAAAGA

Figure 2.13: Sequenced product confirmed as human $VEGF_{189}$. N denotes non-sequenced nucleotide.

$VEGF_{121}$, $VEGF_{165}$, $VEGF_{165}$b cDNA templates were used as positive control for the RT-PCR. These had been expressed in a $pcDNA_3$ expression vector as previously described[180]. Their identity had previously been confirmed by sequencing and they were kindly donated by Dr David Bates (see Acknowledgements).

2.3.16 ELISA

Plasma and BAL VEGF levels were measured using a commercial sandwich ELISA kit according to manufacturer's instructions (R&D Systems, Abingdon, UK). Briefly, a mouse monoclonal antibody specific for VEGF was precoated onto a microplate. 100µl (50µl for BAL samples) of assay diluent was added to each well. 100µl (or 200µl for BAL samples) of standards, controls or samples in duplicate were then pipetted into the wells. After washing in an ELISA plate washer (Tecan, UK), 200µl of polyclonal-labelled detection antibody specific for VEGF conjugated to horseradish peroxidase was added. Following a further wash, a 200µl of substrate solution (equal volumes of hydrogen peroxide and tetramethylbenzidine) was added to the wells yielding a blue colour leaving for 25 minutes in the dark (20 minutes for BAL samples). The reaction was stopped with 50µl of stop solution (1M sulphuric acid) giving a yellow colour proportional to the amount of VEGF present. The intensity of the colour reaction was read spectrophotometrically in a microplate reader at 450nm using Biolinx 2.1 software connected to a MR7000 Dynatec plate reader (Dynex technologies). Wavelength correction at 570nm was used to correct for optical imperfections on the plate. Background intensities in wells containing no sample but ELISA reagents only were subtracted from all values.

This ELISA has a mean intra-assay variability of 5.4% for plasma and 4.7% for BAL samples. The mean inter-assay variability is 7.3% for plasma and 6.7% for BAL samples. Standard curves were created from concentrations varying from 15.6 to 1000 pg/ml (see Appendix). The lower limit of detection was 2.9 pg/ml. All samples were repeated in duplicate. This assay measures biologically free and active $VEGF_{121}$ and $VEGF_{165}$ that is not bound to *sflt*. Previous experiments have indicated there is little/no free VEGF binding capacity within the plasma or BALF of ARDS patients (94% recovery of a known amount of VEGF spiked into plasma and 102% recovery into BAL – Thickett DR, thesis).

2.3.17 Induced Heteroduplex Generator (IHG) Analysis

I used the technique of IHG analysis to allow simple, rapid and unequivocal genotyping[313-316]. Briefly, the principle of heteroduplex analysis (see Figure 2.14 below) depends on formation of heteroduplexes with an altered electrophoretic mobility relative to homoduplexes.

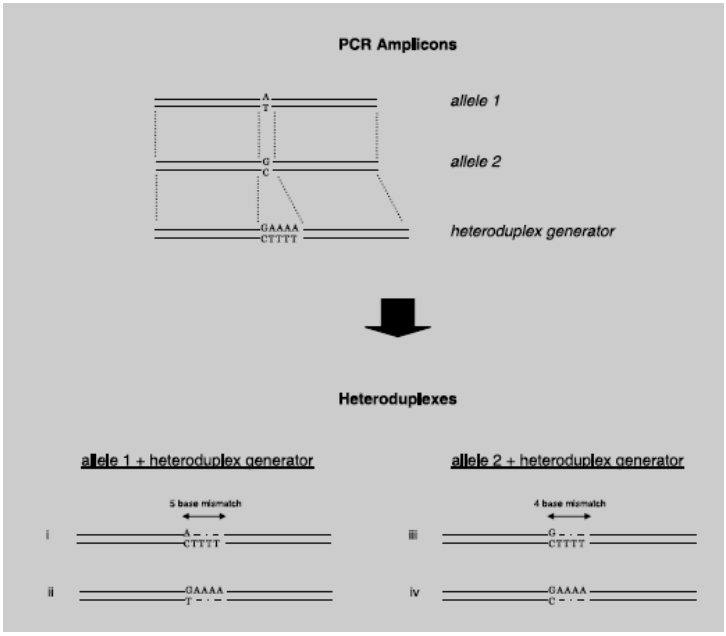


Figure 2.14: Diagrammatic representation of IHG principle. In the above Figure, a single mutation AT/GC is present. The synthesized IHG is an exact copy of allele 2 except for a 4 base insert next to the site of the mutation. Gene fragments containing the polymorphic site are amplified using PCR amplifying the IHG separately. Products from the gene fragments and IHG are mixed, heated and cooled allowing heteroduplex formation. These differ in molecular conformation with a 5-base mismatch on heteroduplexes i and ii due to the presence of the polymorphic base and the 4 base insert compared to a 4-base mismatch on heteroduplexes iii and iv due to the presence of the 4 base insert but no polymorphic site. This therefore allows identification by electrophoresis of allele 1 and 2 in any combination. Adapted from: LJ Keen[314], MJ Bown et al[317].

The mismatch tends to usually retard the migration of DNA during electrophoresis and can result in a large structural perturbation of a "bulge" (due to insertion or deletion) type. "Bubble" (due to single base mutation) types tend to lead to more subtle changes in structure and are hence not usually resolvable by gel electrophoresis.

This technique is ideal for rapid analysis of known mutations. An IHG reagent (either with a 2-5 base pair insert or deletion next to the site of mutation) is amplified separately by PCR as well as the DNA target under study. Products from the target and IHG PCRs are mixed, heated and cooled allowing heteroduplexes to form. The heteroduplexes formed will either have both "bubble" and "bulge" (mutation and insert/deletion) structural perturbations or "bulge" only (no mutation but insert/deletion) resolvable by electrophoresis.

An IHG reagent was synthesized as a long oligonucleotide before purification. The patient samples and IHG reagents were amplified separately by PCR using standard conditions described previously[313]. Briefly, PCR mixes (50mml) contained 0.5mmM each of forward and reverse primers (VEGF forward: 5'-TTTGGGT CCGGAGGGCGAGA-3', VEGF reverse: 5'-TTCCGGGCTCGGTGATTTAGC-3') 2.5mM $MgCl_2$, 200mmM of each dNTP, 1 x *Taq* polymerase buffer (75mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% V/V Tween), 0.5 unit *Taq* polymerase (Advanced Biotechnologies) and either diluted IHG reagent or 500ng genomic DNA.

Following an initial denaturation at 95^{o}C for 5 minutes, 35 cycles of 95^{o}C for 1 minute, annealing at 61^{o}C for 1 minute and 72^{o}C for 1 minute was performed, followed by a final extension at 72^{o}C for 7 minutes. Equal volumes of amplicons from genomic DNA and IHG reagents were mixed, denatured at 95^{o}C for 5 minutes and allowed to cool slowly using controlled ramping to 37^{o}C over a 30 minute period.

Heteroduplexes were resolved by electrophoresis for 90 minutes at 200 V in 15% non-denaturing polyacrylamide "triple-wide" mini-gels (37.5:1 (W/V) acrylamide: bisacrylamide; National Diagnostics, containing 1 x TBE electrophoresis buffer) and visualized on a Kodak digital imaging system using a 302 nm UV trans-illuminator by post-staining in ethidium bromide (5 minutes in 1 x TBE containing 0.5 mg/ml ethidium bromide).

2.3.18 Statistical analysis

Unless stated, all statistical calculations and manipulations were performed using Graph Pad Prism version 4.0 statistical software (Graph Pad, San Diego, California, USA). The Ryan-Joiner test was used to assess the data for normality. Normal data with multiple comparisons were analysed by ANOVA with *post hoc* Bonferroni analysis to add stringency. Nonparametric data with multiple comparisons were analysed by Kruskal-Wallis with *post hoc* Dunn's analysis. Otherwise normal data were compared by student t test (paired or unpaired as appropriate). Nonparametric data otherwise were analysed with Mann-Whitney. Quanto version 1.1 software (http://hydra.usc.edu/gxe/), was used for power calculations in the genetic association study (see Chapter 5)[318 319]. Genotype and allele frequencies were compared by the Fishers exact test to control for low cell values or Chi Squared test if more than 2 x 2 contingency configuration. Hardy-Weinberg equilibrium was assessed using the Chi Squared test. Standard curve data for MTS proliferation assay, RT-PCR and ELISA were analysed by linear regression with plots showing goodness of fit as a dotted line and r^2 values with p values. For all tests, a p value of 0.05 or less was considered significant.

CHAPTER 3:
VEGF ISOFORMS AND RECEPTORS IN ARDS

This chapter describes experiments to examine the expression of VEGF isoform transcripts ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) and its specific receptors in the normal and injured human lung.

Part of this chapter has been published in abstract form in Thorax.

Medford ARL, Armstrong L, Ibrahim NBN, Uppington KM, Millar AB.

Pulmonary vascular endothelial growth factor (VEGF) receptor expression in acute respiratory distress syndrome (ARDS).

Thorax 2002 57(Suppl III): iii69 [P74].

SUMMARY

Background

VEGF has properties that suggest a potential role in ARDS. It was hypothesized that VEGF receptors would be expressed in human lung and that there would be a significant change in VEGF isoform and receptor expression in injured and normal human lung.

Methods

Expression of VEGF and specific receptors was assessed by immunohistochemistry (for protein) and isoform-specific RT-PCR (for RNA) in human normal and ARDS lung.

Results

VEGF and its receptors were expressed at the protein level on alveolar epithelium, macrophages and vascular endothelium and significant changes occurred with the presence and timing of lung injury. In particular, in early ARDS, VEGF protein (and isoform mRNA) and receptor expression were reduced compared to later ARDS. No evidence of isoform switching at mRNA level was detected.

Interpretation

VEGF targets are expressed on both sides of the alveolar-capillary membrane and upregulated in later ARDS consistent with an autocrine role for VEGF in the lung. These changes suggest dynamic alterations in VEGF bioactivity in acute lung injury. Isoform switching does not occur but this does not preclude a role in recovery from lung injury.

3.1 INTRODUCTION

As reviewed in Chapter 1 (sections 1.1.1 to 1.1.7), ARDS is the most extreme form of acute lung injury, characterised by noncardiogenic pulmonary oedema, neutrophilic alveolitis and the development of potentially reversible fibrosis. It continues to have an unacceptable morbidity and mortality[2].

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor of critical importance in vascular development[108]. As discussed in detail in Chapter 1 (section 1.2.1), certain biological properties (especially as a mitogen and permogen) of VEGF have, led to investigating its possible role in ARDS[2].

3.1.1 Background

Observational data show plasma VEGF levels rise and intrapulmonary levels fall in the early stages of lung injury with normalisation of both during recovery[244 247]. These changes in intrapulmonary VEGF have also been noted by other investigators in ARDS and high-altitude pulmonary oedema[248 249]. As reviewed in Chapter 1 (section 1.2.9), cellular and animal studies conflict on the role of VEGF in the alveolar space. Much of the data suggesting a pathological role may indicate secondary responses to more critical events and the overexpression models may have involved variable damage to the alveolar-capillary membrane exposing the underlying endothelium to the higher physiological levels of VEGF in the air space leading to excessive oedema. Timepoints in the animal models and clinical studies also differed. In addition, these data do not account for the consistent findings of abundant VEGF in the normal lung as well as the accumulating data consistent with a protective role for VEGF in the lung.

There are other potential explanations for the observed reduction in intrapulmonary VEGF levels in early ARDS. One mechanism would be by increased expression of VEGF receptors. Another possible mechanism would be a shift towards predominance of cell-associated isoforms or a relative deficiency of soluble isoforms that would reduce freely diffusible detectable VEGF in biological fluids.

VEGF protein is compartmentalised to high levels in normal human epithelial lining fluid and human ATII cells express significant amounts of VEGF protein *in vitro*[128] [239]. As discussed in Chapter 1, (sections 1.2.3), VEGF exerts its biological effect through specific receptors. VEGFR1 may act as a decoy receptor[140] [141]. VEGFR2 is thought to be the main signalling receptor[144] [145]. VEGFR3 is confined to lymphatic endothelium hence was not assessed in this study[148]. Isoform-specific binding of $VEGF_{165}$ to neuropilin 1 co-receptors (NRP-1) augments VEGFR2 signalling activity[149]. As discussed in chapter 1 (section 1.2.4), alternative splicing of the VEGF transcript from exons 5 to 8 leads to the generation of several different length VEGF isoforms with variable diffusibilities depending on their length[160] [165].

3.1.2 Hypothesis

This thesis is based upon the hypothesis that VEGF has an important role in repair and recovery from lung injury. In this chapter, data concerning the following questions is presented and discussed:

- Are VEGF and its specific receptors (VEGFR1, VEGFR2 and NRP-1) expressed in human lung commensurate with a role there?
- Is there a significant change in expression in the injured lung both of functional VEGF isoforms and its specific receptors?
- As ATII cells are key to alveolar repair following injury, is VEGF co-localised with an ATII cell specific marker?

3.2 METHODS

For detailed methods on specimens, immunohistochemistry, paraffin RNA extraction and VEGF isoform RT-PCR see Chapter 2.

3.2.1 Specimens

Archival normal and ARDS lung tissue sections and paraffin blocks were obtained from Frenchay Pathology Department. The North Bristol NHS Trust Local Research Ethics Committee approved this study.

Normal, early and late ARDS lung tissue sections were obtained. Normal lung tissue implied that there was no lung involvement in the cause of death. ARDS lung tissue was subdivided into "early" (within 48 hours of onset) and "late" (after day 7). The underlying cause of ARDS was either remote (gut) or direct (intrapulmonary) sepsis in all cases.

3.2.2 Immunohistochemistry

Detailed methods and concentrations of antibodies are described in Chapter 2 (section 2.3.3). Rabbit polyclonal antibodies to VEGF, VEGFR1, VEGFR2 and NRP-1 (Autogen Bioclear, UK Ltd, Wiltshire, UK) were used as primary antibodies. The VEGF antibody is known to detect $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ isoforms unlike the mouse monoclonal ELISA VEGF antibody (R&D), which detects only the soluble ($VEGF_{121}$, and $VEGF_{165}$) isoforms. Dual staining for alveolar epithelium was performed using Vector VIP substrate. Rabbit polyclonal antibodies to aquaporin-3 (AQP-3, an ATII cell marker) were used as the primary antibody with conditions otherwise as described above.

3.2.3 Paraffin RNA extraction and VEGF isoform RT-PCR

RNA was extracted from formalin-fixed paraffin-embedded tissue sections using the Krafft technique[306]. Detailed methods are described in Chapter 2 (section 2.3.9). VEGF isoform-specific RT-PCR was performed on the RNA as described in Chapter 2 (section 2.3.10). Samples were visualised by agarose electrophoresis with ethidium bromide to allow image capture via a transilluminator and semiquantitative densitometry (section 2.3.11).

3.2.4 Statistical analysis

All statistical manipulations and analyses were performed using Graph Pad Prism version 4.0 software. Data in bar charts are plotted as mean and standard error. Semiquantitative immunostaining Histometrix pixel staining densities were normally distributed as assessed by the Ryan-Joiner test. Because of the necessity for multiple comparisons of the data, ANOVA testing was followed by Bonferroni *post hoc* analysis. Absolute and proportionate RT-PCR densities were normally distributed and the data were analysed by Student's t test. A p value of < 0.05 was considered significant.

3.3 RESULTS

3.3.1 Indirect Immunohistochemistry

Indirect immunohistochemistry revealed no evidence of non-specific staining using isotypic negative control antibodies confirming that any positive staining was specific to the antibody of interest (see Figure 3.1).

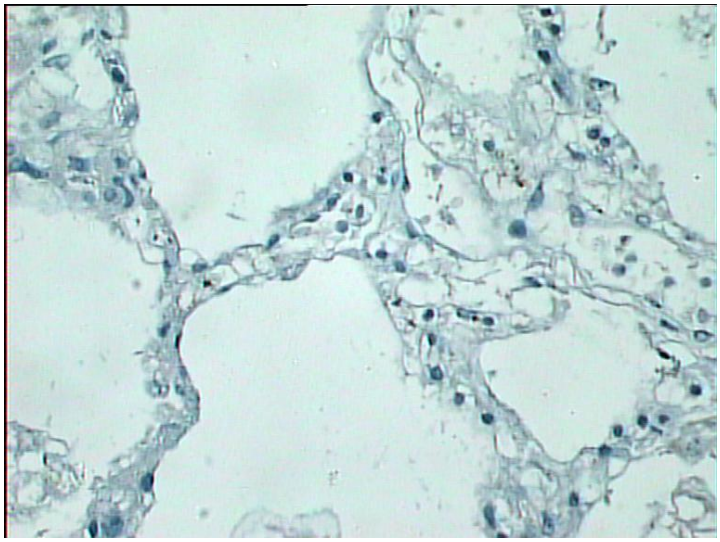


Figure 3.1: Isotypic control staining in normal lung (x 40).

VEGF expression in general was most obviously detected on direct analysis of stained sections on alveolar epithelium and macrophages but also present on vascular endothelium. A typical example section from later ARDS lung is shown in Figure 3.2.

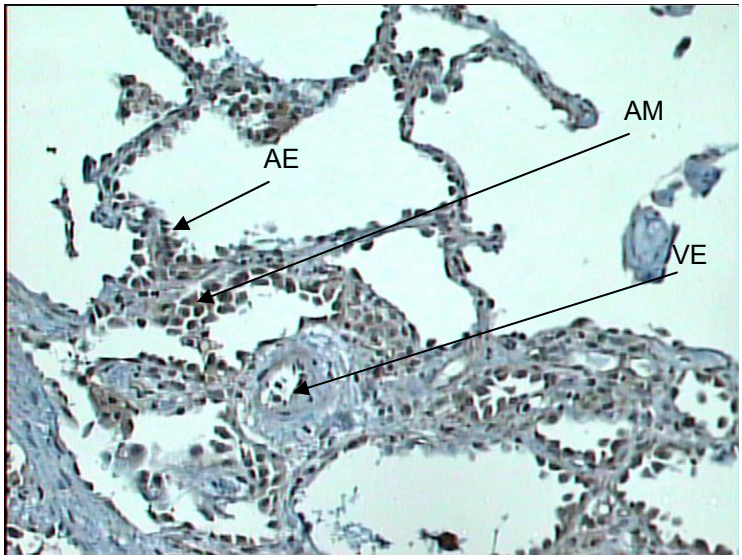
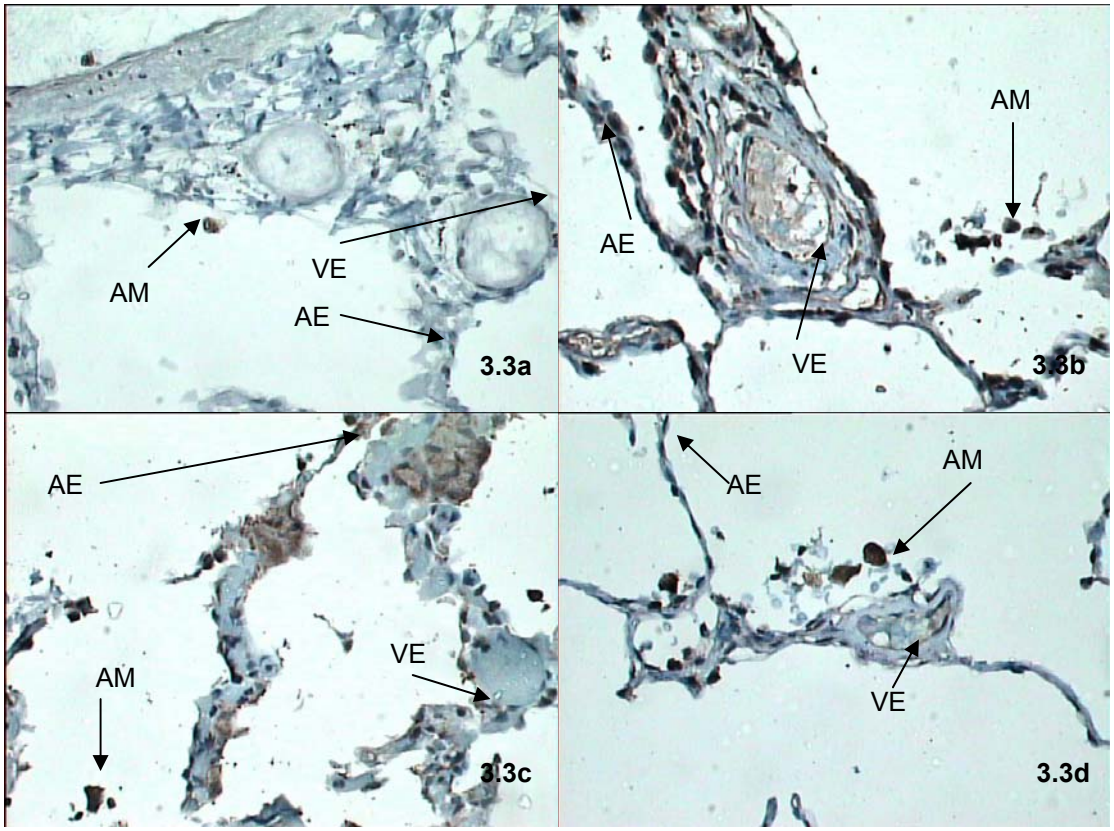


Figure 3.2: VEGF expression in later ARDS lung (x 20). Vascular endothelium, alveolar macrophages and epithelium all positive for VEGF staining as labelled by key below.
Abbreviations:
AE: alveolar epithelium
AM: alveolar macrophage
VE: vascular endothelium

In normal lung sections, VEGF and its receptors (VEGFR1, NRP-1 and VEGFR2) were expressed at apparent varying intensity on alveolar epithelium, macrophages and vascular endothelium (see Figures 3.3 a-d).



Figures 3.3 a-d: VEGF (a) and VEGFR2 (b), NRP-1 (c) and VEGFR1 (d) expression in normal lung (x 40) (apparent varying intensity).

Abbreviations:

AE: alveolar epithelium

AM: alveolar macrophage

VE: vascular endothelium

Densitometry (see Figure 3.4a) confirmed significantly greater staining for VEGFR2 than VEGFR1 in normal lung as suggested on visual inspection of single stain normal lung immunohistochemistry sections (see Figures 3.3a-d).

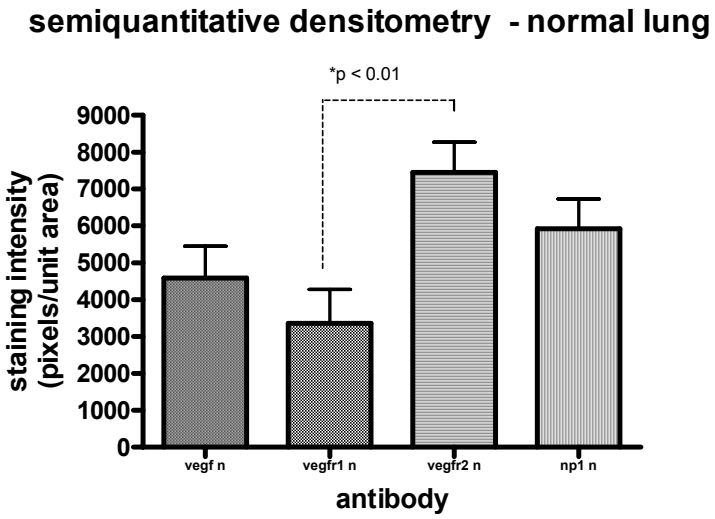


Figure 3.4a: Graph of semiquantitative immunostaining densities for VEGF, VEGFR1 and NRP-1 in normal lung respectively. All data are normally distributed and plotted as mean and standard error. p = 0.008 (ANOVA), p < 0.01 (Bonferroni) for VEGFR1 v VEGFR2 (highlighted*), otherwise all other comparisons not significant.

In early ARDS, reduced alveolar expression of especially VEGF and VEGFR2 was noted. This may partly be due to the well-described loss of alveolar epithelium in early ARDS. However, a significant reduction in staining was confirmed densitometrically for all the molecules studied compared to later ARDS (see Figures 3.5a-d, Figures 3.4a-g).

However, in later ARDS there was marked upregulation of expression and this was confirmed densitometrically (see Figures 3.6a-d, Figures 3.4c-g). The increased staining noted on densitometry suggesting both an increased intensity of staining and an increase in cell number, which reflects the recovery/proliferative phase of "later" lung injury/ARDS.

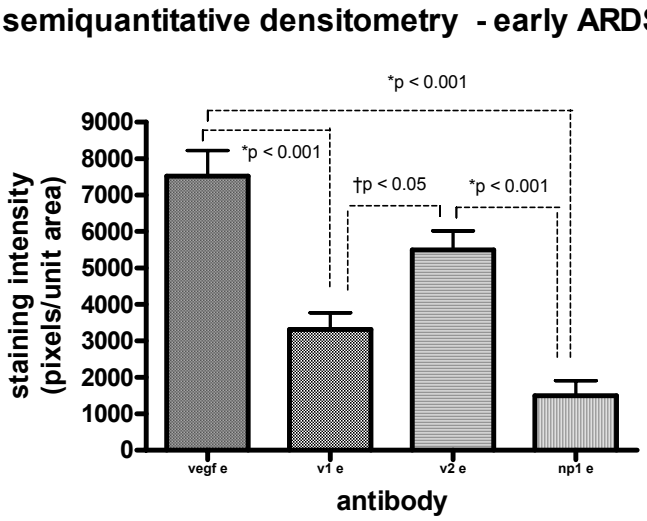


Figure 3.4b: Graph of semiquantitative immunostaining densities for VEGF, VEGFR1, VEGFR2 and NRP-1 respectively in early ARDS lung. All data are normally distributed and plotted as mean and standard error. p = 0.008 (ANOVA), p < 0.001 (Bonferroni) for VEGF v VEGFR1 and NRP-1; VEGFR2 v NRP-1 (highlighted*); p < 0.05 (Bonferroni) VEGFR1 v VEGFR2 (highlighted†), otherwise all other comparisons not significant.

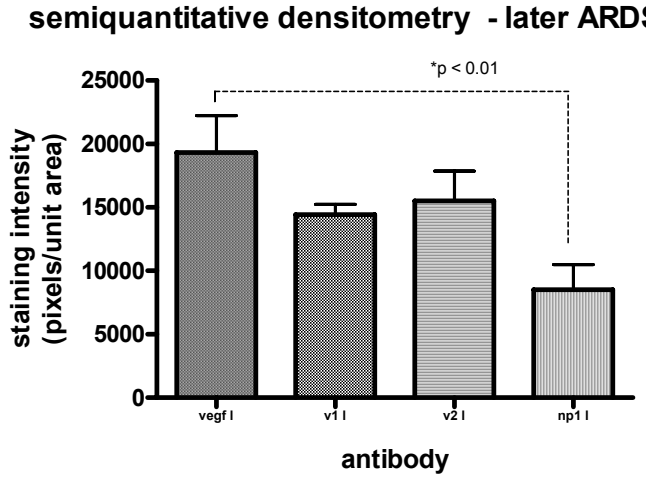


Figure 3.4c: Graph of semiquantitative immunostaining densities for VEGF, VEGFR1, VEGFR2 and NRP-1 respectively in later ARDS lung. All data are normally distributed and plotted as mean and standard error. p = 0.008 (ANOVA), p < 0.01 (Bonferroni) for VEGF v NRP-1, (highlighted*); otherwise all other comparisons not significant.

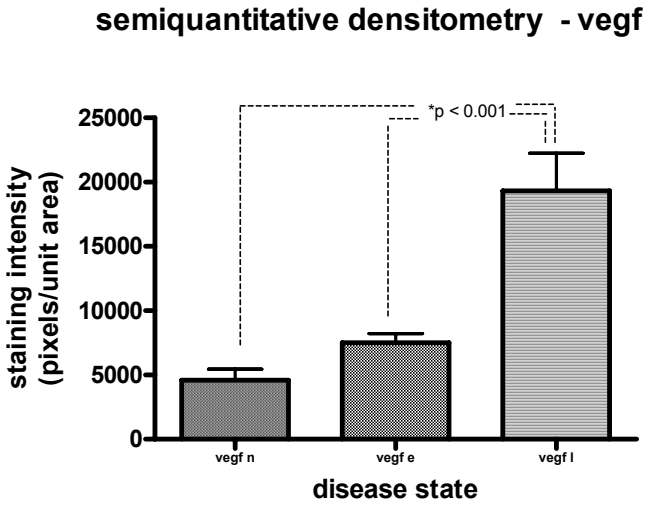


Figure 3.4d: Graph of semiquantitative immunostaining densities according to disease state for VEGF. All data are normally distributed and plotted as mean and standard error. p < 0.0001 (ANOVA), p < 0.001 (Bonferroni) for normal v late, early v late, (highlighted*); otherwise other comparisons not significant.

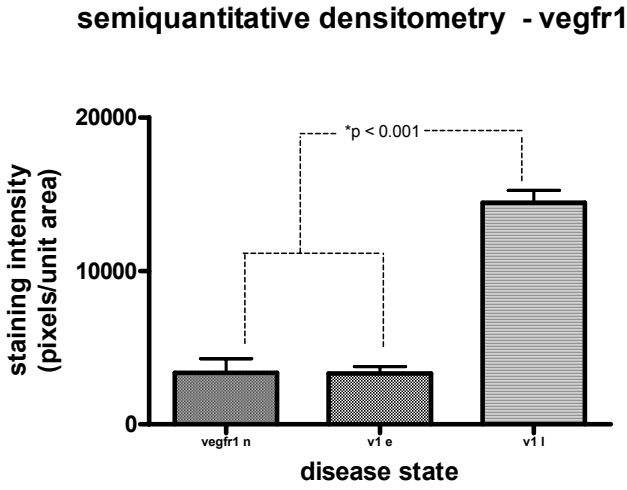


Figure 3.4e: Graph of semiquantitative immunostaining densities according to disease state for VEGFR1. All data are normally distributed and plotted as mean and standard error. p < 0.0001 (ANOVA), p < 0.001 (Bonferroni) for normal v late, early v late, (highlighted*); otherwise other comparisons not significant.

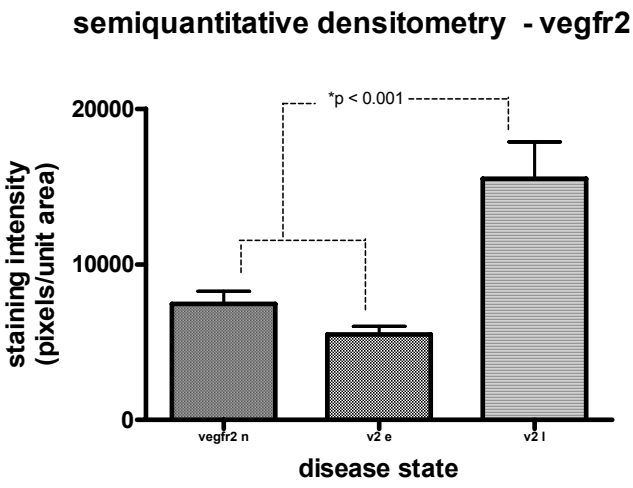


Figure 3.4f: Graph of semiquantitative immunostaining densities according to disease state for VEGFR2. All data are normally distributed and plotted as mean and standard error. p < 0.0001 (ANOVA), p < 0.001 (Bonferroni) for normal v late, early v late, (highlighted*); otherwise other comparisons not significant.

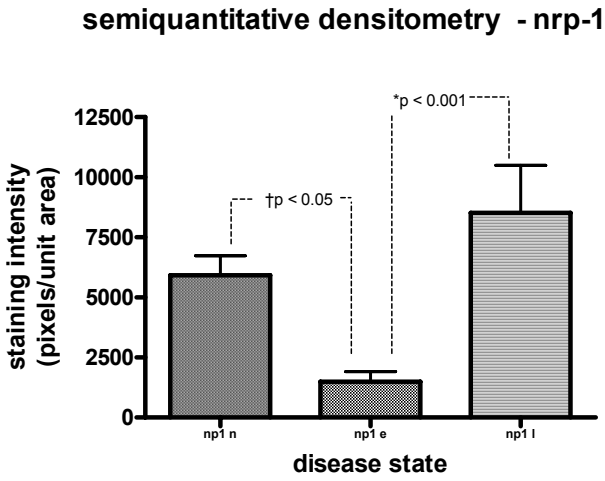
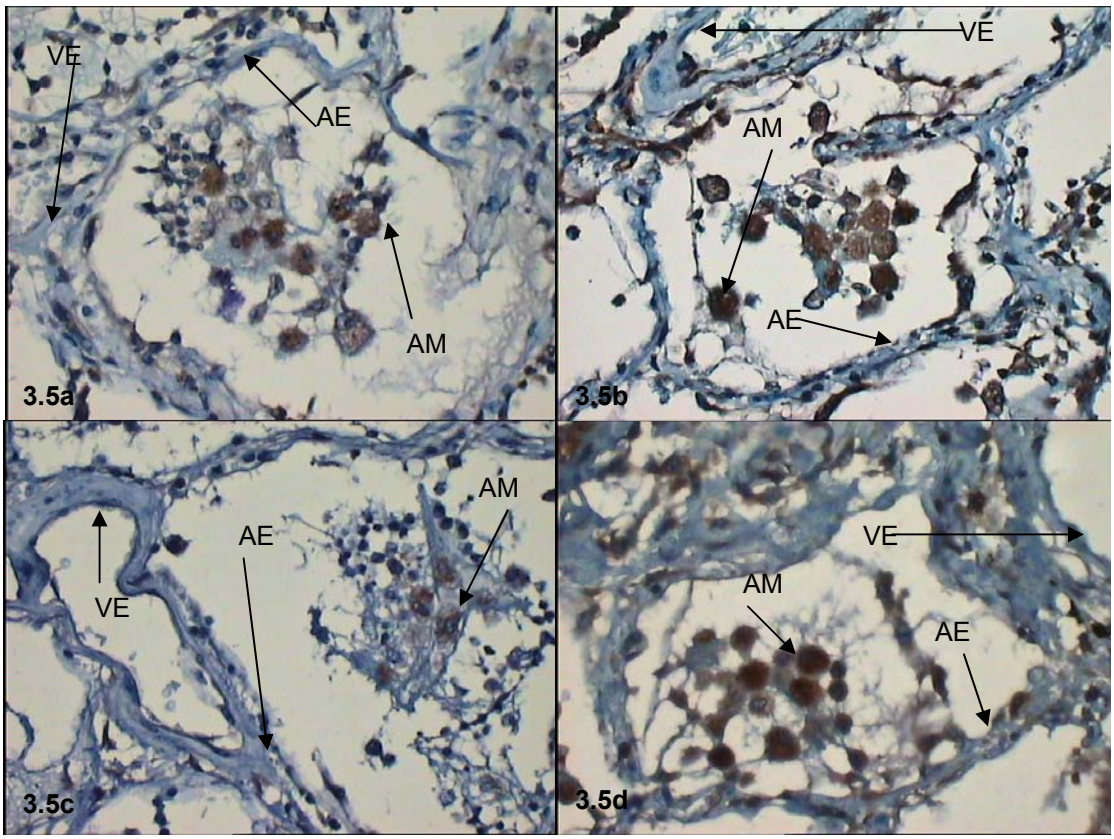


Figure 3.4g: Graph of semiquantitative immunostaining densities according to disease state for NRP-1. All data are normally distributed and plotted as mean and standard error. p = 0.0002 (ANOVA), p < 0.001 (Bonferroni) for early v late (highlighted*); p < 0.05 (Bonferroni) for normal v early (highlighted†); otherwise other comparisons not significant.



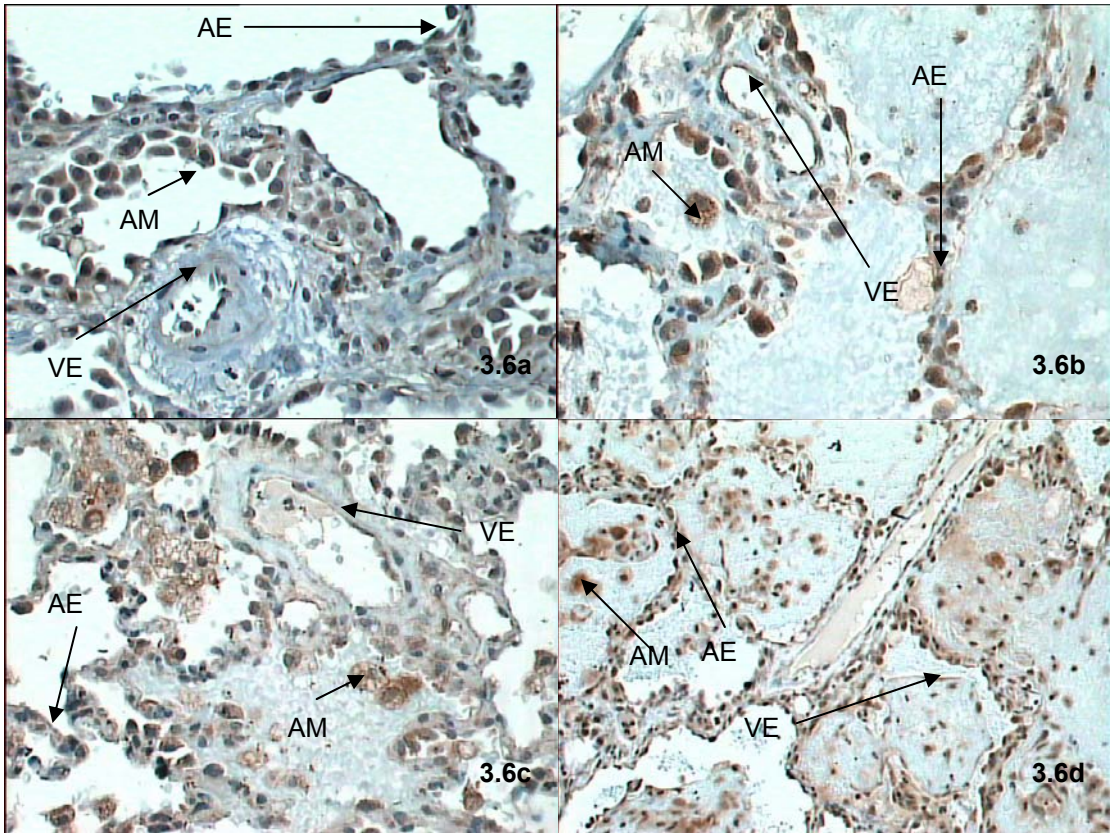
Figures 3.5 a-d: VEGF (a) and VEGFR2 (b), NRP-1 (c) and VEGFR1 (d) expression in early ARDS showing consistently reduced alveolar expression especially for VEGF and VEGFR2 (highlighted) (x 40). Endothelial and macrophage expression as before.

Abbreviations:

AE: alveolar epithelium

AM: alveolar macrophage

VE: vascular endothelium



Figures 3.6 a-d: VEGF (a) and VEGFR2 (b), NRP-1 (c) and VEGFR1 (d) expression in later ARDS lung (x 40). Increased expression of all molecules noted on alveolar epithelium, alveolar macrophages and vascular endothelium. Staining of higher intensity and also increase in cell numbers, specifically alveolar macrophages and alveolar epithelium.

Abbreviations:

AE: alveolar epithelium

AM: alveolar macrophage

VE: vascular endothelium

3.3.2 Immunohistochemistry semiquantitative densitometry

As already displayed, Histometrix analysis and semiquantitative densitometry results are shown in Figures 3.4a-g. They graphically present one set of data in two ways for ease of comparison. Figures 3.4a-c denote the densitometry values for different antibodies in each disease state, acknowledging here conclusions assume no significant differences in antibody affinity and avidity. Figures 3.4d-g denote densitometry values for a specific antibody comparing disease states which are not subject to the same theoretical problems.

Densitometry values were significantly different (see Figures 3.4a-c) between VEGF and the studied receptors for normal, early and later ARDS suggesting heterogeneous expression of the studied molecules at different time points in injury, albeit subject to assumptions of similar antibody affinity and avidity as discussed earlier. Densitometry suggested VEGFR2 expression was highest in the normal lung, significantly higher than VEGFR1 (see Figure 3.4a). In early ARDS, there was a noted significant reduction in VEGFR1 and NRP-1 expression (see Figure 3.4b). Only the latter persisted in later ARDS lung (see Figure 3.4c).

In terms of disease states, there was a significant shift in expression for all the studied molecules (see Figures 3.4d-g). VEGF, VEGFR1 and VEGFR2 were all expressed significantly higher in later ARDS than either early ARDS or normal lung. NRP-1 uniquely was significantly downregulated in early ARDS with a larger significant upregulation in later ARDS but this was not significant compared to normal lung.

3.3.3 Dual staining

Dual staining for ATII cells using AQP-3 demonstrated co-localisation of VEGF on ATII cells (see Figures 3.7a and b).

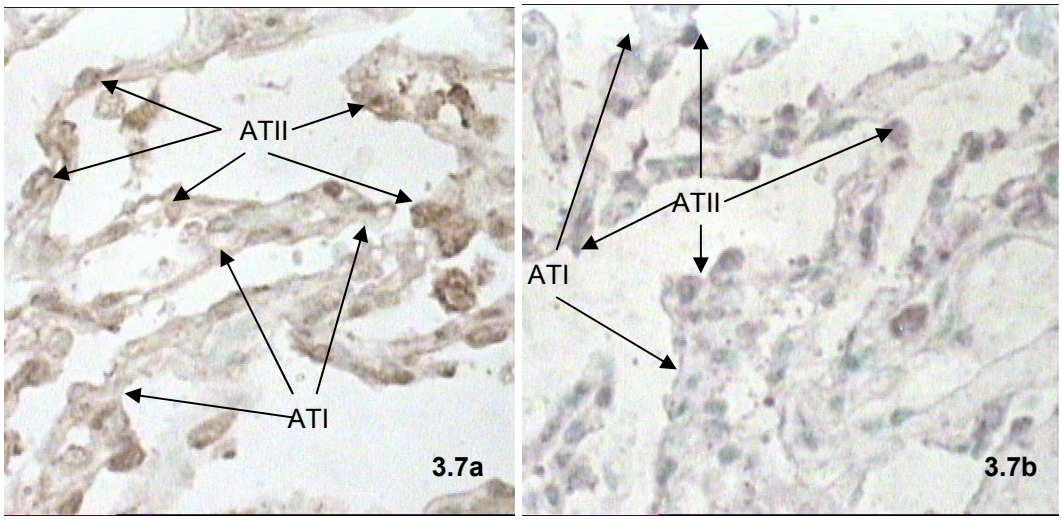


Figure 3.7a: Staining for VEGF (brown, DAB) in normal lung tissue (x 40). ATII cells and ATI cells arrowed. Figure 3.7b: Staining for AQP-3 (purple, VIP) in similar part of normal lung (x40). ATII cells and ATI cells arrowed. Co-localisation of VEGF staining with AQP-3 shown on ATII cells. Note also weak staining for VEGF on ATI cells.

Abbreviations:
ATI: Type 1 alveolar epithelial cell
ATII: Type 2 alveolar epithelial cell

3.3.4 VEGF isoform-specific RT-PCR semiquantitative densitometry

A typical semiquantitative RT-PCR gel from the FFPE tissue is shown in Figures 3.8a (the positive controls are shown in more detail in Figure 3.8b). Because of a problem with cross-linking for longer products (>275 bp), beta-2-microglobulin (B_2M) was used as a housekeeping gene (discussed in section 2.3.10).

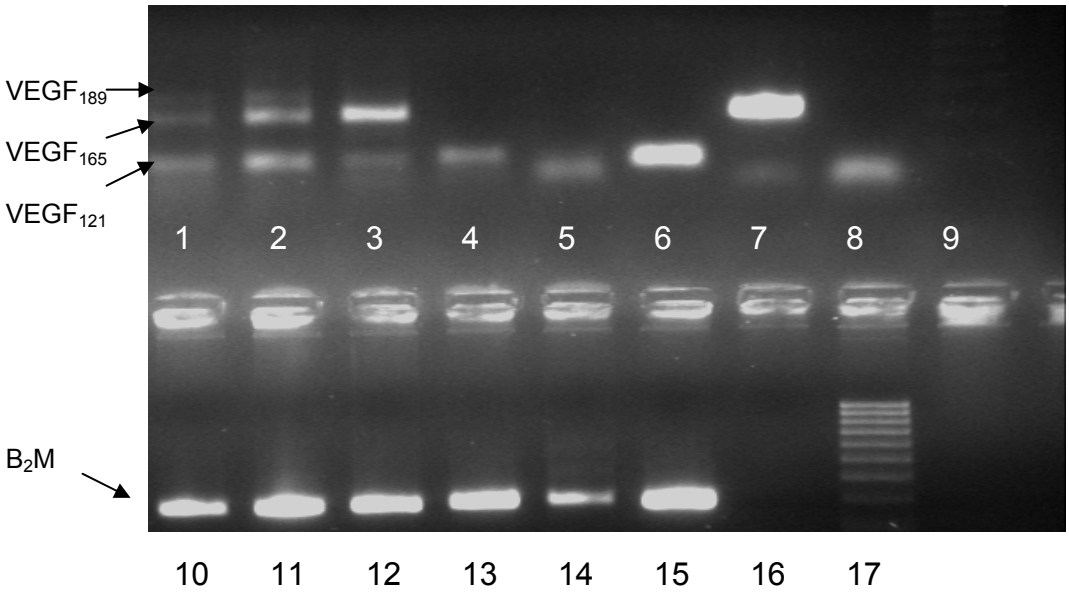


Figure 3.8a: Example RT-PCR gel showing VEGF isoform expression ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) next to 100 bp ladder. B_2M expression shown below. Positive control for $VEGF_{189}$ not shown (see Figure 3.8b for all positive controls).

Lanes as below:

1,10: normal lung

2-3,11-12: later ARDS

4,13: early ARDS

5,8,16: negative control

6: $VEGF_{121}$ positive control

7: $VEGF_{165}$ positive control

9,17: 100bp ladder

14,15: B_2M positive control

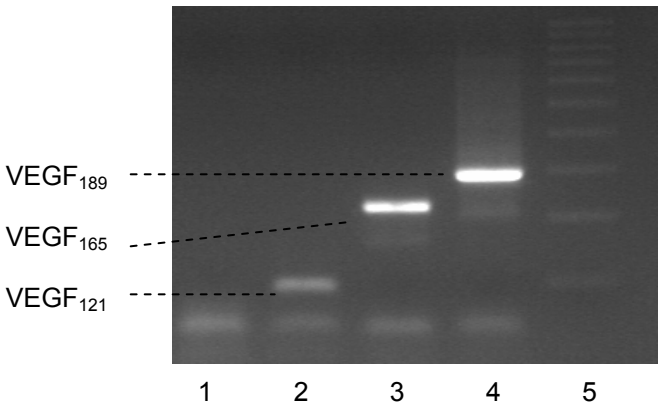


Figure 3.8b: Example RT-PCR gel showing VEGF isoform expression ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) next to 100 bp ladder with all negative and positive controls. Lanes as below:

1: negative control

2: $VEGF_{121}$ positive control

3: $VEGF_{165}$ positive control

4: $VEGF_{189}$ positive control

5: 100bp ladder

Semiquantitative densitometry revealed no differences except a significant increase in $VEGF_{189}$ in late versus early ARDS ($p = 0.006$, t test) (see Figure 3.9).

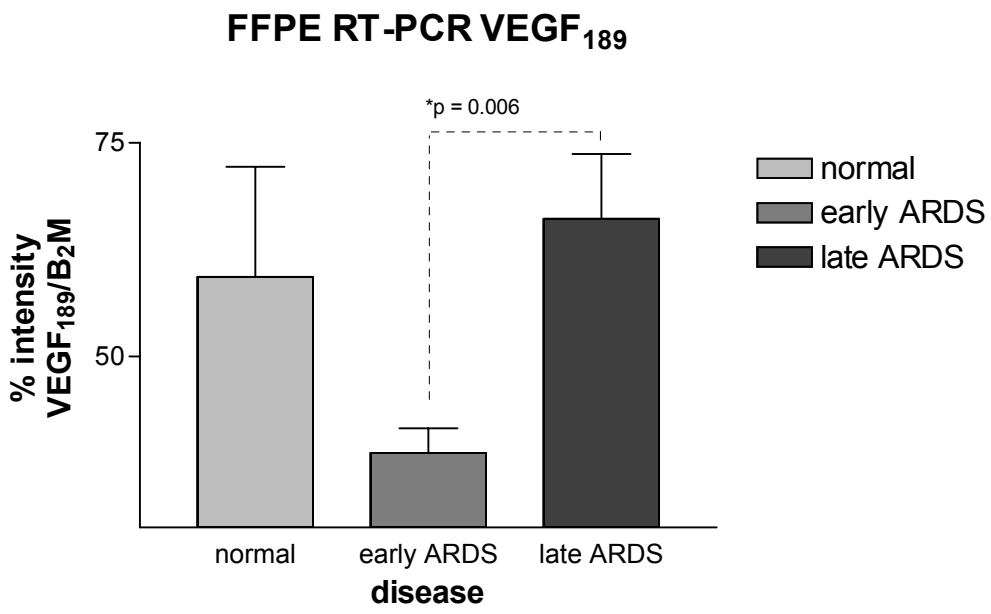


Figure 3.9: Semiquantitative densitometry of $VEGF_{189}$ relative to B_2M (n = 5). Data expressed as mean and standard error. P values: normal v early, p = 0.12; normal v late, p = 0.66; early v late p = 0.006 (highlighted*).

For $VEGF_{165}$, semiquantitative densitometry revealed no differences except a significant increase in $VEGF_{165}$ in late versus early ARDS (p = 0.005, t test) (see Figure 3.10).

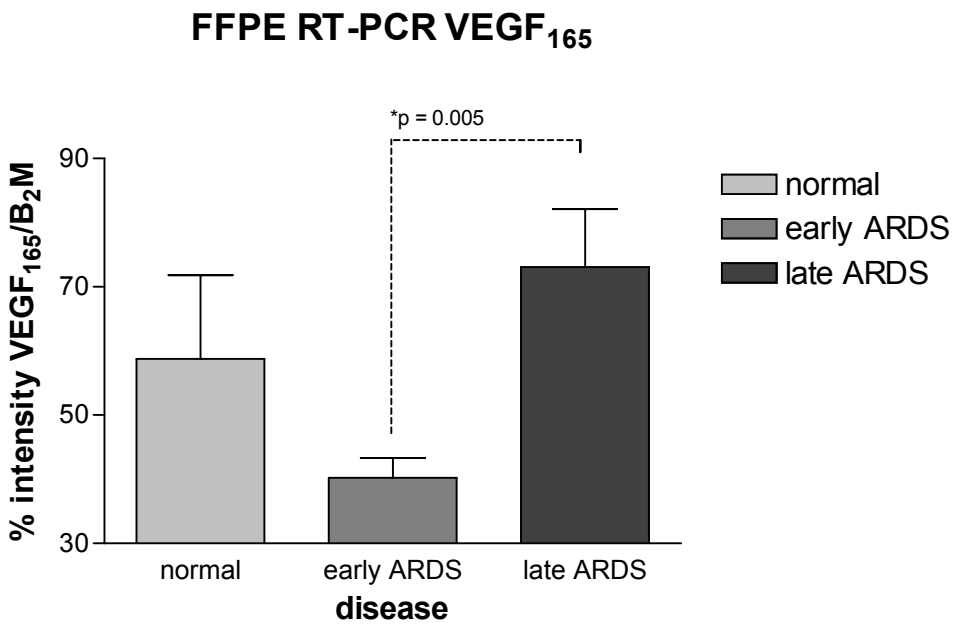


Figure 3.10: Semiquantitative densitometry of $VEGF_{165}$ relative to B_2M (n = 5). Data expressed as mean and standard error. P values: normal v early, p = 0.16; normal v late, p = 0.39; early v late p = 0.005 (highlighted*).

For $VEGF_{121}$, semiquantitative densitometry revealed no statistical differences except a significant increase in $VEGF_{121}$ in late versus early ARDS (p = 0.0005, t test) (see Figure 3.11).

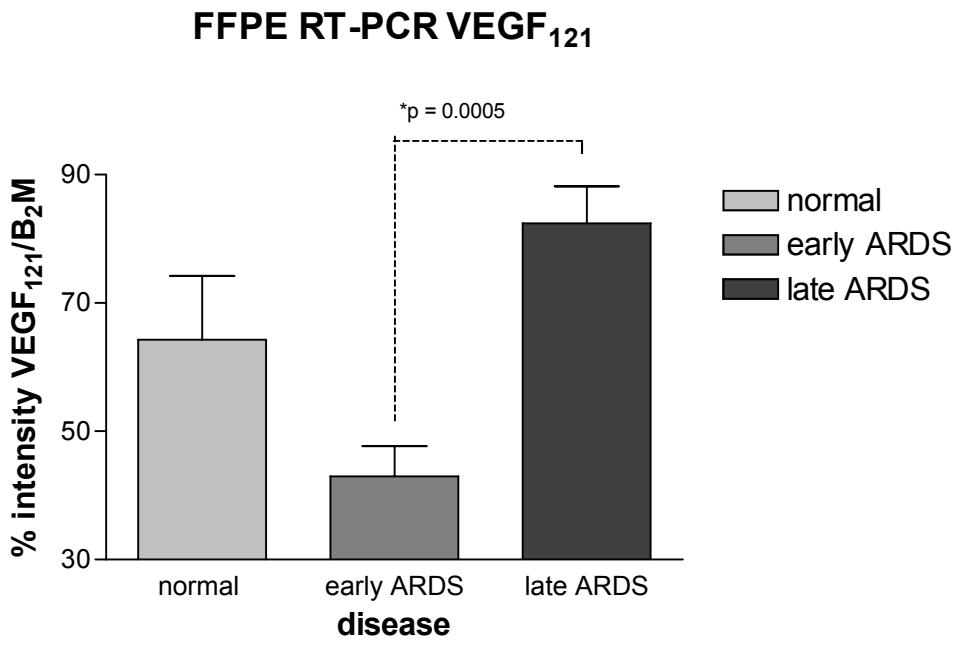


Figure 3.11: Semiquantitative densitometry of $VEGF_{121}$ relative to B_2M (n = 5). Data expressed as mean and standard error. P values: normal v early, p = 0.07; normal v late, p = 0.15; early v late p = 0.0005 (highlighted*).

In summary, a consistent reduction in all measured isoforms was noted in early lung injury that normalised in later ARDS.

In terms of proportionate VEGF isoform transcriptional expression, there was no evidence of isoform switching between disease states (see Figure 3.12, p = 0.93). In all stages of disease, $VEGF_{121}$ was noted to constitute the highest percentage proportion but this was not significant (p = 0.54 normal lung, p = 0.78 late ARDS lung, p = 0.96 early ARDS lung).

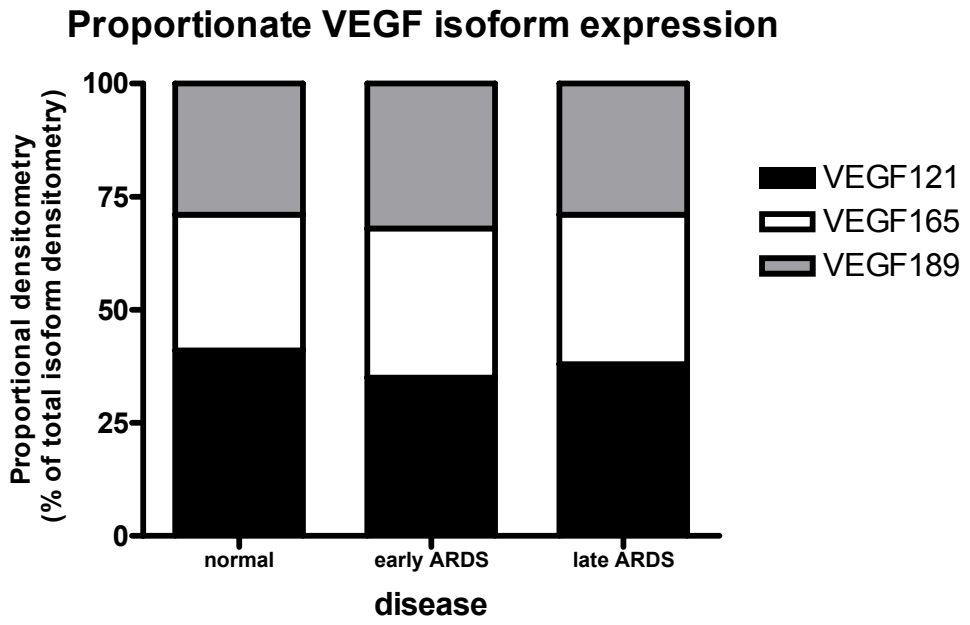


Figure 3.12: Proportionate semiquantitative densitometry for VEGF isoforms in normal, early ARDS and late ARDS lung (n = 5). Data expressed as percentages, p = 0.93, Chi Squared. Note percentage for $VEGF_{121}$ highest for each stage of disease but not significant: normal (p = 0.54), early ARDS (p = 0.96), late ARDS (p = 0.78).

In summary, there was no evidence of isoform switching in any disease state.

3.4 DISCUSSION

3.4.1 Interpretation and current literature

3.4.1.1 Summary of results

These data confirm VEGF receptor expression on additional targets (alveolar macrophages and epithelium in addition to endothelium, their usual site of expression). The data confirm co-localisation of VEGF with AQP3 on ATII cells and a significant upregulation of VEGF (protein and mRNA), VEGFR1 and VEGFR2 protein expression in later ARDS versus normal lung and early ARDS lung. NRP-1 expression is downregulated in early ARDS with upregulation of NRP-1 in later ARDS lung (not versus normal). In normal lung, VEGFR2 expression was significantly higher than VEGFR1. In early ARDS, there was a relative reduction in VEGFR1 and NRP-1 expression (compared to VEGF and VEGFR2) with significantly reduced NRP-1 expression compared to VEGF in later ARDS. In addition, these data show no evidence of VEGF isoform switching at transcriptional level.

These data are consistent with a reduced VEGF signal in early ARDS with up-regulation in later ARDS consistent with a biological role in recovery. In addition, the predominance of the main signalling VEGFR2[144 145] over the "decoy" receptor VEGFR1[114 139-141] in normal lung would tend to optimise VEGF bioactivity in the normal state. This adds to the suggestion of a function in lung recovery from data in previously published observational clinical studies[244 247 249]. An alternative interpretation of these results would be that VEGF has a pathological role in lung injury given the upregulation in necropsy tissue; however, I will go on to justify why this is unlikely in the light of other data.

These data also support a possible autocrine role for VEGF in lung on epithelium in addition to its recognised paracrine function on vascular endothelium (discussed in further detail in Chapter 4). Autocrine VEGF activity has been demonstrated in specialised kidney epithelial cells but as yet not in lung epithelium[106]. Indeed, exogenous VEGF has been shown to act as a growth factor on human foetal pulmonary epithelial cells and is capable of restoring the ability of A549 cells to express VEGF in an acid exposure cellular model of injury raising the possibility of an autocrine alveolar epithelial function[103 255].

However, there are other clear discrepancies with the current literature, which is limited in this area. Based on the receptor expression data, these data would be expected to lead to a reduction in detectable soluble intrapulmonary VEGF (due to increased receptor expression) in later ARDS and possibly a slight increase in soluble VEGF levels (due to reduced NRP-1 expression). This is not in keeping with current observational data in lung injury, ARDS and high-altitude pulmonary oedema which show plasma VEGF levels rise and intrapulmonary levels fall in the early stages of lung injury with normalisation of both during recovery[244 247-249]. However, these VEGF measurements were by ELISA, detecting only the soluble $VEGF_{121}$ and $VEGF_{165}$ isoforms. VEGF expression is significantly upregulated in later ARDS and the immunohistochemistry antibody recognises $VEGF_{189}$ isoforms in addition to the soluble $VEGF_{121}$ and $VEGF_{165}$ isoforms so predicting overall effects on intrapulmonary VEGF levels and bioactivity is complex. In addition, changes in intrapulmonary VEGF isoform expression in acute lung injury may also be contributory but have yet to be clarified.

There are other areas of discrepancy. These data are not consistent with the limited current uncontrolled and observational literature on VEGF receptor expression in acute lung injury. In an observational archival tissue clinical study, Tsokos et al.[320] detected reduced VEGF ($VEGF_{121}$ and $VEGF_{165}$) and VEGF receptor expression on alveolar epithelium in "later" sepsis-induced ARDS. In this study, a reduction in VEGFR2 mRNA (as well as reduced $VEGF_{121}$ and $VEGF_{165}$ mRNA) expression in patients with sepsis was accompanied by reduced alveolar epithelial VEGF protein expression[320]. However, this was an observational study using autopsy material in non-survivors with pre-terminal ARDS due to sepsis but a variety of duration of illness ranging from 4 to 28 days of illness being ventilated for 4 days. The reduction in VEGF signal may reflect failure of alveolar epithelial repair here. Indeed, other observational studies have confirmed that intrapulmonary VEGF levels fail to normalize in non-survivors with ARDS[247].

3.4.1.2 Animal models

3.4.1.2.1 Hypoxia, volutrauma and VEGF intervention models

Animal models of lung injury have increased understanding of changes in VEGF receptor and isoform expression in this context. Available evidence lends support to the concept of VEGFR2 expression in response to injury, whether hypoxia (acute or chronic) or volutrauma. High tidal volume ventilation strategies in an acid-induced murine model of lung injury increase VEGF receptor 2 (VEGFR2) expression with reduction in expression related to a lower tidal volume strategy of ventilation[243]. These early measurements (made only 4 hours after onset of ventilation) would predict a reduction in soluble VEGF in early ARDS, consistent with the data here and previous observational studies[247 249]. Significantly, there was no increase in VEGF noted at the same timepoint in the same study[243 249]. Chronic hypoxia (7-32 days *ex vivo*) also increases VEGFR2 (and VEGFR1 and VEGF) mRNA and protein expression in rat lung[200]. Interestingly, even short term hypoxia (2 hours) increased VEGFR2 (and VEGFR1) transcription removing any confounding causes of vascular shear stress by perfusing the isolated lungs at constant flow and pressure with a priming agent inducing hypoxic pulmonary vasoconstriction. Such an early upregulation in binding sites would be consistent with a reduction in soluble intrapulmonary VEGF. Indeed, a single intraperitoneal injection of LPS led to a significant reduction in measurable lung VEGF mRNA over 2-12 hours consistent with this. There are no histological data to confirm whether there was breakage of the alveolar-capillary membrane or loss of alveolar tissue to explain the reduction in VEGF. The more long-term effects of chronic hypoxia suggest a role for VEGF in recovery via increased receptor activity.

Further evidence that changes in VEGF receptor expression may be more than simply epiphenomena and for the potential importance of VEGF acting through its main functional receptor (VEGFR2), comes from a number of intervention studies using a specific VEGFR2 inhibitor in rats[256 269 271]. In these studies, increased air spaces, loss of alveolar tissue weight, emphysema and alveolarization were consistently noted in newborn, infant and adult rats. There was associated evidence of increased alveolar cell apoptosis in all cases. Although two of these studies did not confirm functional blockade of VEGFR2 signalling activity, Kasahara et al.[256] did demonstrate reduction in VEGFR2 phosphorylation due to the inhibitor and all three studies demonstrated pruning of the pulmonary arteries and right ventricular hypertrophy which are the typical expected findings of VEGF blockade on the pulmonary vasculature as a result of its established functions in vascular development and possible role in pulmonary hypertension (discussed in Chapter 1).

However, some discrepancies persist in the literature in VEGFR2 blockade studies and hypoxia models. Firstly, Kasahara et al.[256], in the aforementioned study, noted no effect on alveolar proliferation in their VEGFR2 intervention study. However, this was only assessed by morphological cell count, with no assessment of thymidine uptake or other proliferation assay used. Therefore, more subtle effects on proliferation cannot be excluded and changes in rates of apoptosis were noted as discussed earlier. Moreover, this does not exclude a protective role in repair or recovery in the lung by anti-apoptotic effect or other non-proliferative survival mechanisms. Secondly, in eNOS-deficient mice, 1-10 days of mild hypoxia in a hypobaric chamber led to a 34-63% reduction in VEGFR2 protein depending on whether the deficiency was partial or total[321]. Conclusions from this study are limited by the fact that there was no significant increase in VEGFR receptor expression in normal mice following mild hypoxia in contrast to many other studies.

3.4.1.2.2 Hyperoxia and prematurity models

The effects of hyperoxia and prematurity in some studies also appear inconsistent and do not always support a protective role for VEGF in the lung[137 322-324]. Hosford et al.[322] demonstrated a reduction in VEGFR2 (VEGF and VEGFR1) mRNA and protein expression in a hyperoxic newborn rat model of lung injury (from day 4 to 14) compared to the normal increase (mRNA only) in normoxic animals. However, no histological analysis was performed in this study and low levels of VEGF and receptor expression did correlate with HIF levels indicating suppression of transcription by hyperoxia. No assessment (histological or otherwise) was made of recovery from injury at a later timepoint. Furthermore, the changes were not all uniform as levels of VEGF protein were actually increased at day 9 following hyperoxic lung injury but fell subsequently.

Klekamp et al.[137] noted a significant reduction in VEGF, VEGFR1 and VEGFR2 mRNA at 48 hours in a hyperoxic adult rat model of lung injury. There was no change in relative isoform expression ($VEGF_{188}$, $VEGF_{164}$) but evidence of substantial loss of VEGF protein at 48 hours from both small and large airway epithelium with increased apoptosis. Again, no assessment was made of recovery from injury here and these decreases in VEGF and receptor expression would be in keeping with previous observational studies in the early stage of lung injury[247-249].

In a newborn and adult rabbit hyperoxic model of lung injury, Watkins et al.[323] detected a relative reduction in $VEGF_{189}$ and parallel increase in $VEGF_{121}$ and $VEGF_{165}$ mRNA expression. This suggests a role for isoform splicing in lung injury (see section 3.4.1.3, next section). The initial reduction in VEGF levels (at 48 hours) is consistent with observational studies in early acute lung injury and there was later normalization of levels and isoform proportions (after 5 days) during recovery although no histological analysis was performed in this study[247-249].

In a premature foetal baboon model of chronic lung disease, Maniscalco et al.[324] noted a reduction in VEGFR1 (and VEGF mRNA and protein) at 125 days (67% of term). Although these results are in contrast to the expected increase in lung VEGF during development, they do not preclude a protective role. Importantly, no reduction in the main signalling receptor (VEGFR2) was noted. In addition, supportive oxygen therapy was given continuously after premature delivery may have impaired VEGF transcription although no measurements of HIF were made. Moreover, the lung injury was heterogeneous with no significant decrease in VEGF histologically at 6 days but decreasing at 14 days, with initial apparent localization of VEGF protein to ATII cells and subsequently thinned alveolar septae histologically consistent with alveolar epithelial loss. Therefore, potentially the failure of VEGF levels to increase may reflect the severity of lung injury in this model and failure of repair of the epithelium.

3.4.1.3 VEGF isoform switching

There is a relative lack of studies in acute lung injury examining isoform switching at the current time. In addition to the data confirming an alteration in absolute isoform expression in early ARDS, the data on proportionate isoform expression confirmed no evidence of isoform switching.

Available data in the literature on isoform switching in human clinical studies are currently sparse and contradictory. On preliminary analysis, these data contrast with an observational study using human post mortem lung tissue from sepsis-induced ARDS patients[325]. In this study, an apparent switch to reduced soluble isoforms during injury was detected at transcriptional level. However, many methodological problems exist in this study preventing further conclusions. The timepoints varied from 4 to 28 days making comparison difficult. Moreover, no proportionate analysis of isoform expression was made, no increase in cell-associated isoform ($VEGF_{189}$) was detected at all in either the control or disease lungs, no assessment of isoform expression was performed at systematic later timepoints in recovery and all the samples were from non-survivors.

Most existing data comes from animal models and the findings appear contradictory. Some data are in agreement with this thesis. In a LPS-induced mouse lung injury model, no change in proportionate VEGF isoform expression was noted following LPS injury from 24 to 72 hours[326]. However, all absolute values of isoform ($VEGF_{120,164,188}$) expression were reduced in keeping with the absolute isoform data in this thesis. Similar changes were noted with age. Klekamp et al.[137] also noted no change in relative isoform expression ($VEGF_{188}$, $VEGF_{164}$) in a hyperoxic adult rat model of lung injury with evidence of substantial loss of VEGF protein at 48 hours from both small and large airway epithelium corroborating observational studies[247-249].

Other studies contrast with the data in this thesis. In a hyperoxic lung injury mouse model, elevated $VEGF_{120}$ and $VEGF_{188}$ isoform expression was noted in BAL fluid after 72 hours hyperoxic injury with no change in $VEGF_{164}$ expression[258]. The apparent switch to both cell-associated and soluble isoform expression in early injury here is not consistent with the observed data in this thesis but may be explained by species differences (mouse versus human) and perhaps by the mode of injury (hyperoxia versus sepsis). In a hyperoxic lung injury rabbit model, a relative reduction in cell-associated isoforms was noted and an increase in soluble isoforms both of which normalized in recovery[323]. However, in rabbit $VEGF_{188}$ is the predominant isoform in the lung so these results indicated a relative switch away from the predominant isoform in early injury with later normalization. In humans, $VEGF_{165}$ is the predominant isoform and species differences again may be relevant. In addition, hyperoxia was applied for 9 days (longer than the other studies) followed by 60% oxygen, which may have impeded transcription via HIF.

In summary, taking into consideration the significant methodological and species differences in published lung injury animal models, this may account for the observed differences in isoform switching compared to the data in this thesis.

In addition to changes in isoform switching and receptor expression, there are other possible mechanisms to explain the apparent reduction in intrapulmonary VEGF levels in the early stages of lung injury. These include damage to the alveolar-capillary membrane and consequent leakage of intrapulmonary VEGF into the vascular bed or an epiphenomenon reflecting damage to the alveolar epithelium as described in normal smokers and patients with idiopathic pulmonary fibrosis (IPF) [252]. However, on the available evidence from current intervention studies the latter would seem unlikely. Further intervention and ultrastructural studies are required to answer these questions.

3.4.2 Limitations

There are several limitations to this work. Only low numbers of samples were obtainable for sectioning and immunohistochemistry, which increases the possibility of error. It also precluded analysing the ratio of predominantly "cell-associated" ($VEGF_{189}$) to predominantly "soluble" isoforms ($VEGF_{121}$ and $VEGF_{165}$) that would have been possible with higher n numbers. The low n numbers are primarily due to the difficulty of finding equal numbers of samples at different timepoints in acute lung injury. Typically, the earlier stages are harder to access due to the reduced tendency for surgical lung biopsy. Ideally, open lung biopsy from living ARDS patients would have been preferred but this is now seldom performed due to the rapidity of onset of ARDS, lack of thoracic surgeons on site and difficulty in achieving consent for research purposes on living tissues.

Necropsy ARDS lung tissue introduces selection bias for a more severe cohort of ARDS and improper control group (although there was no obvious lung disease in the control group). Moreover, this necropsy tissue introduced further selection bias in containing only ARDS related to either direct (lung) or remote (gut) sepsis and not other aetiologies such as trauma. Further selection bias was introduced by those undergoing a post mortem for clinical reasons who are likely to have been a more severe subgroup and also only those where consent to post mortem was possible. Indeed, intrapulmonary VEGF levels are known to be lower in non-survivors with ARDS[247]. Furthermore, the use of non-survivor (necropsy) tissue with the demonstration of upregulation of VEGF and its receptors introduces an alternative interpretation of the results in support of a pathological role in lung injury (although I have presented and interpreted the evidence in the light of other data, especially from intervention studies to conclude that my original interpretation of a role in recovery is indeed more plausible). The use of living ARDS tissue would have potentially avoided this complication.

Semiquantitative immunohistochemistry is primarily a technique for localisation and Histometrix is subject to variability of set criteria for assigned staining. However, Histometrix provides a more objective method of assessing differences in immunostaining than random arbitrary counting by blinded observers. Histometrix cannot assess individual tissues eg) alveolar epithelium or vascular endothelium. It also reflects a composite of pixel staining and will not take account of changes in cell number. Hence, a staining per cell number value cannot be derived.

Significant differences in antibody affinity and avidity limit the strength of any comparison of staining between different antibodies in the same tissue despite the accuracy of Histometrix methodology. It is acknowledged that the conclusions in this chapter from comparisons between different antibody staining have assumed no significant differences in affinity and avidity. Such theoretical concerns should be addressed in future studies.

Ideally, it would have been advantageous to assess individual VEGF isoform protein expression anatomically and functionally but individual isoform-specific antibodies are not currently available for immunohistochemistry although the VEGF antibody used recognises $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ isoforms unlike the antibody used in ELISA (R&D) which merely recognises the soluble isoforms ($VEGF_{121}$ and $VEGF_{165}$). Hence, comparisons between VEGF ELISA and immunohistochemical data are limited in this regard.

Semiquantitative RT-PCR is limited by inferior accuracy compared to real-time PCR and other limitations discussed in Chapter 2. Briefly, there are problems relating to variability in the expression of housekeeping genes, genomic DNA contamination, variable primer efficiency using oligoDT method, and variability due to postPCR manipulations of electrophoresis, imaging and densitometry. There are also technical problems with paraffin RNA extraction with cross-linking for longer product sizes.

3.4.3 Conclusion

In conclusion, the data confirm a significant upregulation of VEGF transcription and translation in later ARDS (versus early ARDS and normal lung). This is accompanied by a similar significant increase in VEGFR1 and VEGFR2 protein expression at the same time point compared to the same disease states. There is, however, no evidence of isoform switching. NRP-1 uniquely is downregulated in early ARDS with a significant upregulation in later ARDS (versus early ARDS). In normal lung and both time points of ARDS lung, VEGF and its receptor expression is heterogeneous and on both sides of the alveolar-capillary membrane. The data suggest significantly higher VEGFR2 versus VEGFR1 expression in normal lung. In early ARDS, there is a relative underexpression of VEGFR1 and NRP-1 compared to VEGF and VEGFR2. NRP-1 remains significantly underexpressed compared to VEGF in later ARDS. These changes do not account for previous published observations on intrapulmonary VEGF levels at this stage. Further studies are required to examine other possible mechanisms for this reduction and account for the observed variability in receptor expression including structural changes to the alveolar-capillary membrane, the alveolar epithelium and VEGF isoform switching. There are discrepancies with previously published studies largely due to significant methodological or species differences. In support of this, these data corroborate a significant number of other observational studies suggesting a biological role in repair following lung injury. An alternative interpretation of the results from necropsy tissue is a pathological role for VEGF but this is not consistent with previous data especially from intervention studies, whereas a protective role is consistent both with previous data and the current results. In addition, VEGF may serve an autocrine function in the lung on alveolar epithelium that is postulated to be its key source here. This will be further discussed in Chapter 4.

CHAPTER 4:
VEGF AND HUMAN ATII CELLS:
RESPONSE AND EXPRESSION

This chapter will describe the isolation, culture and phenotypic characterisation of ATII cells, their response to VEGF in terms of proliferation and their expression of VEGF isoforms and receptors at the transcriptional level.

Parts of this chapter have been published in abstract form in Eur Resp J, Thorax and Am J Resp Crit Care Med.

Medford ARL, Armstrong L, Millar AB.
The effect of vascular endothelial growth factor (VEGF) on primary human cultured type 2 alveolar epithelial (ATII) cell proliferation.
Eur Resp J 2004 24(Suppl 48): 29-30s [S318].

Medford ARL, Armstrong L, Gillespie KM, Millar AB.
The effect of proinflammatory cytokines on primary human cultured alveolar epithelial cell expression of vascular endothelial growth factor (VEGF) isoforms.
Thorax 2003 58(Suppl III): iii18-19 [S58].

Armstrong L, Medford ARL, Thorley A, TD Tetley, Millar AB.
Primary human alveolar type II cells constitutively produce vascular endothelial growth factor.
Am J Resp Crit Care Med 2002 165(8): A372.

SUMMARY

Background

Alveolar epithelial injury is one of the key events in the development of ARDS. The ATII cell proliferates and differentiates to regenerate the alveolar epithelium after injury. I hypothesised that human ATII cells would be a source of and target for VEGF. I also hypothesised that VEGF was an ATII cell mitogen.

Methods

Primary human ATII cells were obtained, phenotyped and cultured from normal resected lung. VEGF expression was assessed by ELISA (for protein) and isoform-specific RT-PCR (for RNA). VEGF receptor expression was assessed by RT-PCR (for RNA). Dose-dependent and specific effects of VEGF ATII cell proliferation were assessed using an MTS assay.

Results

ATII cells expressed all studied VEGF isoforms and receptors at transcriptional level including significant amounts of VEGF protein, which was time-dependent but not affected by LPS. LPS and VEGF (10ng/ml) increased mRNA expression of VEGFR1 and all isoforms except $VEGF_{165}$ (not increased by LPS). No other consistent effects on receptor transcription were noted. $VEGF_{165}$ increased ATII proliferation non-significantly at 10ng/ml with no evidence of dose-dependence. A specific VEGF inhibitor (*sflt*) significantly reduced the effects of 10ng/ml $VEGF_{165}$.

Interpretation

ATII cells are a significant source of the VEGF and its targets consistent with an autocrine action for VEGF here. The pro-inflammatory milieu, especially LPS, increase VEGF isoform and receptor expression here and the effects of VEGF itself are concentration-dependent. LPS has no additional effect on VEGF protein expression suggesting post-transcriptional regulation. The data suggest ATII cell survival may be VEGF-dependent but do not confirm a mitogenic role.

4.1 INTRODUCTION

As already reviewed in Chapter 1, ARDS is the most extreme form of acute lung injury, characterised by non-cardiogenic pulmonary oedema, neutrophilic alveolitis and the development of potentially reversible fibrosis with a considerable morbidity and mortality[2]. In Chapter 1, I have discussed the importance of alveolar epithelial injury in the development of ARDS, although injury on both sides of the alveolar-capillary membrane is necessary.

In Chapter 1, I have extensively discussed the function of VEGF as both a mitogen and permogen, on vascular endothelium. VEGF is compartmentalised to high concentration in the normal human lung. As reviewed in Chapter 1, other cellular, animal and clinical studies indicate that the likely source is alveolar epithelium on the basis of immunolocalisation and expression. The reasons for this compartmentalisation remain poorly understood. Other investigators have found high intrapulmonary VEGF levels in normal or "at risk" subjects before the development of ARDS or compared to the early stages post lung transplant[247 253].

Such findings have resulted in investigation into its potential role in ARDS. As reviewed in Chapter 1, data assessing the role of VEGF in the alveolar space apparently conflicts. Much of the data suggesting a role in injury may indicate epiphenomena and the overexpression models may have involved unrecognised damage to the alveolar-capillary membrane exposing the underlying endothelium to the higher physiological levels of VEGF in the air space leading to excessive oedema. Different timepoints in the animal models and clinical studies were used. The data as a whole do not account for the consistent findings of abundant VEGF in the normal lung as well as the accumulating data consistent with a protective role for VEGF in the lung. In addition, there are potential biologically plausible explanations for the apparent paradoxical data. In addition to the physical factors of failure to prevent fluid efflux by loss of physical integrity, alveolar-capillary membrane damage may potentially contribute to pulmonary oedema by other mechanisms such as exposure of the underlying endothelium to higher (usually intrapulmonary) concentrations of VEGF. In the presence of an intact membrane, VEGF may promote alveolar epithelial proliferation and therefore lung recovery with removal of oedema and restoration of normal alveolar epithelial function. This chapter will concentrate on the evidence for the latter and also endeavour to add to current scientific knowledge on this aspect of VEGF bioactivity.

As the data in Chapter 3 suggest, significant changes in splice variant and receptor expression occur in the time course of lung injury which may partially account for the observed fall in intrapulmonary VEGF levels in early ARDS and normalisation in recovery seen in clinical studies of lung injury[247-249]. Moreover, the VEGF co-localisation with ATII cells data further support the hypothesis that ATII cells are the main intrapulmonary source of VEGF.

However, a number of questions remain. It is not known how the human alveolar epithelium responds to the pro-inflammatory milieu that occurs in ARDS. The effects of local VEGF on human alveolar epithelium are unknown, specifically whether VEGF is mitogenic here stimulating proliferation. The role of the primary cultured human ATII cell (the closest cellular surrogate for human alveolar epithelium) in lung injury is poorly understood as the current literature relates to A549 cell lines only (an adenocarcinoma line originally isolated in 1972 from a single parent cell)[327]. These cells are not functionally equivalent to human ATII cells, differing in many respects including IL-8 production[327]. The paucity of studies on human ATII cells probably reflects the technical difficulties in achieving sufficient pure cells from resected lung tissue, generating such monolayers and obtaining enough normal resected lungs given that diseased lung is more often resected[40 43].

4.1.1 VEGF as an alveolar epithelial mitogen

In the current literature, some studies support the hypothesis that VEGF promotes either recovery and/or proliferation of the alveolar epithelium in addition to its well-described actions on vascular endothelium (see Chapter 1).

Indirect evidence suggesting a possible role in stimulating alveolar epithelial proliferation comes from Kunig et al who administered intramuscular VEGF for 7 days to postnatal rats in a hyperoxic model of lung injury[328]. VEGF prevented loss of alveolar tissue and septal thinning with increased radial alveolar counts and reduced mean linear intercepts, both histological indicators of increase alveolar development. However, this study did not examine specifically direct effects on or cell counts of alveolar epithelium and as there were also clear improvements in vascular development, then it is not entirely clear that these changes in alveolar tissues were primary direct effects.

In human foetal explants, four days exposure to 50-100ng/ml exogenous $VEGF_{165}$ significantly stimulated pulmonary epithelial cell proliferation (as assessed by BrdU incorporation) and increased both ductal lumen volume density and epithelial density (by morphometric analysis)[103].

In a preterm mouse model of respiratory distress syndrome, both intratracheal and intra-amniotic delivery of VEGF facilitated alveolar epithelial maturation and increased surfactant protein expression (within six hours of injection). This was in addition to increasing protection from respiratory failure and death, although there was no reported evidence of a direct assessment or effect on cell number[254].

Indeed, acid injured human alveolar epithelial (albeit A549, not ATII) cells proliferated in response to 24 hours of incubation in increasing exogenous (1 – 250ng/ml) concentrations of VEGF in a concentration-dependent fashion (as assessed by haemocytometry)[255]. In this study, no assessment of thymidine uptake was performed and the assumption is that proliferation was increased rather than cell survival being prolonged.

However, no evidence of proliferation was observed in rat isolated ATII cells in response to 48 hours of 10–100ng/ml $VEGF_{165}$ as assessed by both [^{3}H]thymidine and BrdU incorporation although an increase in SP-B transcription was noted with 50ng/ml and there was no comment on changes in total cell count[259].

Further illustration of the importance of VEGF on the epithelium (rather than the vascular endothelium) comes from work in the kidney. 1nM $VEGF_{165}$ increased epithelial cell proliferation in the kidney (as assessed by [^{3}H]thymidine incorporation, haemocytometry and cytotoxicity (LDH assay)) demonstrating an increase in thymidine uptake[106]. Importantly, this was demonstrated as being due to an increase in cell survival rather than increase in true proliferation as the total cell count was increased but there was no change in thymidine uptake per cell[106]. An autocrine function for VEGF on kidney epithelium has been postulated and such a function may also occur in alveolar epithelium as VEGF receptors have been localised here too[137 329].

In summary, there is a relative lack of data investigating a potential role as a mitogen on alveolar epithelium. None of the existing data has used the gold standard of human ATII cells demonstrating the difficulties of this technique and obtaining sufficient cells. None of the studies in lungs assessed thymidine uptake and cell number as well as cytotoxicity to confirm specificity of the changes seen in cell number either to changes in proliferation or cell death unlike studies in other organs[106]. Existing data has also generally come from either developing human lung or rodent or murine species that limits the strength of any conclusions.

In addition, A549 cells have significant functional differences to ATII cells and this makes translating results more difficult. To date, two of the six studies alluded to did not report a positive effect on ATII cell proliferation or alveolarisation[254 259]. Raoul et al. used relatively less exposure to VEGF (48 hours) and assessed on plastic rather than paraffin embedded tissue[259]. Both of these studies did demonstrate increases in surfactant protein transcription and were conducted in animal (rodent and murine) cells. Both did not assess total cell counts formally and Compernolle et al. did report a positive effect on ATII cell maturation and did not specifically aim to assess proliferation. Moreover, Compernolle et al. used developing rather than mature cells and the much earlier time of analysis at 4-6 hours would not allow time for proliferation[254]. Therefore, some discrepancies may be due to species differences, developmental time factors, different time points and other aspects of methodology.

4.1.2 Hypothesis

This thesis is based upon the hypothesis that VEGF has an important role in repair and recovery from lung injury. In Chapter 3, I demonstrated expression of VEGF and its receptors on both sides of the alveolar-capillary membrane, co-localisation of VEGF in ATII cells but that there was no evidence of VEGF isoform switching. In order to further investigate this hypothesis I explored the following questions:

- Do human ATII cells express significant amounts of VEGF and its receptors?
- How is human ATII cell VEGF expression modified in response to LPS?
- Does VEGF stimulate human ATII cell proliferation?
- Is human ATII cell VEGF isoform and receptor expression modified by pro-inflammatory cytokines and if so in what way?

4.2 METHODS

4.2.1 Rationale

In order to explore this hypothesis, primary human cultured ATII cells were obtained as the closest approximation to the human *in vivo* alveolar epithelium unlike existing studies. The methodology is discussed in detail in Chapter 2 and later on in this chapter but, due to the complex number and order of experiments in this chapter, the essential principles are discussed now for clarity. Firstly, because of the acknowledged and alluded technical difficulties, a previous internationally recognised and accepted protocol (the Witherden and Tetley method) was adopted. Secondly, because of the theoretical problems of low cell number, normal parts of resected lung for cancer were used to achieve enough volume of tissue. An independent pathologist not involved in this work confirmed the presence of normal tissue to ensure lack of contamination.

Having cultured primary human ATII cells, *in vitro* expression of VEGF was examined and response to pro-inflammatory cytokines and ARDS BAL fluid (used as an inflammatory *in vivo* surrogate) that would be anticipated in ARDS. Additionally, the response to increasing concentrations of VEGF was assessed to simulate the changes in local air space VEGF concentration in the early and later stages of ARDS. The functional ATII cell response was characterised by assessing VEGF isoform expression using isoform-specific RT-PCR (in the absence of a current methodology to assess isoform protein expression) and also VEGF receptor expression.

To investigate the possibility of an autocrine function of VEGF and a mitogenic role, proliferation of ATII cells was assessed to increasing concentrations of VEGF simulating again the changes in local air space VEGF concentration in the early and later stages of ARDS. For detailed methods on human ATII cell isolation and techniques, VEGF isoform-specific RT-PCR, MTS assay and VEGF ELISA see chapter 2.

4.2.2 Specimens

Macroscopically normal human lung tissue sections (approximately 15cm x 5cm x 5cm) were obtained from normal parts of resected lung in eleven patients undergoing lobectomy for lung malignancy. The median age was 67 years. Ten donors were ex-smokers and one donor a never-smoker. The North Bristol NHS and United Bristol Healthcare Trust Local Research Ethics Committees approved this study.

4.2.3 Isolation and purification of human ATII cells

Human ATII cells were extracted based on the method of Witherden and Tetley[43], also previously used in this laboratory (see Appendix for Armstrong L et al.[330] illustrating this methodology). Briefly, the tissue sections were washed with normal saline, digested with trypsin and micro-dissected in newborn calf serum and DNAse to remove alveolar macrophages. Following vortexing and filtering with further DNAse treatment, serial adherences were performed to remove residual macrophages. The nonadherent ATII cells were removed and adhered to collagen-coated dishes and either allowed to establish fully confluent or 30% confluent monolayers with ATII morphology (for the MTS assay, see Chapter 2 and below).

It should be noted that cell culturing was performed in the presence of serum (10% newborn calf serum as used in the complete culture medium, see Appendix). Serum starving was considered as an option to allow all the cells to enter G_0 phase of the cell cycle theoretically adding more validity to the proliferation assay experiments. However, serum starving can be unpredictable and potentially harmful to the cells that could have led to potential problems with loss of cell number and limited functional data at RT-PCR. Because cell number was a particular issue (the technical process of ATII cell extraction from resected lung tissue resulted in a significant percentage loss of contaminating cells), serum starving methodology was not employed (see section 4.4 for further discussion).

4.2.4 ATII phenotyping

As reviewed in Chapter 1, ATII cells can be most easily distinguished from other cells by a variety of either physical characteristics or functionally detectable differences. Firstly, they express alkaline phosphatase which can give an easily detectable colouration), surfactant protein (surfactant protein-C, SP-C is most specific for ATII cells) and specific aquaporins (aquaporin-3, AQP-3, is most specific for ATII as ATI also express other aquaporins). Secondly, they are cuboidal in appearance as opposed to the flattened ATI cells and they do not possess the specific proteins of ATI cells including other aquaporins (aquaporin-5, AQP-5; which is expressed by other non-ATII respiratory tract cells, as discussed in Chapter 1) or HTI_{56}. Finally, at electron microscopy, lamellar bodies (the organelles which are the site of surfactant protein synthesis specific to these cells) are noted in ATII cells but not ATI cells[40 41 43-45].

Therefore, ATII phenotype was confirmed by positive staining for alkaline phosphatase (see Chapter 2 Methods, Figure 2.1, page 110) and expression of SP-C and AQP-3 mRNA assessed by RT-PCR (see Chapter 2 Methods, Figure 2.2, page 111). Morphological characteristics with lamellar bodies were confirmed by electron microscopy (see Chapter 2 Methods, Figures 2.3a and 2.3b, p112) and an ATI phenotype excluded by absence of AQP-5 mRNA expression (see Chapter 2 Methods, Figure 2.2, page 111).

4.2.5 RNA extraction

In order to assess the functional response of the ATII cell to the pro-inflammatory milieu in ARDS, ATII cells were stimulated at day 3 for 4 hours (after verification of ATII phenotype, see section 4.2.2) with a variety of pro-inflammatory cytokines which have been implicated in ARDS as well as concentrated BAL fluid from ARDS patients as an *in vivo* surrogate. The concentrations of cytokine are consistent with those used in the literature in other primary cell work (see Chapter 2 Methods, Section 2.3.5, p102). In addition, ATII cells were incubated with increasing concentrations of VEGF based on previous studies to assess the functional importance of VEGF as a possible autocrine factor.

The conditions performed to answer these questions were as displayed in Table 4.1 below (all made up in complete medium, see Appendix).

Incubation no.	Conditions
1	unstimulated control (no cytokine, medium only)
2	concentrated human ARDS BAL fluid (50µl in 1ml complete medium)
3	10µg/ml LPS
4	10ng/ml TNFα
5	1ng/ml IL-1β
6	0.1ng/ml human $VEGF_{165}$
7	1ng/ml human $VEGF_{165}$
8	10ng/ml human $VEGF_{165}$

Table 4.1: Incubation conditions for ATII cell culture experiments.

Total cellular RNA was extracted from 1 x 10^6 ATII cells (at 1 x 10^6/ml) after culture (see Chapter 2) with stimuli for 4 hours at day 3 was completed. Cells were washed in sterile PBS and cellular RNA extracted using RNABee and cellular RNA concentration was measured using a GeneQuant II as previously described in detail in Chapter 2.

4.2.6 Semiquantitative VEGF isoform and receptor RT-PCR

Two-step RT-PCR was performed on the human ATII cell RNA as described in detail in Chapter 2 for the principal VEGF isoforms ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$), and receptors (VEGFR2, VEGFR1, NRP-1) and GAPDH. Products were electrophoresed though agarose gels and visualised using ethidium bromide staining assessing mRNA quantity by digital image densitometry. Primer sequences are described in the Appendix. Positive controls were sequenced VEGF isoforms (see Chapter 2 for details) and whole lung for VEGFR2 and NRP-1 and THP-1 or U937 monocyte cell line for VEGFR1.

4.2.7 MTS proliferation assay

The MTS assay indicates cell viability and has been used a surrogate for proliferation in these experiments. It is important to understand some potential limitations of the MTS proliferation assay used before proceeding further. Any proliferation assay is hampered by the assumption that an increase in the compound of interest is due to increased number of cells rather than increased survival of existing cells. Ideally, any such analysis should be corroborated by performing a DNA incorporation assay (eg thymidine), cell count and apoptosis assay to determine the extent the changes in viability are due to changes in apoptosis, proliferation and functional activity per cell number. The MTS assay was used in these experiments, as it was the only locally available technique.

In order to assess a possible functional role as an alveolar epithelial mitogen, increasing physiological concentrations of VEGF were applied to cultured ATII cells in the proliferation assay experiments. To confirm the specificity of any effect, internal negative (no cells or medium to control for well intensity) and positive (KGF) controls were used as well as an unstimulated (no cytokine) experimental control. In addition, a specific VEGF inhibitor (*sflt*, the soluble VEGF receptor which binds free VEGF) was used in other wells to confirm specificity of any positive effect with VEGF. As an *in vivo* surrogate to ARDS, concentrated BAL fluid from ARDS patients was also used with and without a VEGF inhibitor to assess whether an effect was partly due to VEGF. Again, the concentrations used of VEGF were based on previous studies in the literature (see section 4.2.5) except that 100ng/ml was also used to assess the effects of extremely high intrapulmonary VEGF (as would be attained theoretically by aerosolised delivery of VEGF as a therapy for example) to examine for alveolar toxicity. The concentrations of KGF and *sflt* again correspond to previously published concentrations in primary cell studies.

The conditions performed to answer these questions were as shown in Table 4.2 below (all made up in complete medium, see Appendix):

Incubation no.	Conditions
1	Negative control (no medium or cells)
2	Positive control (50ng/ml KGF)
3	Unstimulated control (complete medium only)
4	10ng/ml *sflt*
5	0.1ng/ml human $VEGF_{165}$
6	1ng/ml human $VEGF_{165}$
7	10ng/ml human $VEGF_{165}$
8	10ng/ml human $VEGF_{165}$ + 10ng/ml *sflt*
9	100ng/ml human $VEGF_{165}$
10	100ng/ml human $VEGF_{165}$ + 10ng/ml *sflt*
11	concentrated human ARDS BAL fluid (50µl in 1ml complete medium)
12	concentrated human ARDS BAL fluid + 10ng/ml *sflt*

Table 4.2: Incubation conditions for ATII cell proliferation experiments.

100µl of 2 x 10^4/ml ATII cells were cultured in complete ATII cell medium until 30% confluent to allow the opportunity for discriminative proliferation. The cells were incubated in either medium with/without mediators (see above) for 48 hours before adding PMS reagents for 4 hours (the precise timepoints as discussed in Chapter 2 were obtained from previous experiments). After proliferation, the confluence levels in the unstimulated cells averaged 50% allowing the possibility of discriminative proliferation. The MTS assay was performed as described in detail in Chapter 2. Briefly, 2mls of MTS solution were added to 100µl of PMS solution. 20µl of this mixture was added to each well in a 96 well plate containing 100µl of 2 x 10^4/ml ATII cells after stimulation for 48 hours with the following (in triplicate).

After a 4 hour incubation, 490nm absorbance was determined. Proliferation was calculated as a % of control proliferation for each experiment ensuring a positive response with the KGF positive control and subtracting background intensities.

4.2.8 VEGF ELISA and response to LPS

Confluent ATII cells were washed at day 3 in culture after removing the medium adding LPS (100ng/ml) and incubating for 3, 6 and 12 and 24 hours (other concentrations of 1, 10 and 1000ng/ml showed no difference – data not shown). VEGF protein levels were measured using a sandwich ELISA kit (R&D Systems, Abingdon, UK) according to manufacturer's instructions as described previously[247]. All conditions were measured in triplicate removing the conditioned medium at each time point and storing at –70^{o}C until analysis.

4.2.9 Statistical analysis

Statistical analysis was performed using Graph Pad Prism software (version 4.0). Data were assessed for normality using the Ryan Joiner test. VEGF isoform RT-PCR data were nonparametric but log transformed (using ln) to normalise them. Graphically, normally distributed (or log transformed data) are represented by plotting means and standard errors of the mean as error bars; nonparametric data are represented by plotting medians and interquartile ranges as error bars. Paired data are graphically represented with interlinking lines and plotted as absolute values. MTS proliferation data (paired and normal) was analysed with the paired t test. Otherwise, groups of normally distributed data were analysed by ANOVA and Bonferroni post test correction if multiple comparisons were used. A p value of less than 0.05 was deemed significant. Groups of nonparametric data (which were not log transformed) were analysed by the Kruskal Wallis test and Dunn's post test correction if multiple comparisons were used.

4.3 RESULTS

4.3.1 ATII VEGF protein levels and response to LPS

Human ATII cells express significant amounts of VEGF constitutively. VEGF protein levels increased with time in human ATII supernatant but there was no increased response to LPS but with the same temporal increase (see Figure 4.1).

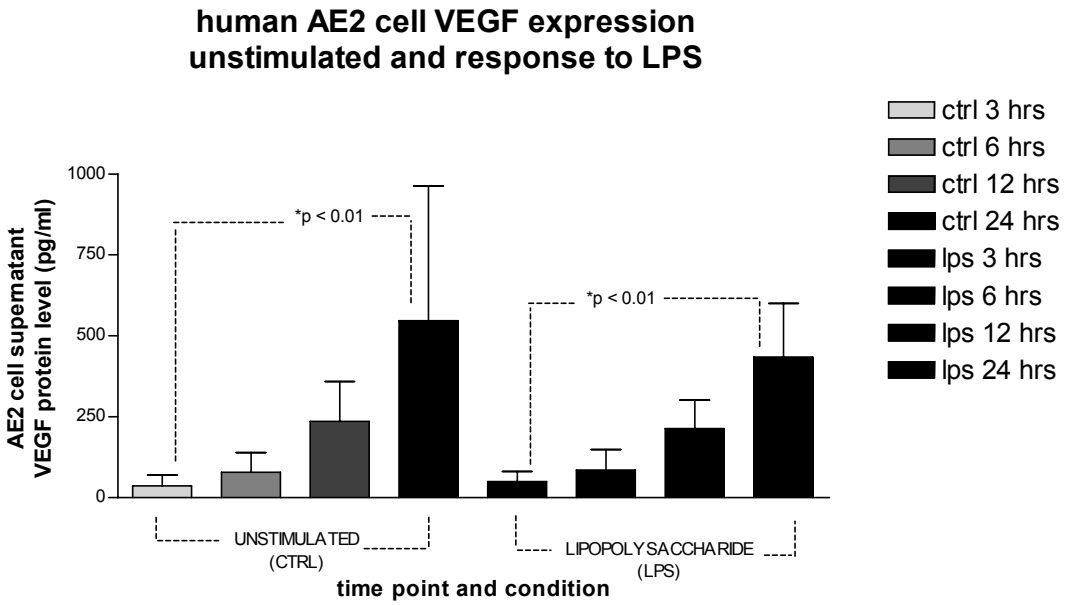


Figure 4.1: ATII cell supernatant levels of VEGF protein unstimulated and in response to LPS 100ng/ml. Data plotted as median values with interquartile ranges (n = 4 for each time point). Unstimulated: p = 0.012 (Kruskal Wallis); lps: p = 0.009 (Kruskal Wallis). *p < 0.01 (Dunns) for 3 hours versus 24 hours, both unstimulated and lps; otherwise p > 0.05 (Dunns) for all other comparisons.

4.3.2 ATII VEGF isoform expression

Due to limitations in ATII cell yield, it was not always possible to perform all the different stimulations in each experiment (see Figures 4.7 – 4.9). ATII VEGF isoform expression was assessed using VEGF isoform-specific RT-PCR (as no protein methodology was available at the time, see Chapters 1 and 2). Semiquantitative RT-PCR demonstrated that ATII cells constitutively express all the three main VEGF isoforms, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (see Figure 4.2).

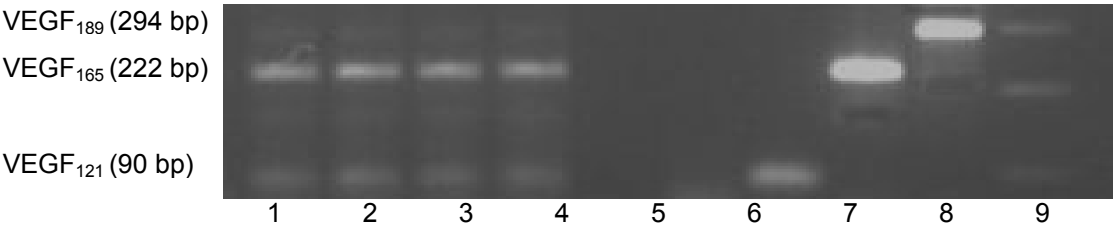


Figure 4.2: Human ATII cell (all unstimulated examples shown above, see lane key below) VEGF isoform-specific RT-PCR gel showing constitutive ATII expression of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (at 90, 222 and 294 base pairs respectively, see Appendix for primer details) with negative and sequenced positive controls and 100bp ladder.

Lane numbers (1-9 above) refer to as follows:

1-4: unstimulated control

5: negative control

6: $VEGF_{121}$ sequenced positive control

7: $VEGF_{165}$ sequenced positive control

8: $VEGF_{189}$ sequenced positive control

9: 100 base pair ladder

Semiquantitative densitometry was performed on the relative ratio of VEGF isoform to GAPDH intensity (see Figure 4.3 for example).

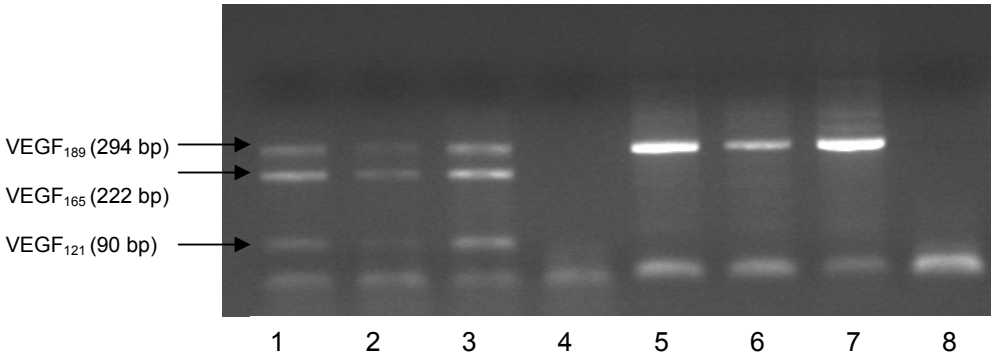


Figure 4.3: Representative stimulated human ATII cell VEGF isoform RT-PCR gel showing isoform and GAPDH bands for densitometric analysis (ladder and positive control lanes not shown).
Lane numbers (1-8 above) refer to as follows:
1: 10ng/ml TNFα
2: unstimulated control
3: 10µg/ml LPS
4,8: negative control
5-7: GAPDH for samples 1-3 respectively (286bp)

Intensities were log transformed (using ln) to normalise the data. Semiquantitative densitometric analyses confirmed ATII cells expressed all the three main isoforms (see Figures 4.4 - 4.6). 10ng/ml $VEGF_{165}$ and LPS significantly upregulated all three isoforms except $VEGF_{165}$, which was upregulated by 10ng/ml $VEGF_{165}$ alone (see Figures 4.4 - 4.6). Other pro-inflammatory stimuli (TNF, IL-1, ARDS BAL fluid) and lower concentrations of $VEGF_{165}$ did not alter VEGF isoform expression.

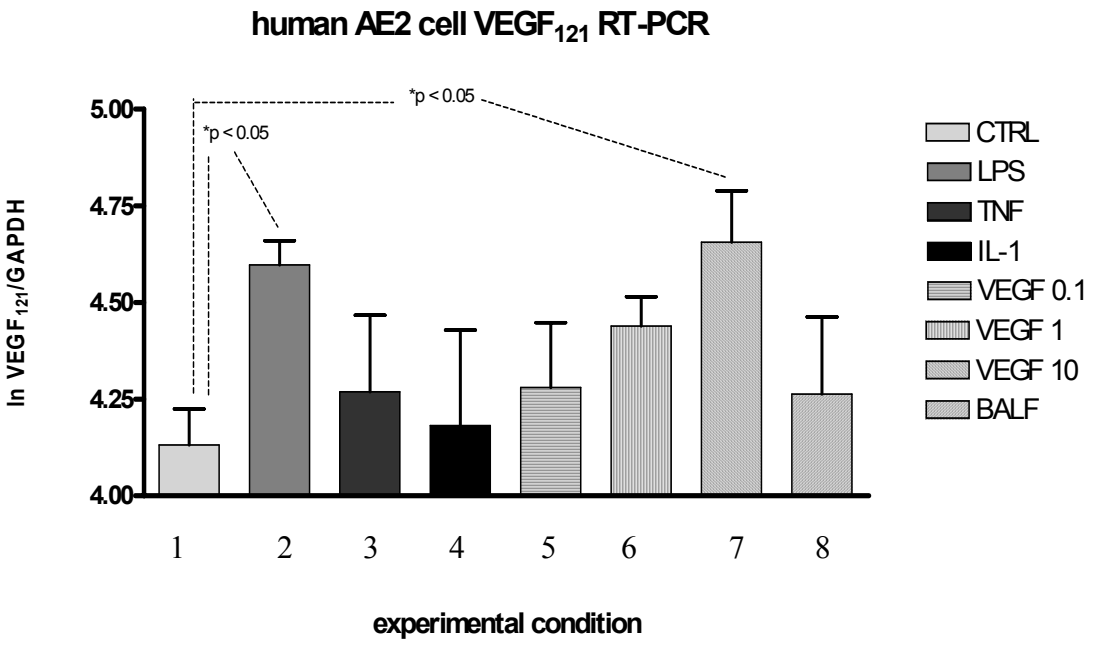


Figure 4.4: Semiquantitative densitometric analysis of ATII $VEGF_{121}$ RNA expression in response to stimulation conditions (see main text and key below; n = 3 except for control, n = 11; LPS, n=4). Y axis denotes log (ln) transform of percentage ratio of $VEGF_{121}$ mRNA intensity to GAPDH. Data are plotted as means and standard errors of mean. p = 0.11 (ANOVA), but *p < 0.05 (Bonferroni) control versus both LPS and 10ng/ml $VEGF_{165}$, otherwise p > 0.05.

Lane numbers (1-8 above) refer to as follows (same lane numbers refer to Figures 4.4-4.6):

1: unstimulated control

2: 10µg/ml LPS

3: 10ng/ml TNFα

4: 1ng/ml IL-1β

5: 0.1ng/ml human $VEGF_{165}$

6: 1ng/ml human $VEGF_{165}$

7: 10ng/ml human $VEGF_{165}$

8: concentrated human ARDS BAL fluid

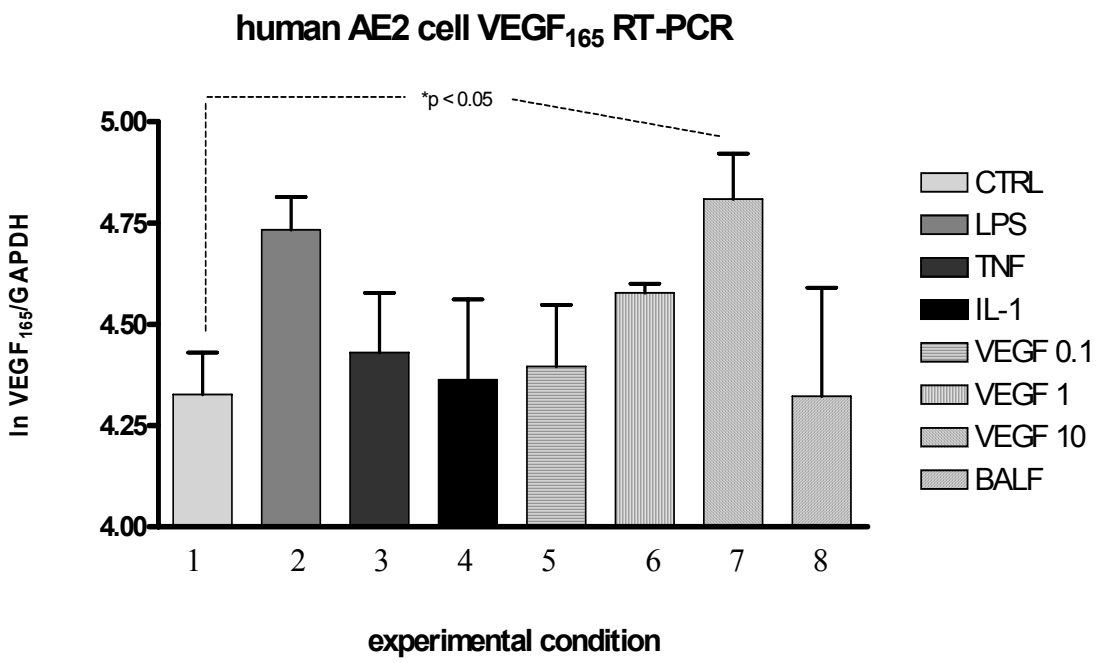


Figure 4.5: Semiquantitative densitometric analysis of ATII $VEGF_{165}$ RNA expression in response to stimulation conditions (see main text and key in Figure 4.7; n = 3 except for control, n = 11; LPS, n=4). Y axis denotes log (ln) transform of percentage ratio of $VEGF_{165}$ mRNA intensity to GAPDH. Data are plotted as means and standard errors of mean. p = 0.19 (ANOVA), but *p < 0.05 (Bonferroni) control versus 10ng/ml $VEGF_{165}$ only, otherwise p > 0.05. Lanes 1-8 as per reference key in Figure 4.4.

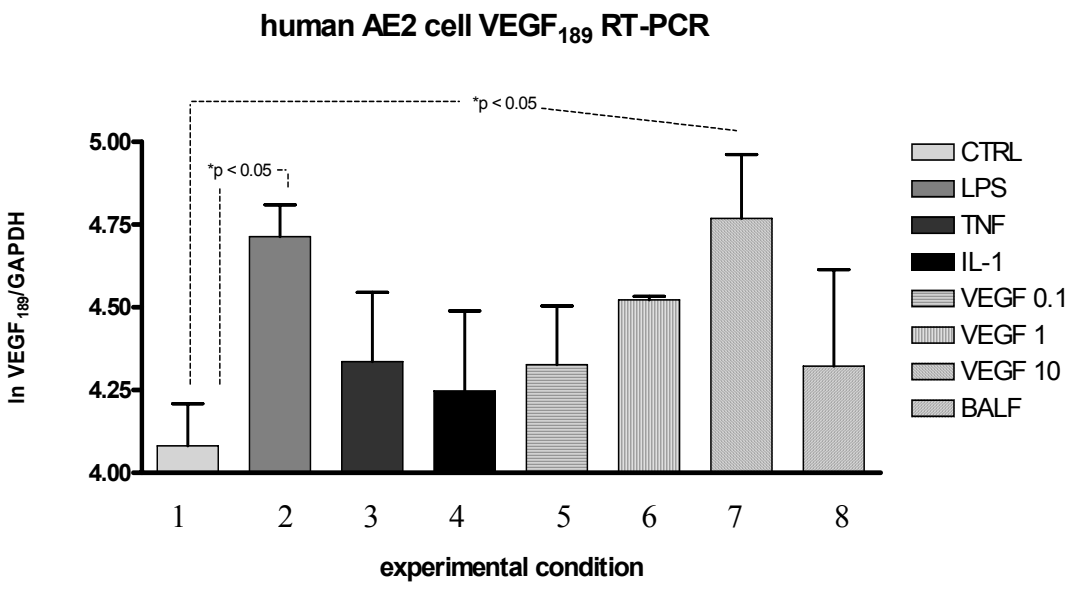


Figure 4.6: Semiquantitative densitometric analysis of ATII $VEGF_{189}$ RNA expression in response to stimulation conditions (see main text and key in Figure 4.7; n = 3 except for control, n = 11; LPS, n=4). Y axis denotes log (ln) transform of percentage ratio of $VEGF_{189}$ mRNA intensity to GAPDH. Data are plotted as means and standard errors of mean. p = 0.09 (ANOVA), but *p < 0.05 (Bonferroni) control versus both LPS and 10ng/ml $VEGF_{189}$, otherwise p > 0.05. Lanes 1-8 as per reference key in Figure 4.4.

4.3.3 ATII VEGF receptor expression

VEGF receptor expression was assessed using RT-PCR. Semiquantitative RT-PCR demonstrated that ATII cells constitutively express the two specific VEGF receptors outside the lymphatic system and the main co-receptor ie) VEGFR2, VEGFR1 and NRP-1 (see Figures 4.7 - 4.9). Due to limitations in ATII cell yield, it was not always possible to perform all the different stimulations in each experiment (see Figures 4.10 – 4.12).

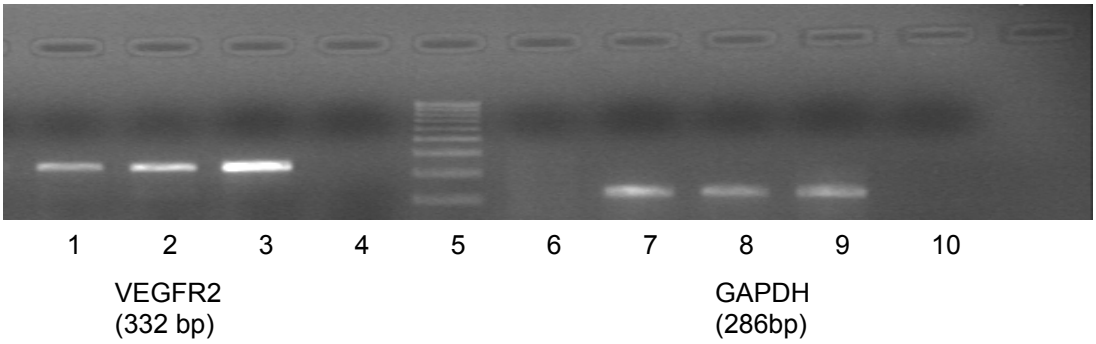


Figure 4.7: Representative stimulated human ATII cell VEGFR2 RT-PCR gel showing VEGFR2 and GAPDH transcripts for densitometric analysis.

Lane numbers (1-10 above) refer to as follows:

1,7: unstimulated control

2,8: 10µg/ml LPS

3,9: positive control (whole lung)

4,10: negative control

5: 100 base pair ladder

6: blank

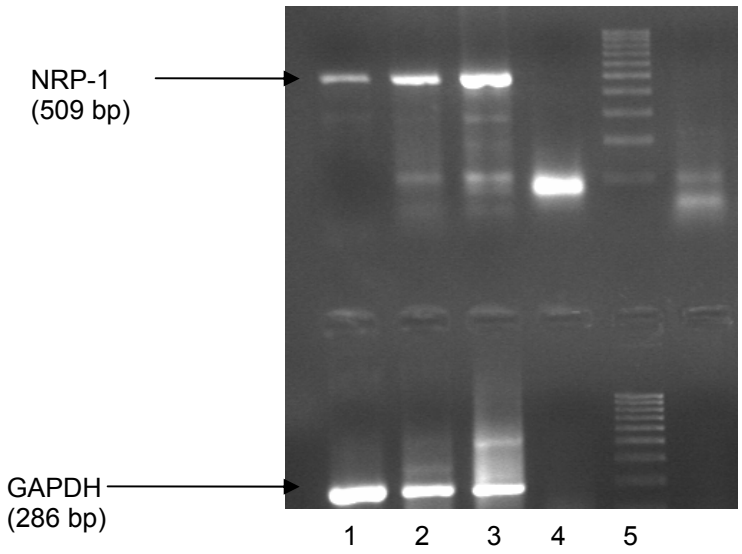


Figure 4.8: Representative stimulated human ATII cell NRP-1 RT-PCR gel showing NRP-1 and GAPDH transcripts for densitometric analysis.

Lane numbers (1-5 above) refer to as follows:

1: 10µg/ml LPS

2: unstimulated control

3: positive control (whole lung tissue)

4: negative control

5: 100 base pair ladder

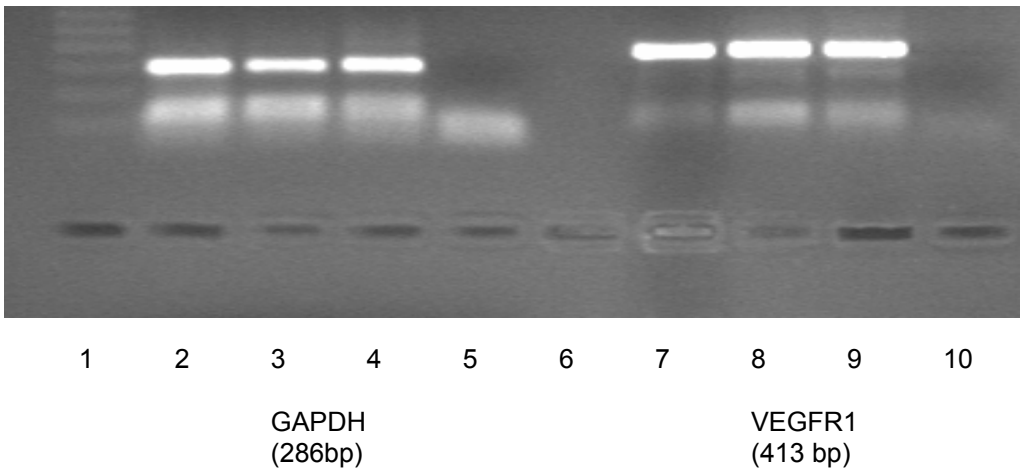


Figure 4.9: Representative stimulated human ATII cell VEGFR1 RT-PCR gel showing VEGFR1 and GAPDH transcripts for densitometric analysis.
Lane numbers (1-10 above) refer to as follows:
1: 100 base pair ladder
2,7: unstimulated control
3,8: 10µg/ml LPS
4,9: positive control (THP-1)
5,10: negative control
6: blank

Semiquantitative densitometry was performed on the relative ratio of VEGF receptor to GAPDH intensity (see Figures 4.10 - 4.12).

Intensities were log transformed (using ln) to normalise the data. Semiquantitative densitometric analyses confirmed ATII cells expressed all the three main receptors (see Figures 4.10 - 4.12). None of the stimulations led to a significant increase in VEGFR2 or NRP-1 in any of the experiments, although 0.1ng/ml $VEGF_{165}$ significantly decreased VEGFR2 expression; and TNF and 1ng/ml $VEGF_{165}$ significantly decreased NRP-1 expression compared to control. In contrast, all tested stimuli increased VEGR1 expression: LPS, 1 and 10ng/ml $VEGF_{165}$. Due to cell yield limitations, not all of the original experiments could be performed so no comment can be made on functional changes in VEGFR1 in response to TNF, IL-1 or ARDS BAL fluid. Other pro-inflammatory stimuli did not alter VEGF receptor expression.

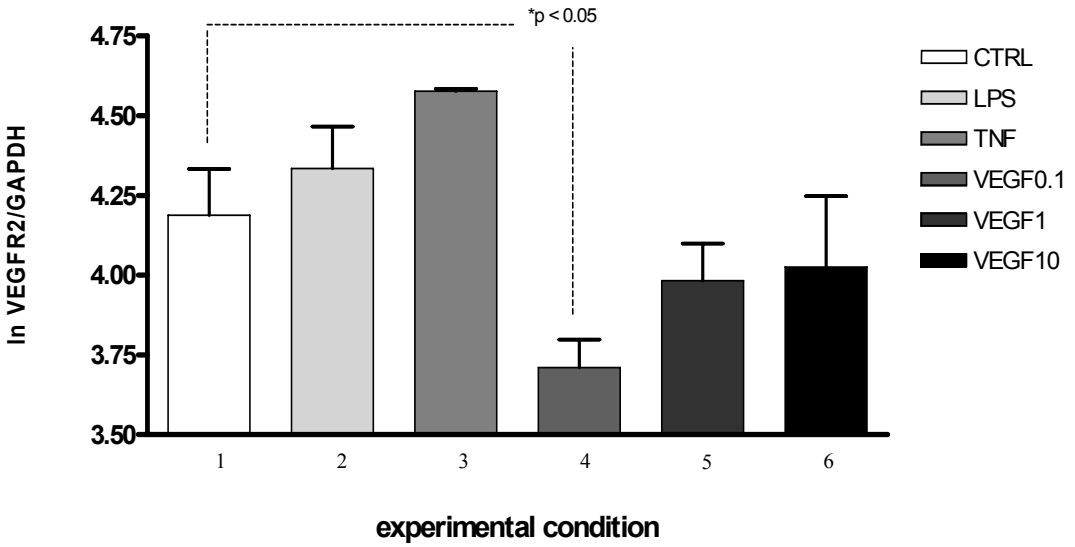


Figure 4.10: Semiquantitative densitometric analysis of ATII VEGFR2 RNA expression in response to stimulation conditions (n = 3 for TNF, n = 4 for 10ng/ml $VEGF_{165}$, n = 5 for 0.1 and 1ng/ml $VEGF_{165}$, n = 7 for LPS, n = 8 for control). Y axis denotes log (ln) transform of percentage ratio of VEGFR2 mRNA intensity to GAPDH. Data are plotted as means and standard errors of mean. p = 0.02 (ANOVA), *p < 0.05 (Bonferroni) control versus 0.1ng/ml $VEGF_{165}$; otherwise, p > 0.05 (Bonferroni) for control versus all other stimulations.

Lane numbers (1-4 above) refer to as follows:

1: unstimulated control

2: 10µg/ml LPS

3: 10ng/ml TNFα

4: 0.1ng/ml human $VEGF_{165}$

5: 1ng/ml human $VEGF_{165}$

6: 10ng/ml human $VEGF_{165}$

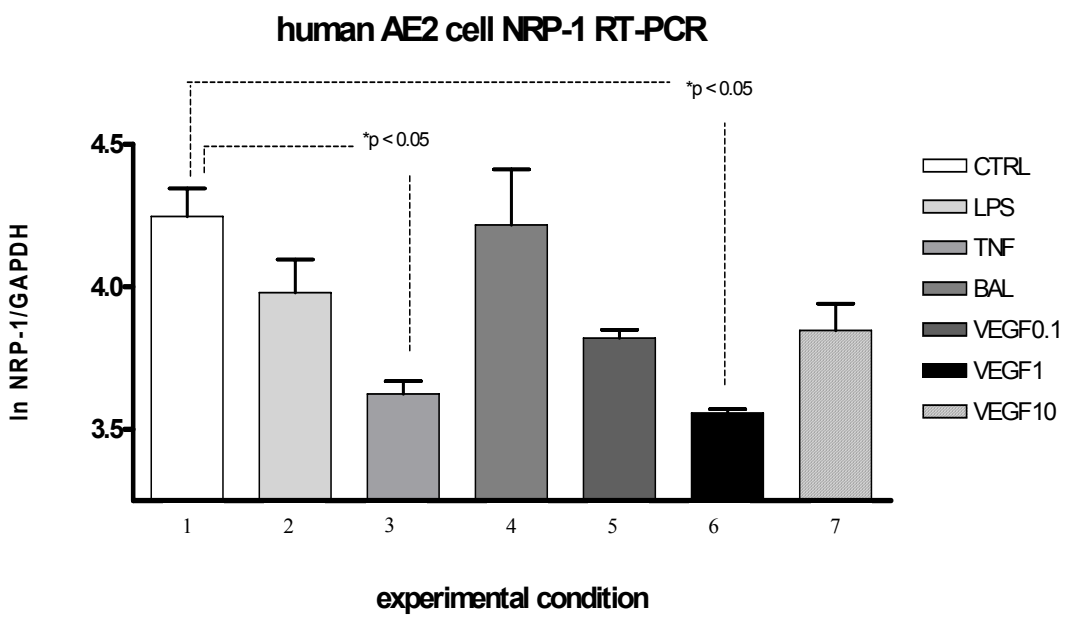


Figure 4.11: Semiquantitative densitometric analysis of ATII NRP-1 RNA expression in response to stimulation conditions (n = 3 except n = 4 for LPS, n = 8 for control). Y axis denotes log (ln) transform of percentage ratio of NRP-1 mRNA intensity to GAPDH. Data are plotted as means and standard errors of mean. p = 0.001 (ANOVA), *p < 0.05 (Bonferroni) control versus TNF and 1ng/ml $VEGF_{165}$.

Lane numbers (1-4 above) refer to as follows:

1: unstimulated control

2: 10µg/ml LPS

3: 10ng/ml TNFα

4: concentrated human ARDS BAL fluid

5: 0.1ng/ml human $VEGF_{165}$

6: 1ng/ml human $VEGF_{165}$

7: 10ng/ml human $VEGF_{165}$

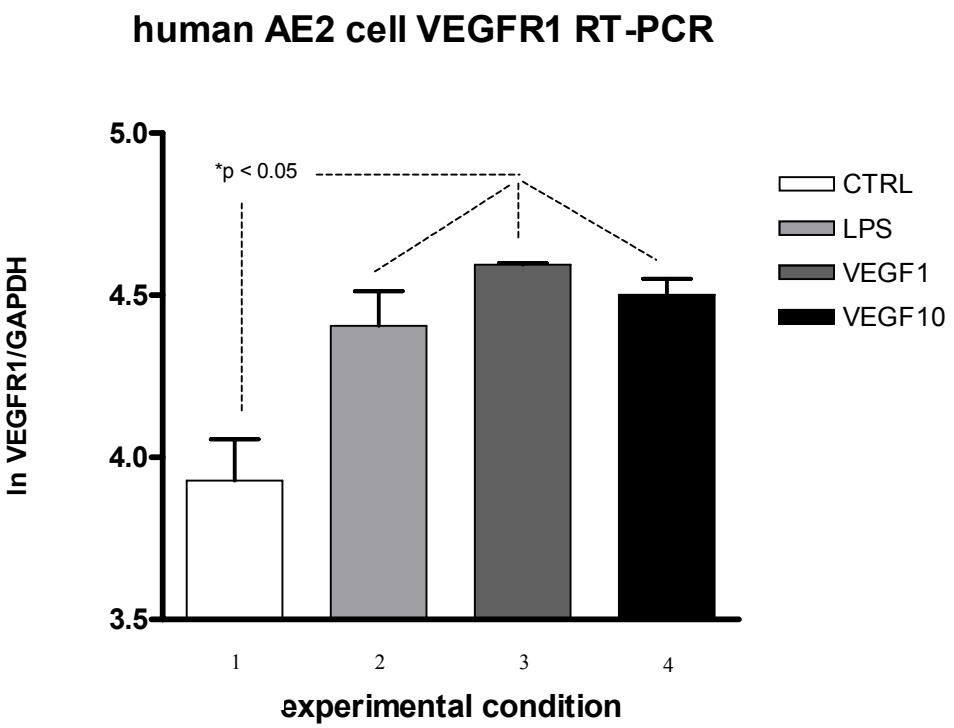


Figure 4.12: Semiquantitative densitometric analysis of ATII VEGFR1 RNA expression in response to stimulation conditions (n = 3 except n = 4 for LPS, n = 6 for control). Y axis denotes log (ln) transform of percentage ratio of VEGFR1 mRNA intensity to GAPDH. Data are plotted as means and standard errors of mean. p = 0.008 (ANOVA), and *p < 0.05 (Bonferroni) control versus all other stimulations (LPS, 1ng/ml and 10ng/ml $VEGF_{165}$).

Lane numbers (1-4 above) refer to as follows:

1: unstimulated control

2: 10µg/ml LPS

3: 1ng/ml human $VEGF_{165}$

4: 10ng/ml human $VEGF_{165}$

4.3.4 Effects of VEGF on ATII proliferation

No significant increase in human ATII cell proliferation was detected with any concentration of VEGF from 0.1 to 100ng/ml although a concentration dependent increase (albeit not significant) was noted plateauing at 10ng/ml VEGF as assessed by MTS proliferation assay (see Figure 4.13). However, KGF (used as an internal positive control) caused significantly higher proliferation than control confirming the ability of the technique to assess response. Due to limitations in ATII cell yield, it was not always possible to perform all the different stimulations in each experiment (see Figures 4.14 – 4.15).

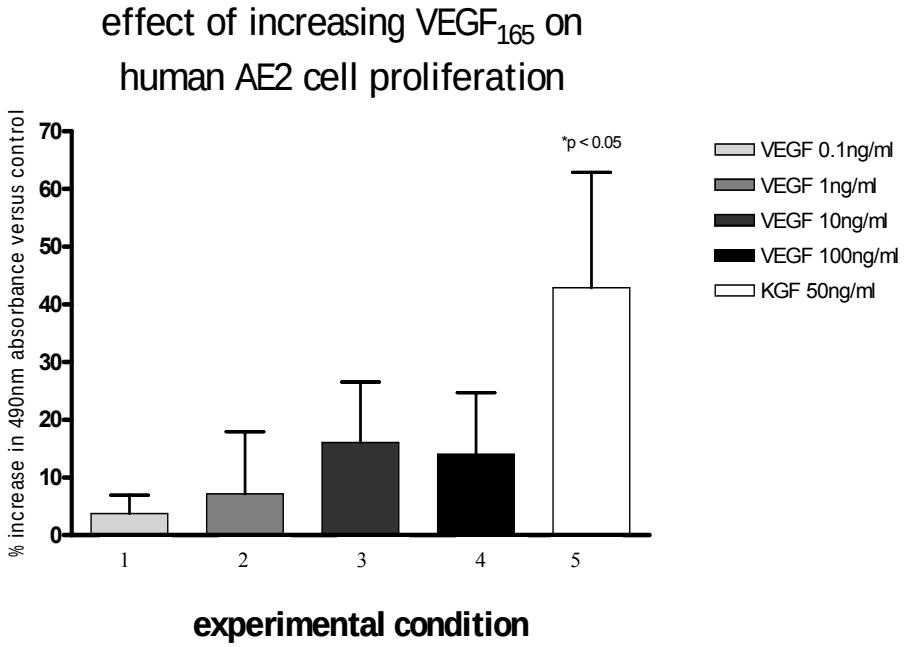


Figure 4.13: ATII cell MTS proliferation assay. Relationship of increasing concentration of $VEGF_{165}$ on MTS proliferation assay in human ATII cells with KGF as positive control (n = 8). Data plotted as means and with standard errors of mean. p = 0.18 (ANOVA) *p< 0.05 (Bonferroni) KGF versus unstimulated control, otherwise all others p > 0.05.

Lane numbers (1-5 above) refer to as follows:

1: 0.1ng/ml human $VEGF_{165}$

2: 1ng/ml human $VEGF_{165}$

3: 10ng/ml human $VEGF_{165}$

4: 100ng/ml human $VEGF_{165}$

5: 50ng/ml KGF (positive control)

With the addition of a natural VEGF inhibitor (10ng/ml *sflt*), there was a significant reduction in proliferation with the higher doses of VEGF (10 and 100ng/ml) (see Figures 4.18 - 4.19) although *sflt* did not significantly alter proliferation versus unstimulated controls (mean change +2.9% (-6.3 - +12.2%, 95% CI; standard error 3.3%) versus control, p = 0.42 (paired t test)).

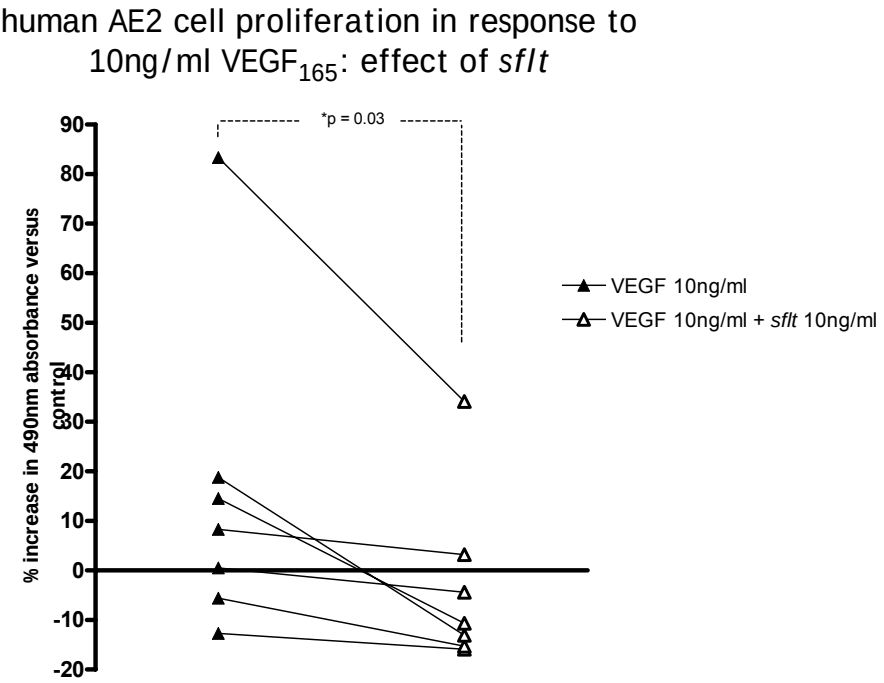


Figure 4.14a: The effect of *sflt* on human ATII cell MTS proliferation in response to 10ng/ml human $VEGF_{165}$ (n = 7). Results displayed as percentage increases or decrease with reference to unstimulated control (not shown as bar but = 0%). Results plotted as paired data *p = 0.03 (paired t test) VEGF 10ng/ml versus VEGF 10ng/ml + 10ng/ml *sflt*. Symbols as in legends.

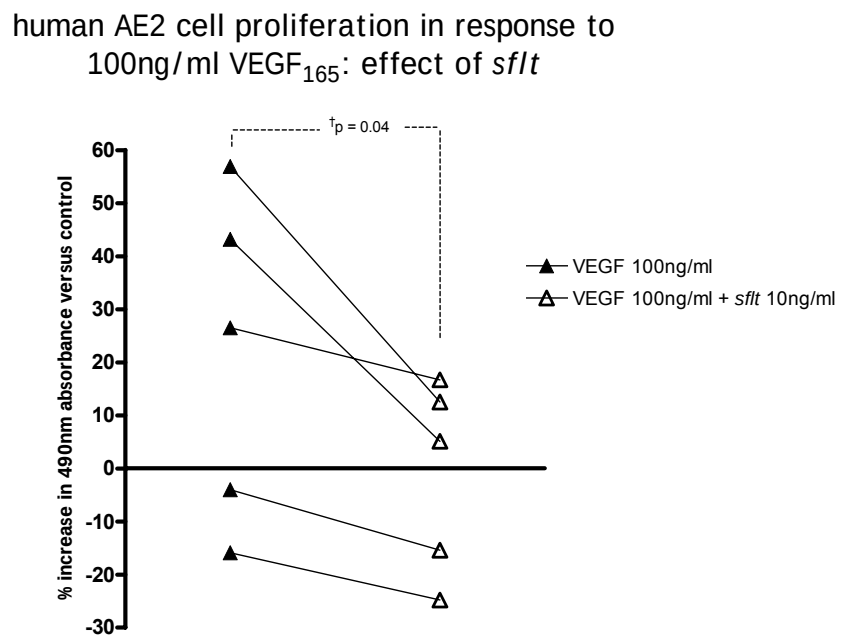


Figure 4.15: The effect of *sflt* on human ATII cell MTS proliferation in response to 100ng/ml human $VEGF_{165}$ (n = 5). Results displayed as percentage increases or decrease with reference to unstimulated control (not shown as bar but = 0%). Results plotted as mean with standard error of mean. †p = 0.04 (paired t test) VEGF 100ng/ml versus VEGF 100ng/ml + *sflt*. Symbols as in legends.

4.4 DISCUSSION

4.4.1 Interpretation and current literature

4.4.1.1 Summary of results

I have demonstrated human ATII cell expression of VEGF at both mRNA and protein level. All the main isoform transcripts ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) are expressed as well as the principal receptor transcripts (VEGFR2, VEGFR1 and NRP-1). Human ATII cells VEGF protein expression is time dependent but not influenced by LPS. A variable response of human ATII cells to stimulation has been demonstrated. LPS increased human ATII cell transcription of $VEGF_{121}$, $VEGF_{189}$ isoforms and VEGFR1. VEGF (10ng/ml) increased transcription of all three isoforms and VEGFR1 (as well as 1ng/ml for the latter) but decreased VEGFR2 (0.1ng/ml) and NRP-1 transcription (1ng/ml). TNF decreased NRP-1 transcription. VEGF did not significantly increase human ATII cell proliferation although the presence of the soluble VEGFR1 *sflt* did significantly reduce proliferation at higher concentrations (10ng/ml and above).

I will now consider the implications and significance of these data in turn in the light of current knowledge.

4.4.1.2 ATII cells as the intrapulmonary source of VEGF

Due to the technical difficulties in human ATII cell culture and obtaining sufficient cells already discussed earlier in this thesis (see introduction, Chapter 4 and Chapter 2), there is no current literature on primary ATII cell expression of VEGF *per se* and these data are therefore novel. As already discussed earlier in this chapter, existing data is based on studies on either human foetal explants, human A549 cells, rat ATII cells, or epithelial cells in other organs eg) kidney.

The ELISA data on human ATII cell supernatant confirms significant expression of VEGF at the protein level. This is in agreement with other studies showing high epithelial lining fluid levels in normal human subjects[128]. The immunohistochemical (see Chapter 3) and RT-PCR data confirm VEGF expression including all the major isoforms on alveolar epithelium.

At the current time, there are no data on the effects of VEGF on human ATII cell expression of VEGF isoforms. VEGF significantly increased expression of all three VEGF isoforms at transcriptional level but only at the higher concentration of 10ng/ml and not at the lower concentrations of 1 and 0.1ng/ml. VEGF concentrations of 10ng/ml are similar to those detected in normal human ELF[122]. These data are therefore compatible with an autocrine action of local intra-alveolar VEGF acting on ATII cells, which has been described in specialised epithelial cells in the kidney[100].

Upregulation of $VEGF_{121}$ and $VEGF_{189}$ isoform expression by LPS was noted but was not detected at the protein level. These data suggest post-transcriptional regulation and further studies should investigate this further. Current literature is conflicting. LPS has been shown to upregulate VEGF expression in airway epithelial cell lines, as well as other cell types including macrophages and myocytes[205 331 332]. In an aerosolised LPS animal model of lung injury, intrapulmonary VEGF protein levels were upregulated[242]. However, these studies did not examine specific isoform expression and the latter study did not specifically assess the direct effects of LPS. Moreover, in human necropsy tissue ($VEGF_{121}$ and $VEGF_{165}$) isoform transcript expression was reduced on alveolar epithelium in "later" sepsis-induced ARDS a situation where high levels of intra-alveolar LPS and TNF would be anticipated particularly in non-survivors[320]. The apparently conflicting results may be explained by the fact this was an observational study using autopsy material in non-survivors with pre-terminal ARDS due to sepsis. Other observational studies have confirmed that intrapulmonary VEGF levels fail to normalize in non-survivors with ARDS[247]. A reduced VEGF signal or presence in non-survivors is consistent with a protective function in the alveolus.

From a biological perspective, a positive response to LPS stimulation would be consistent with a role in recovery, accepting the supportive evidence for a role in recovery rather than injury discussed already in Chapters 1, 3 and 4. Indeed, the reason behind the selective failure of LPS to increase $VEGF_{165}$ transcription is unexplained and surprising as this is the predominant isoform in humans (see chapter 1, section 1.2.4[168]). Further studies are required to examine this further. Specifically, this study has not examined translational effects so this does not exclude the possibility of an increase in $VEGF_{165}$ protein.

Although TNF is well described as upregulating VEGF expression, it was not noted to have any positive effects on VEGF isoform transcription in this study[205 333]. This is perhaps surprising but again this study did not examine post-transcriptional effects and differential isoform expression. Further studies are required to address this question. However, one observational study has reported a reduction in VEGF isoform expression in human alveolar epithelium in the context of sepsis-induced ARDS (where high intra-alveolar TNF levels would be anticipated but were not measured) but this was in non-survivors and necropsy tissue so the failure of an increase in the postulated "protective" alveolar VEGF signal might have been predictable in this study[320].

Because of its novel discovery and methodological difficulties in demonstrating it particularly at the protein level, the isoform expression at translational level has not been studied yet in human ATII cells[180 334]. In addition, a novel inhibitory isoform, $VEGF_{165}b$ has been detected by RT-PCR but is not differentiated by current ELISA techniques due to its structural similarity[180 181]. At the present time, specific antibody techniques and ELISA antibodies are in development to detect this specific isoforms[181 335]. This has clear implications for reappraisal of current VEGF literature as previous ELISA measurements may not have accounted for this isoform particularly if it is demonstrated that there is a significant alveolar source and that isoform switching occurs in injury. This may also apply to the other isoforms in that a $VEGF_{xxx}b$ superfamily may exist on the basis of preliminary studies[181].

4.4.1.3 ATII VEGF receptor expression

In these experiments, I have demonstrated VEGF specific receptor (VEGFR2, VEGFR1 and NRP-1) protein expression on alveolar epithelium and ATII mRNA expression by RT-PCR. This would be consistent with an autocrine role of VEGF in the lung.

The observed effects of VEGF on VEGF isoform and receptor transcription in this study are quite different. In contrast to the increase in VEGF isoform transcription, VEGF increased VEGFR1 transcription (at 1 and 10ng/ml) but decreased VEGFR2 and NRP-1 transcription (at 0.1 and 1ng/ml respectively). As discussed in Chapter 1 (section 1.2.3), evidence points to VEGFR2 (with NRP-1 as a co-receptor) as its main functional receptor with VEGFR1 as a decoy receptor. Therefore, in theory (if VEGFR1 is a decoy receptor), the observed functional effects would tend to favour a reduced VEGF biological positive signal as opposed to the effects of VEGF on its own isoforms. Further studies are required to explore and corroborate these findings.

It is postulated the effects of VEGF on its receptor transcription might serve as a regulatory feedback function to control VEGF activity. However, the fact that this negative feedback appeared to occur at concentrations below 10ng/ml (0.1 or 1 ng/ml) without a clear dose-dependent inhibition suggests the regulation is more complex and non-linear perhaps involving a critical concentration threshold to achieve negative regulation. Alternatively, or in addition, above a certain concentration threshold, a positive feedback regulatory mechanism may occur. Intuitively, this may be a survival mechanism to amplify a positive VEGF biological signal at times of extreme VEGF release but controlling the VEGF signal at lower ambient VEGF concentrations. Only further studies will confirm or refute these hypotheses. In contrast, the effects of VEGF on its isoform expression might serve to maintain intra-alveolar VEGF levels to act on receptors other than those on epithelium.

LPS similarly increased VEGFR1 but not VEGFR2 or NRP-1 expression. TNF did not increase receptor expression but did decrease NRP-1 expression. Both of these effects would act to decrease VEGF biological activity. The reason for these observations is unclear but it is postulated this might be a regulatory feedback mechanism to control VEGF activity. However, protein expression was not assessed or exposure to other concentrations of these cytokines so these data do not exclude a protective role for VEGF as posttranscriptional changes may be important.

There are no specific studies addressing the question of VEGF receptor expression in response to LPS and TNF in the human lung. However, indirect evidence is conflicting. In an observational study on human necropsy tissue, lung tissue VEGFR2 transcript expression was reduced in "later" sepsis-induced ARDS[320]. In such non-survivors, intra-alveolar levels of LPS and TNF would be expected to be significantly elevated. However, conclusions are limited by the fact that specific intra-alveolar measurements of LPS or TNF were not performed, specific localisation was not performed, protein levels were not assessed, and VEGFR1 and NRP-1 were not analysed.

Other indirect evidence has demonstrated increased VEGFR2 (and IL-6) levels in mice in response to high tidal volume ventilation in an acid aspiration model attenuated by low tidal volume ventilation[243]. Such ventilatory strategies again have been associated with pro-inflammatory cytokines release as is thought to occur in ventilator-induced lung injury, and would be expected to increase TNF and LPS levels locally[208]. However, again no specific measurement of lung TNF or LPS levels were made and the stimulus here may have been stretch rather than pro-inflammatory cytokines; additionally, VEGFR1 and NRP-1 expression was not assessed. Moreover, this study was not performed in humans. Further studies are required looking at all the VEGF receptor expression alterations in lung injury with synchronous measurements of cytokine levels and also assessing the effects of stretch.

4.4.1.4 VEGF as an alveolar epithelial mitogen

One of the cardinal functions of VEGF is as a vascular endothelial cell mitogen[100]. Human studies have confirmed high intra-alveolar levels of VEGF in the uninjured state[128]. It is hypothesised that this could be explained by an intrinsic protective role for VEGF in the lung that becomes of greater relevance in lung injury. The postulated mechanism is as an alveolar epithelial mitogen, in addition to its well-known mitogenic role on vascular endothelial cells (reviewed in Chapter 1). This would allow the alveolar epithelium to be repopulated following injury, allow clearance of the alveolar fluid and facilitate restoration of the depleted numbers of type 1 alveolar epithelial cells for gas exchange[2].

Contrary to this hypothesis, I did not demonstrate any significant proliferation effect of $VEGF_{165}$ on ATII cells with the methodology employed. Studies on alveolar epithelium have been conflicting and small in number as already reviewed earlier in this chapter (see introduction, section 4.1.1). These discrepancies may be due to species differences, developmental time factors, different time points and other aspects of methodology. There are also significant limitations in the conclusions that can be drawn from existing studies. These have looked at either developing human lung or rodent or murine species and have not used human ATII cells (the gold standard). The vast majority of existing studies have failed to assess DNA uptake and cell number as well as cytotoxicity and have therefore failed to confirm the noted effects are due to proliferation *per se* rather than reduction in cell death.

The addition of *sflt* to assess the effects of VEGF blockade to higher concentrations of VEGF (10 and 100ng/ml) led to a significant decrease in observed proliferation versus the same VEGF concentrations without the inhibitor. These higher concentrations are compatible with those measured in normal human intra-alveolar VEGF levels but due to limitations on ATII cell yield, the effects of *sflt* on lower VEGF concentrations (which would be compatible with intra-alveolar VEGF levels at varying timepoints in ARDS) could not be explored in these experiments. Nevertheless, these results are compatible with VEGF functioning as a survival factor on human ATII cells, rather than a mitogen *per se*. Current studies provide evidence of this function, albeit on the vascular endothelium. For example, Gerber et al.[118] have demonstrated VEGF upregulates anti-apoptosis proteins bcl-2 and A1 in human umbilical vein endothelial cells. VEGF is also protective of endothelial cells from apoptosis via inhibition of p38 MAP kinase and activation of the phosphatidyl 3-kinase/Akt pathway[147 336].

VEGF blockade alone without VEGF did not significantly alter proliferation. This may indicate that a critical local VEGF concentration is necessary to maintain human ATII cell survival that was observed at VEGF concentrations at or above 10ng/ml in this study. These concentrations equate to those measured in normal human lungs[128]. This does suggest a threshold intra-alveolar concentration may be necessary for human ATII cell survival but only further experiments using *sflt* at these lower VEGF concentrations would answer this question. Alternatively, it is possible that because of either excessive or inadequate VEGF expression by human ATII cells in culture in these experiments, that the addition of *sflt* made no measurable difference either because of an inability to bind all soluble VEGF or because there was no significant soluble VEGF to bind. Further experiments with synchronous measurements of expressed VEGF in the culture supernatants would answer this question. In addition, it would be important to repeat these experiments using different culture time points, different mediator and MTS reagent incubation periods and at different confluence levels with different cell numbers.

4.4.2 Limitations

Before discussing and reviewing the implications of the data in this chapter in the light of current knowledge, it is important to appreciate the limitations to this study. Firstly, it is important to confirm that VEGF is a true alveolar epithelial (as well as endothelial) mitogen, in addition to the established alveolar epithelial growth factors such as KGF[337]. The MTS assay has limitations in this regard. It is based on cell viability and assumes that increased metabolism and generation of a colorimetric formazan product is a valid surrogate for proliferation. However, to confirm true proliferation, corroborative evidence will be required from DNA incorporation assays and morphological assessments of actual cell number in future studies as well as apoptosis assays (as this would also affect DNA incorporation rates). True proliferation would be confirmed by increased DNA incorporation per individual cell rather than solely due to a reduction in apoptosis or increase in cell survival; whereas increased cell metabolism (as detected in the MTS assay) may be due to either increased cell number via an increase in cell survival as demonstrated in podocytes by Foster et al.[106], decline in apoptosis or via increased DNA incorporation and true increase in proliferation as demonstrated by Brown et al.[103]

Demonstration of a direct effect on proliferation is particularly important as accumulating literature suggests that VEGF can promote cell survival via an anti-apoptotic function at least in vascular endothelial cells[118 338]. Indeed, it has been postulated that the apparent beneficial effects of VEGF on the alveolar epithelium may be secondary to its proliferative functions on the vascular bed based on studies demonstrating VEGF blockade profoundly affects the vasculature as well as impairing alveolar growth for example in the developing rat[271]. However, the accumulating wealth of evidence from intervention studies demonstrating profound effects on the epithelium with an emphysema-like appearance in animal studies including rats and adult mice in particular would support the alternative hypothesis of VEGF as a direct alveolar epithelial mitogen[256 257]. Confirmation of true proliferation on ATII cells would add further weight to this hypothesis.

Secondly, the timing of addition of VEGF may have underestimated its proliferative effect. The experimental stimulation with varying doses of VEGF was commenced at 30% confluence but this may not have allowed enough time for discriminative proliferation. Stimulating with VEGF when the ATII cells were less confluent may have allowed more opportunity for proliferation under all the test conditions. The stimulation for 48 hours may not have been optimal for the chosen confluence of 30% ie) it may have been too long to allow adequate proliferation.

Thirdly, ATII culture was performed in serum rich media (10% newborn calf serum, see Appendix). Theoretically, this may make proliferation assay results difficult to interpret, as not all the cells would be in equal phase in the cell cycle and not in G_0 phase. Potentially, it may have led to an underestimate of proliferation because serum starving in a serum free medium would have put the cells in G_0 phase before exposing them to VEGF. Indeed, serum starving was employed both by Ohwada et al.[255] coworkers and Brown et al.[103] who both demonstrated a significant effect on proliferation of alveolar epithelial cells. Raoul et al.[103 259] failed to demonstrate a positive effect on proliferation and used serum rich medium. In addition, use of serum free media can be harmful and might have adversely affected cell survival and negatively interfered with proliferation assays. Given the significant loss of cell number (by virtue of the long and rigorous decontaminating steps in the ATII cell extraction and purification), it was thought to be important not to adversely affect cell numbers in any other way. Therefore, serum rich medium was used in these experiments. It is acknowledged that future experiments should at least in part incorporate serum free techniques when addressing the hypothesis of alveolar epithelial proliferation (discussed further in Chapter 6).

Fourthly, the relatively low n number (reducing the power and increasing the risk of a type 2 error inevitably) is due to the difficulty in obtaining human lung tissue from resection with heightened public anxieties over consent of human tissue research coupled with increased bureaucracy in ethics committees and the technical difficulties in human ATII isolation. In addition, thoracic surgeons in the UK are a scarce resource and there has been a decline in surgical lung biopsies for ARDS. Hence, the lung tissue obtained was from cancer resections, although a pathologist had identified "normal" areas suitable for research as the closest alternative to normal lung. However, it was not possible to obtain human ATII cells from ARDS lung tissue that would have been the optimal comparison to the quasi-normal lung. In addition, inevitably many of the lungs were from current or ex-smokers. Smoking is known to damage alveolar epithelium and is correlated with lower intrapulmonary VEGF levels[252].

Fifthly, neonatal calf serum (NCS) was used in the completed ATII cell medium and it is not known, on the basis of current literature, whether NCS is an exogenous (and confounding) source of VEGF. NCS is known to increase permeability of tight junctions of cultured epithelial cells, a function of VEGF[339]. Future experiments should clarify this issue (although serum starving would circumvent this potential issue, albeit with other potential problems).

Finally, semiquantitative RT-PCR has inherent limitations as described already (see chapters 2, 3). However, at the current time no isoform-specific VEGF antibodies are available and isoform-specific RT-PCR is one of the few methodologies to assess relative isoform expression, albeit at the mRNA level. It would also have been preferable to sequence the receptor RT-PCR products although these receptors are well known to be expressed in the positive controls used and the products correlated well in size on electrophoresis with primers used frequently in the literature.

4.4.3 Conclusion

With regard to VEGF as an intrapulmonary VEGF source, human ATII cells express all the three main isoforms with upregulation in response to VEGF and LPS. Such a mechanism is consistent with a protective role for intra-alveolar VEGF in response to injury. The failure of the other pro-inflammatory cytokines to significantly alter VEGF isoform expression requires explanation; it might serve as a regulatory control mechanism to limit excessive VEGF expression in a pro-inflammatory milieu. These studies did not examine posttranscriptional regulatory mechanisms, which may explain the failure of LPS to increase VEGF protein expression, and they require corroboration in real-time PCR experiments. Moreover, the functional response of human ATII cells in terms of VEGF expression to dysoxic (both hypoxic and hyperoxic environments as occur in ARDS) has not been examined. Further studies are also needed to address whether isoform switching occurs and what the functional effects on $VEGF_{165}b$ production might be in such circumstances.

Intra-alveolar VEGF receptor expression appears to be modified transcriptionally by a pro-inflammatory milieu or VEGF itself. These experiments require corroboration by real time PCR. The observed changes would tend to lead to a reduced VEGF biological signal or increase in the decoy receptor (VEGFR1) and may serve as a regulatory control mechanism to limit VEGF activity in response to injury. Alternatively, posttranscriptional effects might account for the observed increase in VEGF receptor expression (described in Chapter 3) in later lung injury so further studies must examine this possibility further.

Finally, these experiments do not support the hypothesis that $VEGF_{165}$ is a human ATII mitogen *in vitro*. However, the significant impairment of proliferation in a cell viability assay by *sflt* does suggest that VEGF may have a role in human ATII cell survival possibly at a threshold intra-alveolar concentration. Further studies examining apoptosis, cell number and DNA incorporation are necessary to examine these associations further. Certainly, VEGF is known to have an anti-apoptotic effect, at least in vascular endothelial cells[118]. Moreover, these results do not exclude this possibility of VEGF being a true ATII mitogen because as discussed earlier the MTS assay does not only reflect proliferation and only a defined set of time points and confluence settings were used. Further studies assessing the effect of *sflt* at lower VEGF concentrations are required as the effects of VEGF blockade in such environments are less clear. In addition, further studies are necessary to examine the expression of other novel inhibitory isoforms, such as $VEGF_{165}$b, as these may potentially alter human ATII cell proliferation and cell survival given its observed effects on the vascular bed[180 181].

CHAPTER 5:
VEGF +936 CT POLYMORPHISM IN ARDS

This chapter will examine the relationship between the functional +936 CT VEGF polymorphism and the susceptibility to and severity of ARDS. It will also look at the relationship between +936 CT genotype and plasma and intrapulmonary VEGF protein levels.

Part of this chapter has been published in Thorax.

Medford ARL, Keen JL, Bidwell J, Millar AB.

Vascular endothelial growth factor gene polymorphism and acute respiratory distress syndrome.

Thorax 2005 60: 244-48.

SUMMARY

Background

Only some individuals go on to develop ARDS suggesting genetic factors may be important in susceptibility. VEGF may have a role in recovery from lung injury. One functional VEGF polymorphism (+936CT) is associated with significantly lower plasma levels in those with the T allele. I hypothesized possession of the T allele would be associated with susceptibility to and severity of ARDS.

Methods

A cohort of 137 normal subjects, 117 ventilated patients with and 103 "at risk" of ARDS were genotyped. Plasma and BAL VEGF levels in genotyped patients were measured. The relation between VEGF genotype, BAL and plasma VEGF level and physiological severity score and outcome was also determined.

Results

CT and TT genotype frequencies were significantly increased in ARDS patients compared to the other cohorts. In ARDS patients, the T allele was associated with a higher mean APACHE3 score. BAL VEGF levels were lower than plasma levels in both cohorts irrespective of genotype. There was a significant increase in plasma VEGF level in ARDS patients with the T allele.

Interpretation

These data support a role for VEGF in genetic susceptibility to ARDS and its associated physiological derangement. The association of the T allele with a higher plasma VEGF level in ARDS may account for the observed association with increased mortality and physiological derangement. Further studies are necessary to clarify the underlying mechanisms behind this observation.

5.1 INTRODUCTION

As already reviewed in the earlier chapters in this thesis, ARDS is the most extreme form of acute lung injury with a significant mortality and morbidity despite improvements in management of sepsis and ventilatory support[23]. Several properties of VEGF have led to investigation into its potential role in this condition as already discussed. Accumulating evidence is consistent with VEGF having a role in recovery from lung injury. This comes from *in vitro* studies, animal models and clinical observational studies. The apparent discrepancies in the literature which can be attributed to methodology and species differences.

5.1.1 Background

In the presence of a given risk factor for developing acute lung injury, this does not automatically ensue. The incidence of ARDS has been observed as quite low in patients at risk of the syndrome, occurring in only 26% in one large study[340]. Moreover, the proportion of patients developing acute lung injury varied depending on the clinical risk factor, varying from 43% in sepsis to 25% in trauma[340]. In another prospective study, diabetic patients had a significantly lower incidence of ARDS (25% versus 47%)[341]. One of the potential explanations for all these observations is genetic factors. Polymorphic genetic factors controlling cellular and humoral immune responses are known to have a role in explaining the clinical variability in presentation, outcome and duration in other diseases[342].

Genetic polymorphisms occur in at least 1% of the population. They may lead to alterations in the structure and function of proteins by substitution of a single base. Gene expression may be affected by a change in stability of mRNA or altered binding of transcription factors depending on the site of the polymorphism[343]. Polymorphisms that are associated with a biologically plausible and potentially functionally significant effect have been investigated by genetic association studies in particular to try and assess their contribution to susceptibility using a "candidate gene approach".

5.1.2 Genetic association studies in ARDS

The genetic basis for acute lung injury remains poorly understood. This is for a variety of reasons. Firstly, specific allele association studies in acute lung injury have been until relatively recently lacking[344]. Secondly, despite the slow accumulation of evidence from such genetic association studies, genetic factors are likely to be complex in acute lung injury as it arises from diverse triggers in phenotypically variable population and there may well be interactions between the gene and environment as well as variable gene penetrance. Finally, methodological difficulties probably represent the main reason for current low number of studies and level of knowledge rather than the lack of functional polymorphisms with biologically plausible and potentially relevant effects. These include the need for accurate and rigorous phenotyping to remove disease heterogeneity, a sufficient sample size of sufficient statistical power considering the allele frequency, an appropriately matched control group of similar ethnicity (interracial differences in allele frequency may occur), additional appropriate matched controls for a ventilated disease cohort to control for critical illness and ventilation, the lack of confirmation in family-based or linkage association studies which are not feasible in acute lung injury due to its sporadic nature as in other diseases such as asthma, and the failure to often replicate such findings independently in another population as a further stringent test of potential association[345].

Other investigators have extended the "candidate gene approach" used expression profiling with gene chip arrays to help identify novel potential gene candidates for other genetic association studies but these present further problems of large numbers of potential genes. Other attempts to restrict potential candidates have included comparisons of gene expression across species from multiple animal models (rat, murine, canine) using array techniques as well as human cell culture models of stretch-induced lung injury before subsequently confirming the potential importance of the candidate gene in real time PCR and immunohistochemistry studies in human models followed by demonstration of an effect on susceptibility in association studies for the pre-B-cell colony-enhancing factor (PBEF) gene[346]. Ideally, such candidate genes would be confirmed in transgenic and knockout techniques before assessing functional polymorphisms in these genes in rigorously designed genetic association studies.

Currently, the number of genetic association studies in acute lung injury continues to grow but are significantly less than those for sepsis alone[345]. In addition, many of the published studies are hampered by being underpowered, having inadequate ventilated matched controls of similar mixed populations of heterogeneous phenotypes and ethnicities and have not been replicated in independent populations. Existing published studies have only examined a small number of candidate genes: SP-B, angiotensin converting enzyme (ACE), TNF and PBEF as discussed below.

In the C/T 1580 SP-B polymorphism (associated with reduced SP-B activity), the C allele is associated with susceptibility to ARDS although the sample size was relatively small[347]. Similar findings were noted in a study of patients with community acquired pneumonia at risk of ARDS although mixed ethnic groups were used[348]. Another SP-B polymorphism in intron 4 is associated with susceptibility to ARDS in women, in particular by direct pulmonary injury though aspiration or pneumonia although the functional effects here are unknown. However, intrapulmonary SP-B levels were not measured, sample sizes were small and only a ventilated "at risk" group was used as control[349]. In the I/D (insertion/deletion) angiotensin converting enzyme (ACE) gene polymorphism, the D allele (a 287 base pair deletion in intron 16, associated with higher plasma and T cell ACE activity) is associated with both susceptibility and mortality. ARDS patients of mixed aetiologies were used but there was a very large normal subject group, a ventilated non-ARDS respiratory failure group, and a ventilated control group[344]. Presence of the A allele from the G/A 308 TNF polymorphism (associated with increased TNF production) is associated with higher mortality in ARDS particularly in younger patients[350]. Presence of the T allele from the C/T 1543 PBEF polymorphism (associated with reduced transcription) is associated with susceptibility to acute lung injury in Caucasian patients with sepsis[351].

Given its biological properties and existing functional VEGF polymorphisms (reviewed in Chapter 1), extensive genetic association studies have been performed in cancer and vascular disease (not reviewed further here). However, of far greater potential relevance to acute lung injury, other genetic association studies have also confirmed a role in susceptibility in inflammatory diseases. These include rheumatological (rheumatoid arthritis, Behcets disease, Henoch Schonlein purpura, temporal arteritis, ankylosing spondylitis), skin (psoriasis) and kidney disease (nephrotic syndrome)[231 352-358]. In inflammatory lung disease (as discussed in Chapter 1), genetic association studies have examined the VEGF 936 C/T polymorphism in COPD (no association found) and sarcoidosis (reduced susceptibility with T allele)[225 226]. Therefore, it is reasonable to postulate such polymorphisms may also be of relevance in another inflammatory condition affecting the lung, acute lung injury and are hence worthy of study.

As reviewed in Chapter 1, considerable variation in plasma VEGF levels has been noted amongst healthy subjects and functional VEGF polymorphisms are well described but at the current time have not been investigated as a potential explanation for susceptibility to the development of acute lung injury or critical illness. A CT substitution at position 936 distal to the start of translation in the 3'-untranslated region of the VEGF gene on chromosome 6p21.3 has been associated (by as yet uncertain mechanisms but thought to involve transcription factor AP-4) with a 75% reduction in plasma levels in subjects with the T allele, both CT and TT genotypes[220]. It should be noted that, currently, the recognised functional consequences of this polymorphism are not known despite the known significant effects on plasma VEGF level.

5.1.3 Hypothesis

This thesis is based upon the hypothesis that VEGF has an important role in repair and recovery from lung injury. In Chapter 3, I confirmed VEGF and VEGF receptor expression on both sides of the alveolar-capillary membrane including ATII cells but with no evidence of VEGF isoform switching. In Chapter 4, I confirmed ATII cells as a significant alveolar source of VEGF and its receptors. The data also suggest VEGF has an autocrine action here serving as an ATII cell survival factor. In order to further investigate this hypothesis, I explored the following questions:

- Does a functional VEGF polymorphism (the +936 CT polymorphism) contribute to genetic susceptibility to ARDS?
- In addition to susceptibility to ARDS, is there a relationship between VEGF +936 genotype and severity of ARDS?
- Are any observed relationships with susceptibility and severity of ARDS, specific to this syndrome rather than critical illness *per se*?
- What is the relationship between VEGF +936 genotype and plasma and broncho-alveolar fluid (BALF) VEGF levels?

5.2 METHODS

For detailed methods on severity scores, plasma, BALF, DNA extraction and genotyping see Chapter 2.

5.2.1 Participants

Blood samples were obtained from 137 normal subjects and 215 ventilated patients in Southmead Hospital Intensive Care Unit (ICU) both "at risk" (n = 103) of and with ARDS (n = 117) as defined in 1994 by the American-European Consensus Conference Committee[11]. "At risk" patients were classified strictly as per previous established definitions used in previous studies, also discussed in Chapter 2[98].

Patients in the "at risk" cohort who went on to develop ARDS (see section 5.3.1 in Results, Chapter 5) were included in the ARDS cohort for statistical analysis (as they had developed ARDS) but also excluded from either cohort for a second analysis of an ARDS cohort referred to as "ARDS excluded" in the Results (section 5.3.1) to control for the change in disease phenotype and assess the impact of this sub-cohort.

Normal subjects were in good health, of identical ethnicity and of similar gender ratio and age. Plasma and BAL samples (with matched genotypes) were obtained only in a proportion of patients because of failure of genotyping or DNA extraction, decline of consent, haemodynamic instability, timing of admission or unpredicted patient movements off unit. Whole blood sampling (and successful matched genotyping) was performed in 78 ARDS and 45 ventilated "at risk" patients. Fibreoptic bronchoscopy and broncho-alveolar lavage was performed in the right middle lobe (as described previously) with matched genotypes in 59 ventilated ARDS 32 "at risk" patients[247]. Flexible bronchoscopy was already performed as part of routine clinical practice as a standard of care in the intensive care unit (to detect possible nosocomial infection). Sampling was performed at 72 hours on average from the time of ITU admission (see results for sampling range).

Exclusion criteria were patients with an extensive smoking history (> 20 pack years), IPF, COPD or malignancy. The North Bristol NHS Trust Local Research Ethics Committee granted full ethical approval for the study. Informed consent was obtained from the patient or a surrogate.

5.2.2 Clinical data

Murray Lung Injury, Acute Physiology and Chronic Health Evaluation II (APACHE II), Acute Physiology and Chronic Health Evaluation III (APACHE III) and Simplified Acute Physiology (SAPS II) Scores were recorded for each ICU patient[7] [288-290]. 28 and 60 day mortality were also recorded.

5.2.3 VEGF Measurements

Plasma and BALF VEGF were measured using a sandwich ELISA kit (R&D Systems, Abingdon, UK) according to manufacturer's instructions as described previously[244] [247]. The detection limit was 2.9 pg/ml with 5% intra-assay and 7% inter-assay variability.

5.2.4 Statistical Analysis

Using Quanto version 1.1 software (http://hydra.usc.edu/gxe/), assuming a T allele frequency of 0.16 as per existing studies, a type 1 error rate of 0.05, with two-way statistical significance at $p < 0.05$, and assuming a 1:1 control:case ratio, 142 cases and controls would be required to have 80% statistical power to demonstrate to detect an odds ratio of 2 difference in allele frequency[220 318 319]. Given the recruited numbers in this study, with a control: case ratio varying between 1.17:1 (for ARDS) to 1.33:1 (for "at risk"), the study was estimated to have 75.4% statistical power according to detect such a finding.

Data were analyzed using Graph Pad Prism version 4.0. The Ryan-Joiner test was used to assess normality of data. Nonparametric data were normalised using log transformation. ITU severity scores and ELISA data were compared by ANOVA with a *post hoc* Bonferroni correction to control for multiple group comparisons. Genotype and allele frequencies, age distributions and risk factor distributions were compared by the Fishers exact test to control for low cell values. The Chi Squared test was used for contingency table analysis of these when a subsequent analysis including the "ARDS excluded" cohort was performed (see section 5.2.1 for discussion). Data in bar charts are plotted as mean and standard error. Hardy Weinberg equilibrium was assessed using the Chi Squared test for allele frequencies. For all tests, a p value of 0.05 or less was considered significant.

5.3 RESULTS

5.3.1 Baseline Characteristics

Table 5.1 shows baseline characteristics for normal (N) subjects, ventilated "at risk" (AR) patients and ARDS patients.

Group	No	Mean (SE) age (years)	Sex (F:M)
Normal	137	52*† (1.7)	70:67
"At risk"	103	64* (1.4)	44:59
ARDS	117	61* (1.4)	48:69
"ARDS excluded"	100	61† (1.5)	42:58

Table 5.1: Baseline characteristics (modified from Medford et al.[359]). "ARDS excluded" refers to ARDS cohort excluding those who developed ARDS from the "at risk" cohort (n = 17) during the period of study (see Methods section 5.2.1 for further discussion). *p < 0.001 Bonferroni N versus AR and ARDS, p < 0.01 Bonferroni †N versus "ARDS excluded", p < 0.0001 (ANOVA)

There were no significant variations between groups in ethnicity or gender distribution, but the age of the normal group was significantly lower than the "at risk" and ARDS groups (mean age 52 yrs v 64 yrs and 61 yrs [$p < 0.001$ and $p < 0.01$ Bonferroni]).

The risk factor profile of the "at risk" and ARDS cohorts is shown in Table 5.2 and overall significantly different ($p < 0.02$, Chi Squared). They were well matched for most aetiologies and subtypes of sepsis except a higher proportion of total and chest sepsis ($p < 0.01$ and $p < 0.02$ respectively, Chi Squared) and a lower proportion of massive transfusion in the ARDS cohort ($p < 0.0002$, Chi Squared).

Risk factor	"At risk"	ARDS	"ARDS excluded"	p value
Sepsis (combined)	72 (69.9)	99 (84.6)	85 (85)	0.01*
Sepsis (chest)	37 (35.9)	61 (52.1)	54 (54)	0.02†
Sepsis (abdomen)	22 (21.4)	30 (25.6)	24 (24)	0.76
Sepsis (unknown site)	10 (9.7)	6 (5.1)	5 (5)	0.29
Sepsis (nervous system)	3 (2.9)	2 (1.7)	2 (2)	0.82
Massive transfusion	22 (21.4)	7 (6)	6 (6)	0.0002**
Acute pancreatitis	5 (4.9)	9 (7.7)	8 (8)	0.61
Inhalational injury	4 (3.9)	2 (1.7)	1 (1)	0.34
Total	103	117	100	0.02†

Table 5.2: Risk factor profiles for ventilated cohorts. Values are given as number (%). †Overall p < 0.02 (Chi Squared). *p < 0.01 sepsis (combined) AR v ARDS; †p < 0.02 sepsis (chest) AR v ARDS; **p < 0.0002 massive transfusion AR v ARDS. (Modified from Medford et al.[359])

Table 5.3 shows the "at risk" and ARDS ventilated cohorts were matched in terms of generalized physiology severity scores (APACHEII, APACHEIII and SAPSII), but Murray Lung Injury scores were higher in the ARDS cohort as expected (2.84 or 2.92 v 1.36 [$p < 0.001$ (Bonferroni)]).

Group	Genotype	Murray LIS	SAPS II	APACHE II	APACHE III*
"At risk"	All	1.36 (0.08)	42.2 (1.31)	17.0 (0.78)	67.9 (2.34)
	CT,TT	1.21 (0.20)	39.9 (2.85)	18.0 (1.42)	62.2 (4.87)
	CC	1.40 (0.09)	42.8 (1.47)	17.0 (0.78)	69.3 (2.65)
ARDS	All	2.84 (0.06)	45.7 (1.32)	19.0 (0.75)	73.0 (2.47)
	CT,TT	2.74 (0.11)	46.8 (2.63)	19.0 (1.30)	80.9* (4.33)
	CC	2.90 (0.07)	45.2 (1.51)	18.5 (0.92)	69.3* (2.92)
"ARDS excluded"	All	2.92 (0.06)	46.0 (1.49)	20.5 (0.85)	73.4 (2.81)
	CT,TT	2.86 (0.11)	48.7 (2.99)	22.2 (1.49)	83.8* (4.94)
	CC	2.94 (0.08)	44.8 (1.69)	19.7 (1.02)	68.6* (3.26)

Table 5.3: ICU severity scores. LIS = Lung Injury Score. Column 3 contains all relevant severity scores for all groups and genotypes expressed as mean values with standard error in parentheses. NB: data for APACHE2 $\log_{10}$ transformed to normalise prior to statistical analysis. *p < 0.05 (Bonferroni) ARDS CT,TT v ARDS CC (ANOVA, p = 0.02). (Modified from Medford et al.[359])

16.5% (n = 17) of the "at risk" group subsequently developed ARDS, pulmonary and abdominal sepsis being the commonest predisposing factors in these individuals (see Table 5.4 below).

Risk factor	Number (%)
Sepsis (combined)	14 (82.4%)
Sepsis (chest)	7 (41.2%)
Sepsis (abdomen)	6 (35.3%)
Sepsis (unknown site)	1 (5.9%)
Massive transfusion	1 (5.9%)
Acute pancreatitis	1 (5.9%)
Inhalational injury	1 (5.9%)
TOTAL	17 (100%)

Table 5.4: Risk factor profile of "at risk" cohort subsequently developing ARDS.

5.3.2 CT, TT Genotype and T Allele Frequencies in Patient Groups

Figure 5.1 shows a typical example of IHG technique for genotyping (see Figure 5.1).

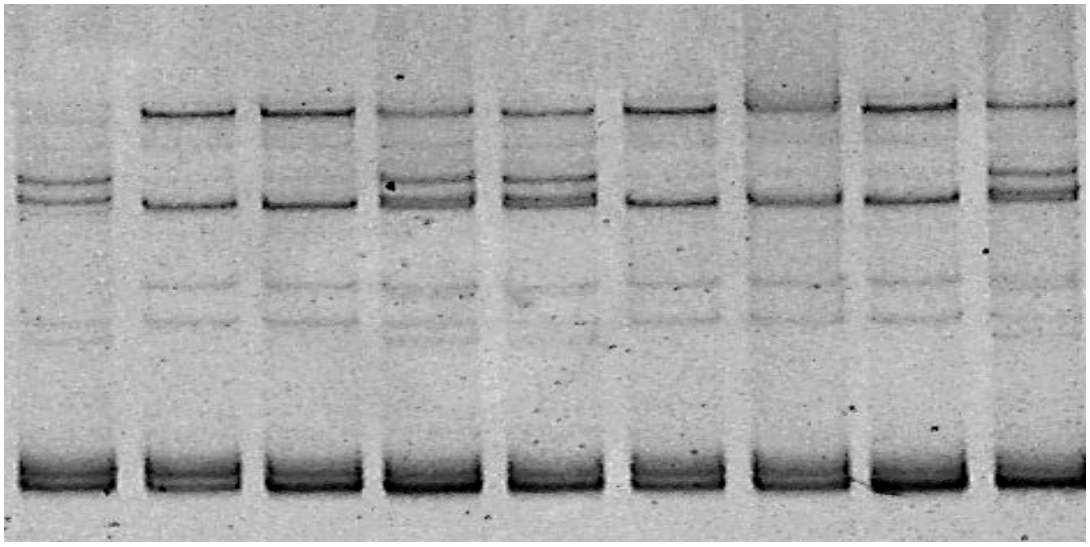


Figure 5.1: Typical digitally captured image of IHG gel showing three easily differentiated VEGF +936 genotypes: CC, CT and TT. Please see Chapter 2 for detailed methods.

Table 5.5 shows the genotype and allele frequencies for the three different groups. For all samples, the genotype distribution was in Hardy-Weinberg equilibrium (see Tables 5.6 a-d) (χ^2 = 1.418, p = 0.50 for normal; χ^2 = 0.729, p = 0.70 for "at risk"; and χ^2 = 0.173 for ARDS, p = 0.92; χ^2 = 1.766, p = 0.41 for "ARDS excluded").

Group	No	Genotype frequencies (%)		Allele frequencies (%)	
		CT, TT (polymorphic)	CC (normal)	T (polymorphic)	C (normal)
Normal	137	27 (19.7)	110 (80.3)	30 (10.9)	244 (89.1)
"At risk"	103	20 (19.4)	83 (80.6)	22 (10.7)	184 (89.3)
ARDS	117	38 (32.5)*	79 (67.5)	41 (17.5)†	193 (82.5)
"ARDS excluded"	100	32 (32)**	68 (68)	33 (16.5)††	167 (83.5)

Table 5.5: Genotype and allele frequencies. (Modified from Medford et al.[359])
Genotype frequencies: *ARDS v N: OR 2.01, 95% CI 1.13 to 3.58, p = 0.02 Fishers exact; *ARDS v AR: OR 2.05, 95% CI 1.02 to 2.20, p = 0.03 Fishers exact; AR v N: OR 0.98, 95% CI 0.52 to 1.87, p = 1.00 Fishers exact; **"ARDS excluded" v N: OR 1.89, 95% CI 1.04 – 3.43, p = 0.05 Fishers exact; **"ARDS excluded" v AR: OR 1.92, 95% CI 1.00 – 3.67, p = 0.05 Fishers exact.
Allele frequencies: †ARDS v N: OR 1.77, 95% CI 1.06 to 2.91, p = 0. 04 Fishers exact; †ARDS v AR: OR 1.82, 95% CI 1.04 to 3.18, p = 0.04 Fishers exact; AR v N: OR 0.97, 95% CI 0.54 to 1.74, p = 1.00 Fishers exact; ††"ARDS excluded" v N: OR 1.59, 95% CI 0.93 – 2.71, p = 0.10 Fishers exact; "ARDS excluded" v AR: OR 1.63, 95% CI 0.91 – 2.92, p = 0.11 Fishers exact.

VEGF +936 Genotype	Genotype frequency		$(Obs - Exp)^2/Exp$
	Expected (Hardy Weinberg)	Observed	
TT	1.63	3	1.151
CT	26.6	24	0.254
CC	108.8	110	0.013
TOTAL χ^2			1.418

Table 5.6a: Normal subjects, Hardy Weinberg equilibrium calculation.

VEGF +936 Genotype	Genotype frequency		$(Obs - Exp)^2/Exp$
	Expected (Hardy Weinberg)	Observed	
TT	1.179	2	0.57
CT	19.68	18	0.15
CC	82.14	83	0.009
TOTAL χ^2			0.729

Table 5.6b: Ventilated "at risk" subjects, Hardy Weinberg equilibrium calculation.

VEGF +936 Genotype	Genotype frequency		$(Obs - Exp)^2/Exp$
	Expected (Hardy Weinberg)	Observed	
TT	3.79	3	0.165
CT	34.5	35	0.007
CC	78.7	79	0.001
TOTAL χ^2			0.173

Table 5.6c: ARDS subjects, Hardy Weinberg equilibrium calculation.

VEGF +936 Genotype	Genotype frequency		$(Obs - Exp)^2/Exp$
	Expected (Hardy Weinberg)	Observed	
TT	2.51	1	0.908
CT	26.3	31	0.840
CC	69.1	68	0.018
TOTAL χ^2			1.766

Table 5.6d: "ARDS excluded" subjects, Hardy Weinberg equilibrium calculation.

CT and TT genotypes occurred significantly more frequently in the ARDS group than the normal group (OR 2.01, 95% CI 1.13 to 3.58, p = 0.02 Fishers exact), and those "at risk" (OR 2.05, 95% CI 1.02 to 2.20, p = 0.03 Fishers exact). This was consistent even when analysing the "ARDS excluded" cohort (excluding those "at risk" who developed ARDS): OR 1.89, 95% CI 1.04 to 3.43, p = 0.05 Fishers exact versus normal, OR 1.92, 95% CI 1.00 to 3.67, p = 0.05 Fishers exact versus "at risk" and did not significantly differ from the original ARDS cohort: OR 0.94, 95% CI 0.53 to 1.68, p = 0.88. The polymorphic T allele occurred significantly more frequently in the ARDS group (OR 1.77, 95% CI 1.06 to 2.91, p = 0.04 Fishers exact) than in the normal group and the ventilated "at risk" group (OR 1.82, 95% CI 1.04 to 3.18, p = 0.04 Fishers exact). There were no differences in either genotype (OR 0.98, 95% CI 0.52 to 1.87, p = 1.00 Fishers exact) or allele frequencies (OR 0.97, 95% CI 0.54 to 1.74, p = 1.00 Fishers exact) between the "at risk" and normal cohorts. Analysing the "ARDS excluded cohort", none of the allele frequencies were significantly different versus normal (OR 1.59, 95% CI 0.93 – 2.71, p = 0.10 Fishers exact), versus "at risk" (OR 1.63, 95% CI 0.91 – 2.92, p = 0.11 Fishers exact) and versus the original ARDS cohort (OR 0.89, 95% CI 0.54 – 1.49, p = 0.70 Fishers exact).

5.3.3 CT, TT Genotypes and Mortality (28 and 60 day)

Table 5.7 shows 28 and 60 day mortality according to disease group and genotype. Although there was a non-statistical trend to higher mortality in the ARDS (and "ARDS excluded" cohorts, especially with the latter) as would be expected, there were no significant differences in mortality between the ARDS and "at risk" cohorts as a whole either for 28 day mortality (ARDS v "at risk" OR 1.55, 95% CI 0.87 to 2.76, p = 0.15 Fishers exact; "ARDS excluded" v "at risk" OR 1.79, 95% CI 0.99 – 3.22, p = 0.06 Fishers exact) or 60 day mortality (OR 1.45, 95% CI 0.82 to 2.55, p = 0.25 Fishers exact; "ARDS excluded" v "at risk" OR 1.61, 95% CI 0.90 – 2.58, p = 0.06 Fishers exact).

Group	N	Genotype	28 day mortality (%)	60 day mortality (%)
"At risk"	103	All	28/103 (27.2)§	31/103 (30.1)‡
	20	CT,TT	6/20 (30)	7/20 (35)
	83	CC	22/83 (26.5)	24/83 (28.9)
ARDS	117	All	41/117 (35.0)	43/117 (36.8)
	38	CT,TT	17/38 (44.7)*	18/38 (47.4)†
	79	CC	24/79 (30.4)*	25/79 (31.6)†
"ARDS excluded"	100	All	40/100 (40)§	41/100 (41)‡
	32	CT,TT	16/32 (50)**	16/32 (50)††
	68	CC	24/68 (35.3)**	25/68 (36.8)††

Table 5.7: 28 and 60 day mortality (modified from Medford et al.[359]).
28 day mortality: *ARDS CT,TT v CC OR 1.81, 95% CI 0.80 to 4.05, p = 0.21 Fishers exact; **"ARDS excluded" CT,TT v CC OR 1.83, 95% CI 0.78 – 4.30, p = 0.19 Fishers exact; § "ARDS excluded" v "at risk" (all) OR 1.79, 95% CI 0.99 – 3.22, p = 0.06 Fishers exact.
60 day mortality: †ARDS CT,TT v CC OR 1.90, 95% CI 0.85 to 4.23, p = 0.15 Fishers exact); ††"ARDS excluded" CT,TT v CC OR 1.72, 95% CI 0.73 – 4.03, p = 0.28 Fishers exact; ‡"ARDS excluded" v "at risk" (all) OR 1.61, 95% CI 0.90 – 2.58, p = 0.11 Fishers exact).

28 and 60 day mortality rates did not differ between CT/TT and CC genotypes in the "at risk" cohort (28 day mortality OR 1.19, 95% CI 0.41 to 3.48, p = 0.78 Fishers exact; 60 day mortality OR 1.32, 95% CI 0.47 to 3.72, p = 0.60 Fishers exact). However, a non-significant trend was noted for higher 28 and 60 day mortality rates for CT/TT genotypes in the ARDS cohort (28 day mortality OR 1.90, 95% CI 0.80 to 4.05, p = 0.21 Fishers exact; 60 day mortality OR 1.90, 95% CI 0.85 to 4.23, p = 0.15 Fishers exact), also noted in the "ARDS excluded cohort" (28 day mortality OR 1.83, 95% CI 0.78 to 4.30, p = 0.19 Fishers exact; 60 day mortality OR 1.72, 95% CI 0.73 to 4.03, p = 0.28 Fishers exact)

5.3.4 CT,TT Genotypes and Physiological Scores

Table 5.3 shows the ICU severity scores according to disease group and genotype. As expected, the Murray Lung Injury score was significantly higher for the ARDS cohort than the "at risk" cohort (mean score 2.84 v 1.36, $p < 0.001$ Bonferroni).

There was no association between genotypes and Lung Injury, APACHE II or SAPS II scores. However, ARDS patients with CT or TT genotypes had significantly higher APACHE III scores those with CC genotypes (mean score 80.9 v 69.3, $p < 0.05$ Bonferroni).

5.3.5 +936 Genotype, plasma and BALF VEGF Levels

5.3.5.1 Genotype, demographics and mortality

Sampling to obtain plasma and BALF was performed at 72 hours on average from the time of ITU admission (mean 2.8 days, 95% CI 2.25 – 3.36, sd 0.24 for "at risk"; mean 3.18 days, 95% CI 2.69 – 3.66, sd 0.276 for ARDS). Of the cohorts analyzed for VEGF genotype and plasma and BAL VEGF protein level, they were not significantly different in terms of gender, race or age according to VEGF +936 genotype (see Table 5.8). As predicted, the ARDS cohorts had higher mortalities and all 60 day mortality rates were higher than the 28 day rates. However, there was no relationship between the presence of the T allele and either 28 or 60 day mortality (see Table 5.8).

It should be noted that the allele frequencies and mortality rates in Table 5.8 differ from Table 5.7 due to selection of those from the original cohorts able to undergo BAL. As such, it is acknowledged this is a potential source of selection bias for those "fit" enough to undergo BAL (see section 5.4.2 in Discussion, Chapter 5). Nevertheless, the observational data on relation between BAL, plasma VEGF level and genotype are novel and important, with this proviso.

	"At risk" (n = 45)		p value	ARDS (n = 78)		p value
	CC (n = 26)	CT, TT (n = 19)		CC (n = 48)	CT, TT (n =30)	
Age (yrs)	60 (3.20)	65.8 (4.08)	0.28	60.4 (2.23)	61.1 (2.76)	0.84
Gender (F:M)	9: 17	8: 11	0.76	19: 29	16: 14	0.25
28 day mortality	23.1% (6/26)	31.6% (6/19)	0.75	37.5% (18/48)	43.3% (13/30)	0.83
60 day mortality	23.1% (6/26)	36.8% (7/19)	0.54	37.5% (18/48)	43.3% (13/30)	0.83

Table 5.8: Demographic data and mortality according to VEGF +936 genotype in "at risk" and ARDS cohorts. Age data plotted as mean with standard errors in brackets.

5.3.5.2 Acute physiology score, oxygenation and VEGF protein level according to VEGF +936 genotype

The relation between the T allele and markers of oxygenation and physiological disturbance is shown in Table 5.9. As expected, the oxygenation fraction was lower in the ARDS cohorts with higher Murray Lung Injury scores. Other generalized acute physiology scores were also expectedly higher in the ARDS cohorts. However, there was no relationship between the presence of the T allele and oxygenation, Lung Injury or acute physiology score.

	"At risk" (n = 45)		ARDS (n = 78)	
	CC (n = 26)	**CT, TT (n = 19)**	**CC (n = 48)**	**CT, TT (n =30)**
PaO_2/FiO_2 (mmHg)	236.1 (16.9)	234.8 (26.8)	148 (11.2)	146 (11.24)
LIS	1.59 (0.16)	1.21 (0.21)	2.88 (0.09)	2.70 (0.12)
SAPS2	42.0 (2.82)	39.9 (2.85)	45.7 (1.82)	47.0 (2.88)
AP2	18.0 (1.36)	17.7 (1.42)	19.7 (1.79)	20.9 (1.39)
AP3	64.6 (4.69)	62.7 (4.87)	70.4 (3.76)	81.2 (4.79)
Plasma VEGF (pg/ml)	**443.0 (120.4)***	**313.8 (88.7)****	**333.0 (56.7)***,†**	**482.4 (101.4)***,††**
BAL VEGF (pg/ml)	218.9 (42.5)*	125.3 (31.3)**	179.0 (39.9)†	244.8 (60.4)††

Table 5.9: Acute physiology score, oxygenation and VEGF protein level according to VEGF +936 genotype in "at risk" and ARDS cohorts. All data plotted as means with standard errors in brackets. "At risk", p < 0.0001 (ANOVA), Bonferroni p values all > 0.05 except *p < 0.05 plasma VEGF CC versus BAL VEGF CC; **p < 0.001 plasma VEGF CT,TT versus BAL VEGF CT,TT. ARDS, p < 0.0001 (ANOVA), Bonferroni p values all > 0.05 except ***p < 0.001 plasma VEGF CC versus plasma VEGF CT,TT; †plasma VEGF CC versus BAL VEGF CC; ††plasma VEGF CT,TT versus BAL VEGF CT,TT.

The relation between the T allele and plasma/BAL VEGF protein levels is shown in Table 5.9 and Figures 5.2a-b for both "at risk" and ARDS cohorts. As in keeping with previous observations, BAL VEGF protein levels were significantly lower than plasma in the ARDS cohort irrespective of the presence of the T allele[247]. The same observations were noted for the "at risk" cohort. There was no observed relationship between the presence of the T allele and BAL VEGF levels for either cohort. However, the presence of the T allele was associated with a significantly higher plasma VEGF level in the ARDS cohort (but not the "at risk" cohort).

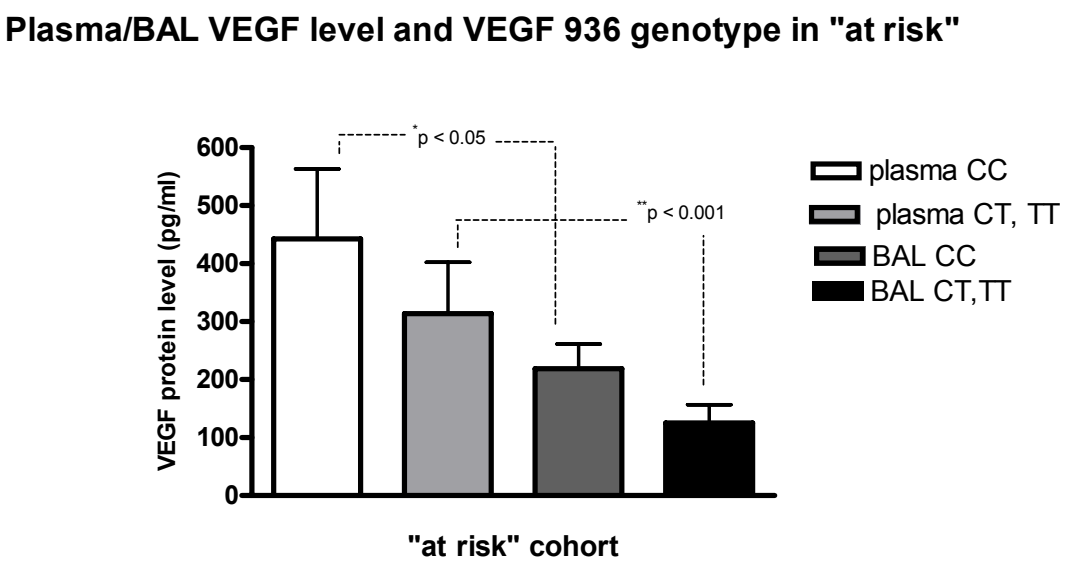


Figure 5.2a Relationship between T allele and plasma/BAL VEGF protein level in "at risk" cohort: *p < 0.05 plasma versus BAL CC, **p < 0.001 plasma versus BAL CT, TT. Data represented as mean with standard errors as horizontal bars.

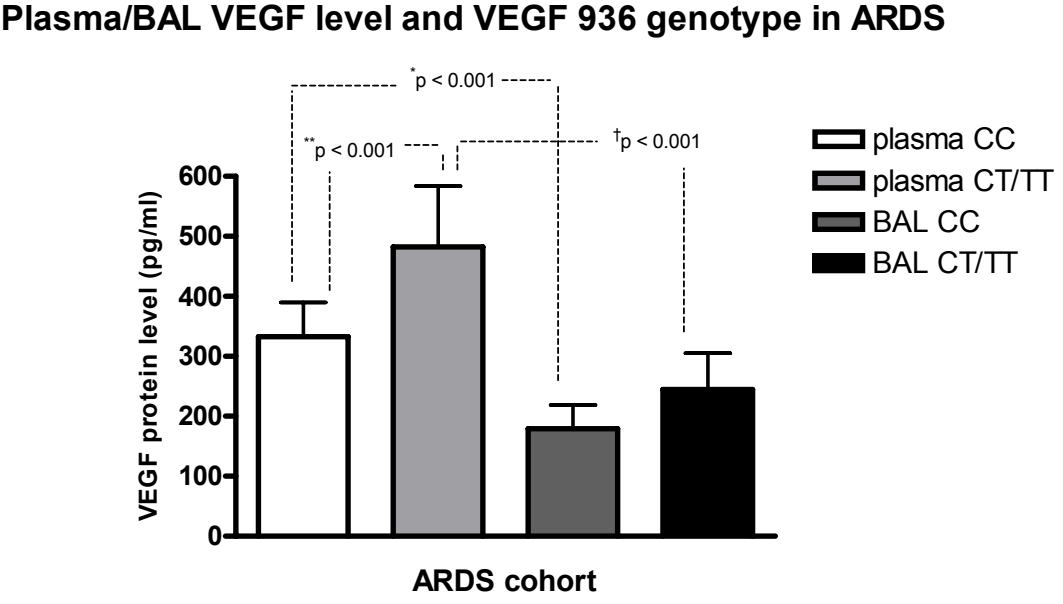


Figure 5.2b Relationship between T allele and plasma/BAL VEGF protein level in ARDS cohort: *p < 0.001 plasma CC versus BAL CC; **p < 0.001 plasma CC versus plasma CT,TT; †p < 0.001 plasma CT,TT versus BAL CT,TT. Data represented as mean with standard errors as horizontal bars.

5.4 DISCUSSION

5.4.1 Interpretation and current literature

This study suggests an association between a specific allele (the VEGF +936 T allele) and susceptibility to ARDS within ARDS subjects and associated physiological disturbance (APACHE III score). This was specific to the ARDS cohort and not demonstrated in the "at risk" cohort, which controlled for ventilator-induced injury suggesting that these findings are specific to ARDS, accepting the fact that an equivalent ventilated non-ARDS respiratory failure cohort and ventilated non "at risk" control cohort would have added further weight to this but were not feasible in this study. These data do lend support to the concept that VEGF may be an importance gene in influencing susceptibility to and severity of ARDS adding to published data suggesting a role for VEGF in ARDS[244 247 249].

As already reviewed, there is a relative paucity of genetic association studies in acute lung injury for a variety of reasons that have shown a positive association. This study is the first examining the contribution of VEGF to possible susceptibility in acute lung injury although other association studies on functional VEGF polymorphisms have been implicated in a variety of inflammatory conditions including rheumatological disease, kidney disease, skin disease and sarcoidosis as already reviewed earlier, see Section 5.1. Currently, with the exception of the current study in this thesis, published important genes in acute lung injury are only SP-B, ACE, TNF and PBEF[344 346-351].

Previously an association between the T allele and 75% reduction in plasma levels has been reported in normal subjects[220]. In this study, the presence of the T allele was also associated with a significant increase in plasma VEGF levels in the ARDS cohort. There are no previous published report of associations between the T allele and plasma VEGF levels in ventilated "at risk" or ARDS patients. As already reviewed in Chapters 1 and 3, Thickett et al.[244] have reported a significant elevation in plasma VEGF in early ARDS that fails to normalise in non-survivors. The association of the T allele with higher plasma VEGF levels in ARDS might be one explanation for the association with greater physiological disturbance (APACHE III score). The failure to demonstrate a mortality increase with the T allele in the ARDS cohort may reflect the underpowering of the study to demonstrate such a difference. However, other studies conflict as already reviewed in Chapter 1. Maitre et al.[249] found no difference in serum VEGF levels between early ARDS patients and controls whereas Hanaoka M et al.[248] have reported a rise in serum VEGF levels in high altitude pulmonary oedema with recovery. These differences can be attributed to differences in methodology and differences in aetiology of lung injury.

In normal human lungs, VEGF is compartmentalized with intrapulmonary VEGF levels (measured in fluid obtained at bronchoalveolar lavage) 500 times higher than plasma levels[128]. As already reviewed in Chapter 1, epithelial and endothelial injury occur in ARDS with a significant reduction in the alveolar-plasma concentration VEGF gradient in early ARDS but re-establishment of this in recovery[244]. Therefore, it is likely that the lung is a source of VEGF and that the rise in plasma VEGF level in early ARDS due to leakage of VEGF via a disrupted alveolar-capillary membrane exposing the underlying endothelium to its physiological effects including increased microvascular permeability as seen in ARDS.

In this study, the T allele was not associated with a change in intrapulmonary VEGF levels, which were consistently lower than plasma levels for all genotypes in both cohorts. This may be due to the timing of the sampling as observational studies have confirmed time-dependent changes in intrapulmonary VEGF following ARDS (as reviewed in Chapter 1), an effect on cell-associated VEGF isoforms ($VEGF_{189}$ and $VEGF_{206}$ not detected by the ELISA) or possibly a functional effect via some other mechanism than change in VEGF protein (eg transcriptional stability). There are no previous reports of the effects of the T allele on intrapulmonary VEGF levels and if the lung is the main source of VEGF how the T allele might affect intrapulmonary production. Previous observational studies have demonstrated a reduction in intrapulmonary VEGF levels in early ARDS (within 48 hours of diagnosis) and an increase in these levels in survivors at 72 or more hours[247-249]. In this study, BAL was taken off slightly later at 72 hours and hence not comparable. A relation cannot be therefore discounted between the T allele and intrapulmonary VEGF levels at earlier timepoints in the illness. As already discussed, epithelial lining fluid concentrations could not be derived and dilution effects may have led to a type 1 error here also.

In assessing the relationship between the VEGF polymorphism and other ARDS parameters only the APACHE III physiological score was associated. This may be related either to the non-statistical trend to increased plasma VEGF levels or the superior sensitivity of the APACHE III score over other scoring systems. This system uses statistical modelling techniques to weight and select the variables, and multiple logistic regression to estimate risk of death unlike the other used scoring systems, which use a more subjective method with weights and variables, selected by expert opinion. There is some evidence to suggest that the APACHE III is a superior prognostic model[295 360]. However, further larger studies are required to confirm the absolute specificity of this association.

So why is there an apparent relation between the T allele, ARDS susceptibility, and severity of physiological dysfunction? Possible (but as yet untested) mechanisms include alterations in transcript stability or enhancement leading to alterations in VEGF expression, alteration in alternate splicing leading to isoforms switching or even alterations in VEGF receptor expression. These hypotheses would assume VEGF to have a protective role in recovery from lung injury and hence the more important role of this polymorphism in the lung. Studies to determine the effect of these genotypes on resident lung cells such as the alveolar epithelium are required to enable the mechanism by which they may influence ARDS pathogenesis.

As already reviewed in Chapter 1, the role of VEGF in the normal human lung remains uncertain; but evidence from a variety of studies suggests a possible protective role in resolution from lung injury. Cellular, animal and clinical studies have confirmed the significant intrapulmonary VEGF concentrations in normal lung. They also suggest alveolar epithelium is a predominant intrapulmonary source and that VEGF is capable of acting as a mitogen here. Cellular and animal lung injury models as well as intervention studies with specific inhibitors, overexpression models and knockout models also add weight to the hypothesis of a protective role. Data in humans are scarcer and less conclusive but do indicate significant alterations in VEGF levels on both sides of the alveolar-capillary membrane both in the normal and injured states whether in ARDS or other forms of noncardiogenic pulmonary oedema such as high altitude pulmonary oedema. There are some apparent discrepancies in the literature with the potentially adverse effects of VEGF overexpression and stretch on VEGF expression in certain animal models and cellular studies but the majority of the discrepancies can be accounted for by differences in methodology, time points, species differences, or undetected changes in soluble receptors or isoform switching. VEGF levels may simply reflect damage to the alveolar epithelium as described in normal smokers and patients with idiopathic pulmonary fibrosis (IPF). However, more compelling evidence in the intervention and knockout studies argues against this.

5.4.2 Limitations

There are several limitations to this genetic association study in this thesis. In any genetic epidemiological study, cohort size is paramount and the sample size in this study is modest compared to other similar studies although consistent with the initial power calculation considering the initial expected allele frequency. However, this study was not powered to show a difference in mortality or outcome. Moreover, as reviewed already, acute lung injury is a complex disease likely to be polygenic, and this study has examined only one candidate gene necessitating further increases in sample size.

The availability of plasma and BAL samples with successful genotyping was a limiting factor for the reasons already mentioned of clinical instability, failure of genotyping or DNA extraction, decline of consent, haemodynamic instability, timing of admission or unpredicted patient movements off unit. This led to an inadvertent subpopulation from the original cohorts who were able to undergo BAL, which theoretically may have been a less severe group and had different mortality and allele frequencies from the original cohort (accounting for the differences in Tables 5.7 and 5.8). This limits the strength of conclusions from this subpopulation introducing a potential selection bias although the data provide observational information about the relation between BAL and plasma VEGF level and genotype with this proviso. Ideally, it would have been preferable to sample both plasma and BAL fluid both early and later during the period of illness for both genotypes and in all patients to assess the possibility of a relation between genotypes and levels earlier in the course of the injury. In addition, it was not possible to sample at exactly the same point in the duration of illness, as there was some variability in date of admission to the ICU between patients with some inter-hospital transfers between ICUs. Finally, ideally it would have been preferable to obtain BAL urea values to derive epithelial lining fluid concentrations of VEGF to control for variation in BAL volume and dilution effects[361 362].

The cohorts were not completely matched for age or risk factor profile. *Post hoc* analysis demonstrated a lower age in the normal group. Intrapulmonary VEGF levels have been reported to decline slightly with age (between the ages of 10 to 80 years up to 20 pg/ml), but vary minimally between the ages of 50-70 years, the age range of our cohorts[277]. The higher proportion of patients with sepsis and lower proportion of transfusion-related injury in the ARDS cohort limits the strength of our conclusions although sepsis is commonly the most prominent risk factor in "at risk" cohorts in such studies.

Genetic confounding can cause population specific differences in allele frequencies. In this study, the T allele frequency in the normal group (0.11) was lower than that of previous studies in the same ethnic group (0.16)[220]. This is difficult to explain other than having occurred by chance. The same frequency was noted in the "at risk" cohort. Importantly, the T allele frequency in the ARDS group was higher than all of these suggesting an important difference (0.18). Moreover, an attempt was made to minimise genetic confounding by rigorous phenotypic classification (using an accepted international definition of ARDS and previously published definition of "at risk" although the latter is not an internationally accepted definition). However, acute lung injury is a heterogeneous condition and it is often not possible to narrow the phenotype of the "at risk" cohort. In this study, this was also not possible although sepsis was by far the largest constituent. Other confounders known to affect VEGF levels either positively (cancer) or negatively (increasing age, smoking, emphysema, interstitial lung disease) were excluded if possible (reviewed in section 1.2.10 in Chapter 1).

Well-matched controls in adequate number are also essential to any well-designed genetic association study. A ventilated "at risk" cohort was used for comparison in whom genotype frequencies were comparable to normal subjects to exclude the possibility of a false association with critical illness. However, it would have been preferable to have a ventilated non-ARDS respiratory failure control group in addition to control for the same level of critical respiratory illness. In addition, a ventilated non-"at risk" control group would have been preferable to control for ventilation itself as well as standard cohort of normal subjects. In this thesis, the ventilated normal control and non-ARDS respiratory failure control groups were not accessible weakening the association of any findings. All three groups of controls have been employed in select well-designed studies[344].

Prospective recruitment to each cohort was undertaken over the same time period reducing the possibility of recruitment bias as a cause of chance variation in genotype frequencies. The cohorts were all of Caucasian ethnicity to remove the possibility of altered genotype frequencies in different ethnicities. However, because different ethnicities have not been examined for the same polymorphism, the role of ethnic factors in polymorphism frequency variation cannot be resolved at the present time. Of note, a higher VEGF +936 CT genotype frequency has been reported in a Japanese cohort than would be expected in a normal Caucasian population[224].

It is also advantageous to be able to demonstrate a functional, biologically relevant effect of the polymorphism that might explain an association with susceptibility to the disease in question. The polymorphism study in this thesis fulfils these criteria, although the relation between genotype and intrapulmonary VEGF levels (potentially more relevant in lung injury) in normal subjects is not known.

Finally, there is the possibility that the findings here are not causal and that the VEGF +936 CT polymorphism might be in linkage disequilibrium with another functional polymorphism, although this has yet to be demonstrated[220]. If future studies confirm such findings in independent populations and also for other functional VEGF polymorphisms, then linkage disequilibrium would be unlikely.

To control for multiple comparisons, *post hoc* Bonferroni statistical analysis was used. For contingency data, to remove errors related to low cell values with Chi-squared test, the Fishers exact test was used for analysis. The polymorphism under study is associated with a functional effect on the gene product and our results are biologically plausible.

5.4.3 Conclusion

In conclusion, individuals with the T allele are more susceptible to ARDS than normal subjects and ventilated "at risk" subjects. Those with the T allele develop ARDS of a higher physiological severity (higher APACHE III score) and increase plasma VEGF levels. However, presence of the T allele does not affect intrapulmonary VEGF levels or outcome. The lower intrapulmonary VEGF levels than plasma in both groups are consistent with previous observational data in ARDS and may reflect greater injury than suspected in the "at risk" group. These data, therefore, suggest a potential role for VEGF gene polymorphism in the development of ARDS in humans. In the future, intrapulmonary delivery of VEGF to those particularly susceptible according to the presence of the T allele may have a potential therapeutic role either in reducing the risk of ARDS or reducing severity of disease in those with established disease and genotyping may help target such therapy.

Before such therapies become reality, further studies are required to assess the effects of the T allele on resident lung cells especially alveolar epithelium, isoform switching, receptor expression and alteration in VEGF transcripts. Moreover, further gene microarray experiments including the VEGF gene amongst other candidates in cellular, animal and human models will help to refine the functionally important and relevant genes and clarify the strength of any possible association. Amongst other alleles, the possible role of other functional VEGF polymorphisms needs to be appraised[220-222]. Using the "candidate gene" approach, future studies also incorporating transgenic and knockout models of the VEGF gene and other relevant candidates will add further clarity to the genetics of acute lung injury. Further rigorously designed genetic association studies on the functional VEGF polymorphic genes are required adequately powered to detect changes in outcome.

CHAPTER 6: CONCLUSIONS

This chapter will summarise the significant findings from the results Chapter 3-5 in this thesis and highlight in the conclusions that can be drawn but also the limitations of the experiments. In addition, I will suggest how the experiments could be optimised, if repeated, what other questions are unanswered and what experiments are required in the future, Finally, I conclude with a revised hypothesis based on all the above for the role of VEGF in repair and recovery from acute lung injury.

Part of this chapter has been published in Thorax.

Medford ARL, Millar AB.

Vascular endothelial growth factor (VEGF) in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS): paradox or paradigm?

Thorax 2006 61: 621-6.

6.1 Conclusions

Research into acute lung injury in human studies is hampered by the lack of a specific definition until recent times, the lack of a specific biomarker to confirm the diagnosis, the heterogeneity of inducing factors and outcomes, the lack of an animal model mimicking acute lung injury exactly and the lack of understanding of any relevant genetic factors[363 364]. As well as the endothelium, the alveolar epithelium is of paramount functional importance with recovery being essential to establish fluid clearance, prevent fibrosis and restore gas exchange in ARDS[337].

As I have reviewed earlier in this thesis, VEGF is a potent permeability, angiogenesis and survival factor, known to act on vascular endothelium. VEGF is known to be abundant in the lung on the basis of cellular, animal and clinical studies, especially alveolar epithelium. Initial clinical and animal overexpression studies had led to the initial hypothesis that VEGF may be responsible for the noncardiogenic pulmonary oedema in ARDS.

However, human clinical studies in normal subjects have clearly demonstrated high intra-alveolar VEGF levels in the absence of ARDS or angiogenesis that would not be consistent with this hypothesis. In addition, as reviewed in Chapter 1, a wealth of accumulating cellular, animal and clinical studies have demonstrated recovering intra-alveolar VEGF levels following lung injury. Moreover, intervention studies of VEGF blockade in animals have demonstrated profound alveolar architectural abnormalities similar to emphysema as well as defects in the pulmonary vasculature. These studies have led to the revised hypothesis that VEGF is protective and that it has a role in recovery and repair in acute lung injury. As I have also reviewed, despite the apparent conflicts in the literature, a lot of these differences can be explained by methodological differences, species differences, and variations in alveolar-capillary membrane injury.

6.1.1 Alterations in VEGF receptor expression and isoform switching

To explore this reparative VEGF lung hypothesis further, I have explored possible reasons for the observed alterations in intra-alveolar VEGF levels following injury. One possible such explanation would be a significant alteration in available VEGF binding sites (receptors) and also changes in isoform transcription (isoform switching); both leading to significantly altered VEGF bioactivity.

In Chapter 3, I have demonstrated VEGF protein and receptor (VEGFR2, VEGFR1 and NRP-1) expression on both sides of the alveolar-capillary membrane; alveolar epithelium, alveolar macrophages and vascular endothelium. However, expression is heterogeneous in normal, early and later ARDS lung. In normal lung, VEGFR2 (the main functional receptor for VEGF bioactivity) expression is increased relative to VEGFR1 (the functioning decoy receptor) consistent with a protective non-injurious function in the normal lung. VEGFR1 and NRP-1 expression is decreased in early ARDS with persistent decreased expression of NRP-1 in later ARDS (although significantly higher than its expression in early ARDS). In later ARDS (albeit in necropsy tissue), I have demonstrated an increase in VEGF, VEGFR2 and VEGFR1 expression versus normal and early ARDS lung. These changes would facilitate a greater VEGF biological signal in the recovery stage of lung injury (albeit in necropsy tissue where there has been failure of the recovery mechanism) although the functional consequences of the changes in expression in early ARDS are more difficult to interpret.

I have also demonstrated no evidence of isoform switching at transcriptional level. Changes in isoform switching cannot therefore account for the observed reduction in soluble intra-alveolar VEGF levels in early ARDS although post-transcriptional mechanisms cannot be exlcuded[247 249]. In addition, there is a consistent increase in transcription of all three VEGF isoforms in later ARDS compared to early lung injury. Despite the use of necropsy tissue, these data can be interpreted as showing an increase in VEGF transcription in "failed" recovery (necropsy tissue), which supports the findings of increased VEGF and receptor protein expression at this stage. These data are also consistent with published animal and clinical observational studies on higher intra-alveolar VEGF levels in recovery (albeit "failed" recovery, in necropsy tissue) from lung injury[247 249]. An alternative interpretation of a pathological hypothesis can potentially be based on the necropsy tissue data. However, if all existing data (especially intervention studies) are considered, then the data are still consistent with the VEGF reparative hypothesis, demonstrating "failed" repair with increased VEGF activity in necropsy tissue.

6.1.2 Human ATII cells as the predominant cellular source of VEGF and their response to injury

I have co-localised VEGF protein expression with type 2 alveolar epithelial cells by dual staining immunohistochemistry indicating that ATII cells in the alveolar epithelium are the principal intrapulmonary source of VEGF. To explore this further, I have conducted further experiments in primary cultured human ATII cells, the closest available cellular surrogate to the human *in vivo* alveolus.

6.1.2.1 Human ATII cell isoform and receptor expression in response to pro-inflammatory cytokines

Having established ATII cells as the important cellular intrapulmonary VEGF source, it was further hypothesised that ATII cells express significant amounts of VEGF protein, that VEGF acts as an ATII cell mitogen and that ATII cell VEGF isoform and receptor expression is profoundly altered in a pro-inflammatory milieu simulating the alveolar micro-environment in ARDS.

I have demonstrated human ATII cells are a principal source of VEGF in the lung expressing significant amounts of VEGF protein and the three predominant isoform transcripts ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) and specific receptors (VEGFR1, VEGFR2 and NRP-1) *in vitro*. As discussed in Chapter 4, I have demonstrated a variable response of human ATII cells to stimulation. LPS increased human ATII cell transcription of $VEGF_{121}$, $VEGF_{189}$ isoforms and VEGFR1. However, LPS did not increase VEGF protein expression in ATII cell culture over control. VEGF (10ng/ml) increased transcription of all three isoforms and VEGFR1 (as well as 1ng/ml for the latter) but decreased VEGFR2 (0.1ng/ml) and NRP-1 transcription (1ng/ml). TNF decreased NRP-1 transcription.

These data confirm expression of VEGF binding sites on the alveolar epithelium, which facilitates an autocrine role for VEGF in the injured alveolus. This would be an important mechanism for locally secreted VEGF to exert its protective effect on the epithelium (discussed further in section 6.1.2.2). Such an autocrine role has been demonstrated in epithelial cells, albeit in the kidney and not in the lung as yet[106].

It is postulated the differential response of ATII cells to these cytokines in terms of isoform and receptor expression may indicate a strategy by the alveolus to maximise VEGF release from the epithelium but to act more distally. The effects of VEGF in particular on its isoform and receptor expression are variable and intriguing. VEGF appears to positively regulate its isoform expression especially at 10ng/ml which would amplify a positive VEGF biological signal at times of significant VEGF release eg) following recovery from lung injury. At the same time, VEGF positively regulates VEGFR1 expression that would theoretically regulate VEGF activity assuming VEGFR1 functions as a decoy receptor. VEGF also negatively regulates VEGFR2 and NRP-1 but only at specific and lower concentrations suggesting a more complex, non-linear control mechanism of negative and possibly positive regulation involving a threshold. Ultimately, such mechanisms may serve to deliver maximal VEGF biological signal at times of recovery from injury in a temporally and spatially regulated fashion. However, only further experiments will confirm this question (see section 6.3).

The failure of LPS to increase VEGF protein expression (as opposed to the increase in transcription noted) requires explanation but may either indicate post-transcriptional regulatory mechanisms or reflect high protein expression in the normal lung in the non-injured state. Transcriptional rates in these states and post-transcriptional effects must be the subject of further study (see section 6.3).

6.1.2.2 The effects of VEGF on human ATII cell proliferation

I have found no clear evidence of a significant increase in human ATII cell proliferation with VEGF although the addition of *sflt* to higher doses of VEGF (10 and 100 ng/ml) resulted in a significant decrease in proliferation. These data can be interpreted as supporting the role for VEGF at least as an alveolar survival factor but these experiments were limited in number with significant heterogeneity. VEGF is well known to act as a survival factor for ATII on vascular endothelial cells via upregulation in anti-apoptosis proteins but this has not yet been described in alveolar epithelium[118]. The failure of *sflt* to inhibit proliferation versus control without VEGF suggests that either endogenous VEGF levels in the experiments were too high or too low to be affected.

As reviewed in Chapter 4, studies investigating alveolar proliferation are conflicting. These discrepancies may be due to cellular differences (A549 cells rather than ATII cells), species differences (developing human lung, rodent and murine lungs), developmental time factors, different time points and other aspects of methodology (not human ATII cells). In addition, there has been a consistent failure to assess DNA uptake and cell number as well as cytotoxicity to confirm an effect on proliferation rather than cell survival.

6.1.3 Functional VEGF polymorphisms as potential genetic susceptibility factors to the development of acute lung injury

As reviewed in Chapter 1, differential susceptibility to developing ARDS or acute lung injury exists but the mechanisms behind this are unclear. Functional polymorphisms are one possible explanation to these observations. As an extension to the VEGF reparative hypothesis, it was hypothesised that functional VEGF polymorphisms would alter susceptibility to the development of and severity of ARDS. As reviewed in Chapter 5, I have investigated the possible role of a functional VEGF polymorphism (+936 CT) in clinical cohorts.

Presence of the VEGF +936 T allele confers increased susceptibility to ARDS and is also associated with increased physiological severity of injury, as defined by an increased APACHE3 score. In normal subjects the T allele is associated with a 75% reduction in plasma VEGF levels although the mechanisms behind this remain unclear. Altered transcription factor binding has been postulated as one such mechanism[220]. The relationship with intra-alveolar levels in normal subjects is not known at the present time. In ARDS, I have demonstrated a significant effect of the T allele, different to that reported in normal subjects, with an increase in plasma VEGF levels (unlike the "at risk" cohort where no effect was demonstrated). I did not detect an effect of the T allele on BAL VEGF levels. The higher plasma VEGF levels in the T allele group may partially explain the associated higher physiological disturbance and potentially the increased susceptibility to developing ARDS. These higher levels may indicate greater leakage of intrapulmonary VEGF levels and greater damage to the alveolar-capillary membrane. The exact mechanisms by which the T allele facilitates greater injury and its effects on intra-alveolar isoform switching remain unclear. The exact mechanisms by which these changes occur and the effects (if any) on VEGF isoform expression remain to be elucidated.

6.1.4 Potential role for $VEGF_{165}b$ and other inhibitory isoforms

This novel discovery has lead to a requirement for a reappraisal of all VEGF research. At the current time, the only technique able to distinguish between these isoforms is by RT-PCR using isoform-specific primers that flank the exon 9-containing region. More recently, specific $VEGF_{165}b$ protein methodologies have been developed[181 334]. Specialised epithelial cells (podocytes) have been found to express this isoform and to display isoform switching[334].

There is the intriguing possibility of a whole $VEGF_{xxx}b$ family of inhibitory isoforms renamed as $VEGF_{xxx}$-A isoforms as there is no true intron between exon 8 and 9 (Figure 1.17)[181 334].

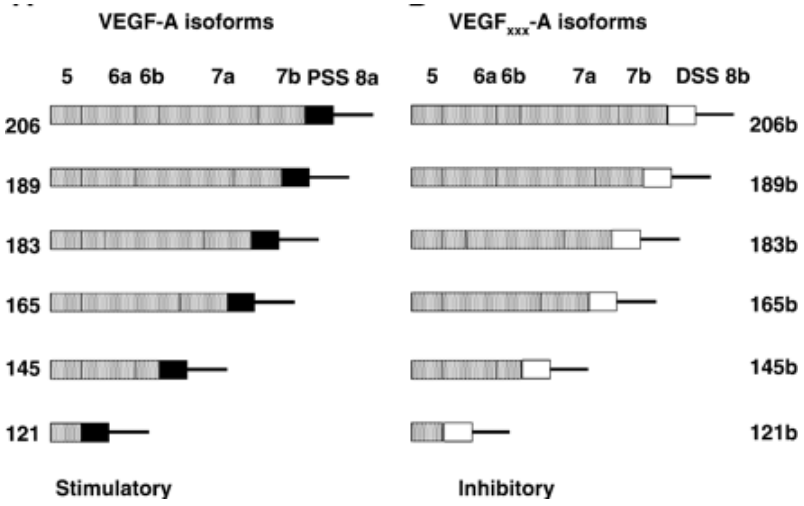


Figure 6.1: Putative $VEGF_{xxx}$-A family of isoforms. Exon 9 has been renamed exon 8b with distal splice site selection (DSS) as opposed to the usual exon 8a with proximal splice site selection (PSS) associated with the normal VEGF isoform family. Source: Cui TG et al.[334]

More recent data has shown expression of a $VEGF_{xxx}b$ superfamily in human podocytes as well as inhibition of VEGF–mediated VEGFR2 signalling and angiogenesis in the eye and viscera[181]. Mimicking a splicing switch (using A375 melanoma cells) from $VEGF_{165}$ to $VEGF_{165}b$ inhibited tumour growth *in vivo* in mice[181]. The possible existence of $VEGF_{165}b$ or a $VEGF_{xxx}b$ superfamily in alveolar epithelium remains currently unknown, whether switching occurs between stimulatory and inhibitory isoforms and the relationship of switching to acute lung injury.

6.2 Caveats and suggested repeat experiments

The conclusions in this thesis must be assimilated with the acknowledgement of some limitations with the techniques and experimental design used. I will now review some of the key limitations and how I would repeat such experiments again in the light of these issues.

6.2.1 Whole lung tissue experiments

The immunohistochemistry experiments are limited by the fact that the experiments were conducted in necropsy ARDS lung tissue (introducing an alternative interpretation potentially of the results, discussed in Chapter 3) and also by the low patient number. Moreover, the use of only sepsis-related ARDS cases, with time for 6 hours of necrolysis and using post mortem tissue consented on clinical grounds only introduce further confounders. These include protein damage, and selection bias based on aetiology and case severity. Ideally, *in vivo* surgical lung biopsy ARDS lung tissue would be the gold standard to investigate VEGF biology to minimise necrolysis and assess a variety of case severities without post mortem selection issues. Surgical biopsies are scarcely performed now due to difficulties in patient consent, rapidity of illness in ARDS or lack of on site thoracic surgical expertise. As reviewed in Chapters 1 and 3, VEGF levels have been observed to correlate with survival with failure to increase intra-alveolar and reduced plasma levels associated with poorer outcome[247].

Indeed, immunohistochemistry is an excellent technique for localisation but can only give limited information on quantitation and functional changes. Assumptions about similar affinity between different antibodies limit the strength of conclusions comparing staining of different antibodies in the same lung tissue. In addition, data on individual VEGF isoform protein expression are not currently available, as isoform specific antibodies are not yet developed for ELISA, Western blotting or immunohistochemistry. The immunohistochemistry antibody detects $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$. The ELISA antibody detects both $VEGF_{121}$ and $VEGF_{165}$. Hence, the immunohistochemistry data provide a composite of all three isoforms.

Although I have demonstrated expression of VEGF receptor protein on alveolar epithelium, it would be important to demonstrate by dual staining techniques, co-localisation of VEGF receptors (in addition to VEGF) with ATII cell markers. This would add weight to the hypothesis of VEGF having an autocrine role on such cells and a protective role in recovery from injury.

In addition, $VEGF_{165}b$ specific antibodies are not currently available to allow anatomical localisation of $VEGF_{165}b$ in the lung. This has important functional implications in terms of overall VEGF bioactivity, given its profound functional differences to the other VEGF isoforms (reviewed in Chapter 1).

Semi-quantitative RT-PCR has many limitations as discussed in chapter 2 including variations in housekeeping gene expression and a variety of limitations during RT-PCR, imaging and densitometry. In addition, the process of RNA extraction from paraffinised lung tissue is technically challenging and theoretically can lead to loss of product structural integrity at higher sizes due to cross-linking (see Chapter 3). Real-time PCR would allow more precise quantification of changes at the transcriptional level[309]. This methodology was not locally available at the time of performing these experiments.

In summary, the optimal design of the immunohistochemistry experiments would use higher n numbers of *in vivo* surgical biopsy lung tissue. Comparisons of staining would ideally be performed only of different antibodies with similar or known affinities. To assess VEGF isoform transcript localisation in the absence of isoform-specific antibodies for all the main isoforms as well as $VEGF_{165}b$), in situ hybridisation using isoform-specific primers would give some information on pre-translational lung isoform expression providing some anatomical data. Real time PCR analysis of extracted RNA from non-paraffinised whole ARDS lung tissue with isoform specific antibodies would provide functional data on isoforms expression at stages of injury negating the problems of paraffinised tissue RNA extraction and the other complications with this technique.

6.2.2 Human ATII cell experiments

The ATII cell experiments are limited by low n number which reflects the difficulty in obtaining such lung tissue and the difficulties of the technique with regard to cell yield because of the purification and extraction process. Lung tissue is limited because of the reluctance for patient consent for research purposes and also the requirements for macroscopically normal lungs, devoid of gross emphysema or cancer which would profoundly affect VEGF levels (as reviewed in Chapter 1).

It is difficult to ensure complete cell purity with ATII cell culture despite stringent efforts to remove other cell types in the extraction process, especially alveolar macrophages. Indeed, the cells were phenotyped as ATII cells using all available methods. It is impossible to exclude small areas of micro-emphysema (given that the all the patients were ex-smokers), which would be expected to alter VEGF levels (as discussed in Chapter 1).

The MTS assay has many limitations as reviewed in Chapter 4. There are assumptions that detected increased cell viability is due to increased cell proliferation but thus does not exclude effects on increased cell survival or reduced apoptosis. Such effects which are described for VEGF in vascular endothelium[118]. To confirm specificity and verify an effect on proliferation, an assessment of DNA incorporation, apoptosis and cell count is necessary.

It is possible that the cell number used, the time points of cell culture, degree of confluence and incubation time with cytokines may have underestimated the effects seen on proliferation. In addition, serum starving was not used to confirm the cells were all in G_0 phase although this was because of the concern of further injury to the cells, low cell yield and limitations in lung tissue supply.

As discussed earlier in section 6.2.1 and Chapter 2, semi-quantitative RT-PCR has many limitations at the level of housekeeping gene expression, genomic DNA contamination, RT-PCR, imaging and densitometry processes. Real time PCR would allow more precise quantification of changes at the transcriptional level in ATII cells[309]. This would allow, for example, the exploration and confirmation of the apparent non-linear (not dose-dependent) effects of VEGF at different concentrations on receptor and isoform expression. It would also confirm the presence of complex, non-linear negative and positive feedback regulatory transcriptional mechanisms. When isoform-specific antibodies are available, this will allow functional studies at transcriptional and translational levels.

It would be important to measure ATII cell supernatant VEGF levels to assess endogenous VEGF protein expression in response to the various experimental conditions. Significant extremes of endogenous VEGF levels in the supernatants might account for the failure of *sflt* to alter proliferation compared to control (see Chapter 4).

Another potential confounder is exogenous VEGF in neonatal calf serum (NCS) in the ATII cell experiments. It would be important to assess VEGF presence and concentration in NCS, as although not described in the literature, other sera have permeability-inducing effects (as discussed in Chapter 4).

In summary, higher numbers of bigger volume lung tissue are needed, devoid of microscopic cancer or emphysema, from never-smokers to repeat these experiments. This would allow extraction of higher cell numbers to assess proliferation by concurrent cell viability assay DNA incorporation assay, apoptosis assay, cell count and assessment of cell cycle stage using *sflt* with all VEGF concentrations. RNA extraction followed by real time PCR would provide more accurate functional data on isoform expression with concurrent measurements of supernatant (and NCS) VEGF levels with isoform-specific antibodies when available.

6.2.3 Genetic association study

It was not possible to perform matched plasma and BAL VEGF measurements in this study for all the patients due to a variety of haemodynamic, ethical, procedural and temporal factors. As discussed, this led to an inevitable alteration of the total "at risk" and ARDS cohort as not all patients underwent BAL. This limits the applicability of conclusions from this "sub-cohort" to the original cohort. It is impossible to exclude a degree of genetic confounding leading to population specific differences in allele frequencies in this genetic study despite my stringent attempts to characterise the cohorts carefully. I also attempted to minimise other confounders of VEGF levels including age, smoking, cancer, critical illness and recruitment bias. However, it was not possible to measure matched plasma and BAL VEGF levels in normal subjects to assess the relation between the T allele and BAL VEGF levels. In addition, a matched non-inflammatory pulmonary oedema cohort was not included to exclude a confounder of non-specific hypoxaemia and pulmonary oedema.

In summary, the optimal design of a repeat genetic association study looking at the 936 CT polymorphism would have adequate statistical power to demonstrate a significant difference in susceptibility which might necessitate a multi-centre study. Measurements of both matched BAL and plasma VEGF would be performed simultaneously to assess the relation between the T allele and intra-alveolar VEGF levels with the whole cohort undergoing BAL to avoid inadvertent subpopulation issues. Such a repeat study would include a non-inflammatory pulmonary oedema cohort (on the basis of a clinical diagnosis of cardiogenic pulmonary oedema, elevated PAWP or low oedema fluid protein ratio) cohort of equal impaired oxygenation to control for the severity of illness to confirm the specificity of these findings. This would be in addition to cohorts of normal subjects, ventilated controls, ventilated "at risk" and ventilated ARDS patients.

6.3 Future additional experiments

In addition to repeating some of the experiments to try and reduce the limitations discussed in section 6.2 (and reviewed in more detail in Chapters 3 to 5), I will now discuss the key future questions that remain. I will now discuss what further future experiments are necessary to answer the questions. Perhaps the most important information will be obtained from relevant transgenic overexpression and knockout animal models.

The exact function and relative expression of $VEGF_{165}b$ remains to be clarified because of limitations in detecting it and differentiating it from VEGF itself although more recent data are encouraging in this regard suggesting a $VEGF_{xxx}b$ isoform superfamily[181]. Specific transgenic animals overexpressing these isoforms or with knockouts may clarify their role. Indeed, the mechanisms behind isoform switching (if it occurs) are crucial as there is evidence that mimicking a $VEGF_{165}$ to $VEGF_{165}b$ switch can have anti-tumour effects *in vivo* for example[181]. This may have therapeutic relevance in the injured lung.

Selected VEGF isoform knockout animal models of lung injury may help to further define their relative functional importance in terms of development, susceptibility, and response to lung injury. Animal models of lung injury will also provide testing of possible local intrapulmonary VEGF therapies once the relative importance of the isoforms has been evaluated including the $VEGF_{xxx}b$ family. Functional genomic and proteomic analysis in such models will help define other important molecules in this regard. It is important to assess the functional effects of all the different VEGF (and $VEGF_{xxx}b$) isoforms on alveolar epithelial (ideally human ATII) cells in terms of isoform expression and proliferation.

More studies are required to assess alveolar epithelial repair in acute lung injury. ATII proliferation takes 24-48 hours to become significant in recovery from lung injury[365]. Therefore, other mechanisms such as cell migration and spreading may contribute to alveolar epithelial repair[337]. The potential role of VEGF in these other repair functions in the alveolus remains currently poorly understood and requires clarification.

It would be advantageous to assess other key cells (including lung microvascular endothelial cells) involved in the alveolar-capillary membrane ie) ATII cells in co-culture with microvascular endothelial cells. In particular, the response of lung microvascular endothelial cells to VEGF and pro-inflammatory cytokines is important to assess given the apparent differential response of ATII cells in terms of isoform and receptor expression. This would clarify whether intra-alveolar VEGF exerts a more distal effect in ARDS in addition to its proximal effect on alveolar epithelium. This would also involve assessing the relationship between endothelial and epithelial injury and the fibrogenic response. ATI cells are notoriously difficult to culture but would also be key cells to investigate. It would be important to evaluate isoform and receptor expression at the posttranscriptional level as discussed previously to determine the nature and extent of post-transcriptional regulation especially in response to pro-inflammatory cytokines in addition to $VEGF_{165}b$ expression.

When isoform-specific and $VEGF_{xxx}b$ antibodies become available, this will allow anatomical localisation and functional analysis of their expression in normal and ARDS lung tissue as well as ATII cells to complement the experiments performed so far at transcriptional level.

The importance of VEGF gene studies would be strengthened by microarray studies assessing the important upregulated genes in ARDS to confirm that this is a polymorphic gene which requires further investigation in case control studies. Assuming this to be the case, there are many other functional VEGF polymorphisms requiring study that have biologically plausible effects. More work is required on the particular mechanisms of the functional effects seen with the polymorphic alleles, in terms of changes in transcription factor activity, isoform switching or other signal transducer effects.

Finally, any appraisal of the role of VEGF will have to take into account the relative contribution of other relevant molecules such as *sflt* (whose levels are known to be altered in acute lung injury), angiostatin and the Tie family as well as other possible relevant ATII mitogens such as EGF, HGF, KGF and TGF to allow a better understanding of VEGF signalling mechanisms[158 337 366]. The importance and interaction of other factors such as mechanical and physical forces must be appraised and their relative contribution and effects on VEGF activity.

6.4 Postulated action of VEGF in the alveolus: a revised hypothesis

As reviewed, much of the literature on VEGF in lung injury is conflicting but these differences can be attributed to different methodologies, species and time points. With this in mind, the most convincing interventional studies point to a protective role for VEGF in the lung.

In the light of the experiments in this thesis, I conclude that VEGF is indeed an ATII cell survival factor acting in an autocrine manner and ATII cells are the predominant intrapulmonary source (see Figure 6.2). Although isoform switching does not occur, there is significant VEGF receptor upregulation in later ARDS. ATII cells exhibit a differential response to the inflammatory milieu in ARDS with an amplified isoform expression response but relative underexpression of VEGF receptors. Functional VEGF polymorphisms also have a role in determining susceptibility to and severity of lung injury.

I hypothesise[367] (see Figure 6.2) in the light of the above, that these changes maximise VEGF protective biological actions in the alveolus especially in later ARDS with a reduced VEGF signal in early ARDS via reduction in isoform transcription, coupled with the reduced intra-alveolar VEGF levels noted in observational studies at this time. This hypothesis explains the high intra-alveolar VEGF levels in normal subjects despite no ongoing angiogenesis in the normal lung. In my revised hypothesis, I also speculate that VEGF has a more distal action on targets other than ATII cells via the observed relative down-regulation of ATII VEGF receptors in the inflammatory state that may also act as a regulatory control mechanism to limit VEGF release in a controlled fashion in response to injury. In addition, VEGF is an ATII cell survival factor and I speculate this would involve down-regulation of apoptosis, but the exact mechanisms remain to be clarified. Although the experiments in this thesis did not support a contribution as an ATII cell mitogen, I hypothesise that there may be a contributory role here too which was not confirmed because of methodological limitations discussed in Chapter 4. VEGF genotypes may account for the observed differential susceptibility to acute lung injury involving a "two hit" theory of susceptible genotype followed by other direct (aspiration) or remote (gut sepsis) injury triggering the cytokine cascade leading to full blown acute lung injury.

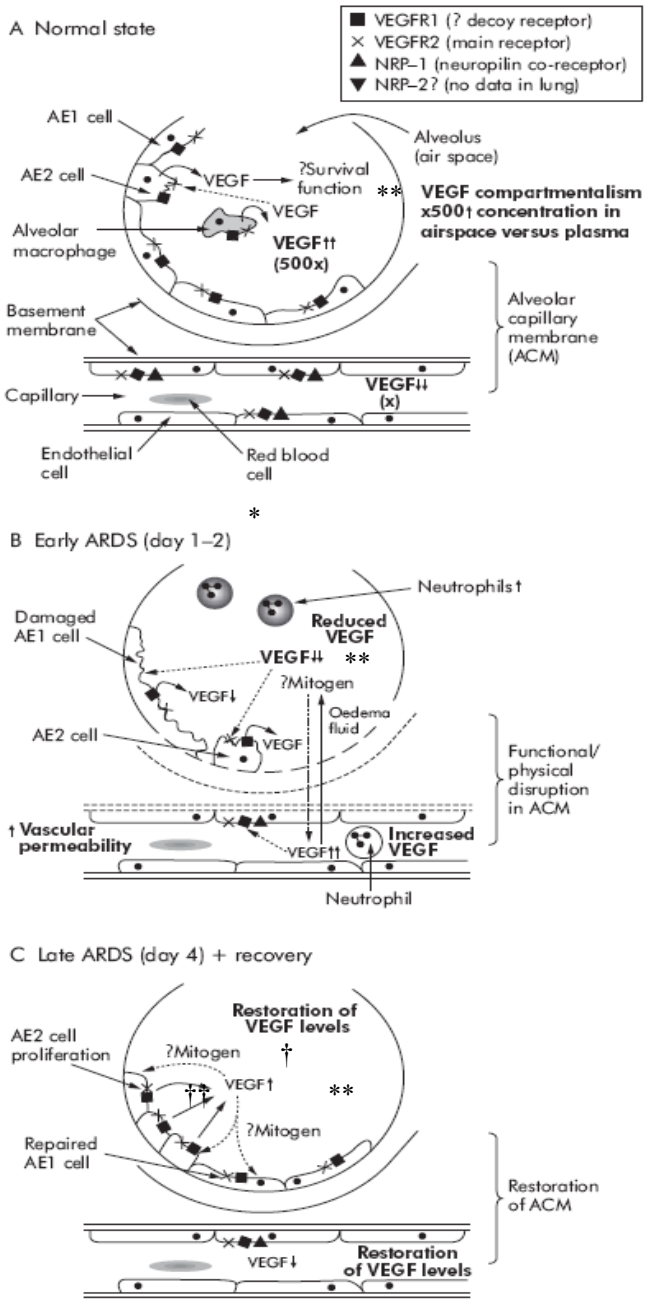


Figure 6.2: Revised hypothesis of function of VEGF in the alveolus (see text for details, section 6.4). Adapted from Medford et al.[367] (see Appendix 1). Symbols: *"Two hit" theory: 1. Susceptible VEGF genotype eg) 936 T allele; 2. Injury (direct/remote). **Increased ATII cell survival: ?mechanism ↓apoptosis? †Differential response to inflammation: ↑VEGF isoform expression but limited ATII VEGFR upregulation: distal target and regulatory control? ††Autocrine loop on ATII cells.

6.5 Final comment

In conclusion, much still has to be learned about VEGF before therapy can be contemplated in ARDS as illustrated by the paradoxical results highlighted in Chapter 1 in animal models using VEGF delivery. However, VEGF has been identified as a potentially important mediator in repair and recovery from ARDS and has been utilised as a potential therapy in respiratory distress syndrome. Despite apparent discrepancies, accumulating cellular, animal and clinical studies suggest a protective role for VEGF in the lung. Whether VEGF is simply an ATII survival factor or actually a mitogen remains to be clarified and the exact mechanisms behind its alveolar protective function still need to be clarified. The future challenge will be to better understand the basic mechanisms underlying its role allowing generation of more targeted and effective therapies conferring additional information on response to treatment, prognosis, severity and susceptibility.

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APPENDIX 1: PAPERS

I enclose papers published in peer-reviewed journals relevant to the material in this thesis. The first paper is a review article summarising some of the literature reviewed in Chapter 1 and also some of the hypotheses discussed in further detail in Chapter 6. The second paper is a genetic association study relates to the data discussed in Chapter 5. The third paper illustrates the technique of human ATII cell culture used in Chapter 4.

Papers

1. Medford ARL, Millar AB. Vascular endothelial growth factor (VEGF) in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS): paradox or paradigm? *Thorax* 2006 61: 621-6.

2. Medford ARL, Keen LJ, Bidwell J, Millar AB. Vascular endothelial growth factor gene polymorphism and acute respiratory distress syndrome. *Thorax* 2005 60: 244-48.

3. Armstrong L, Medford ARL, Uppington KM, Robertson J, Witherden IR, Tetley TD, Millar AB. Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. *Am J Resp Cell Mol Biol* 2004 31(2): 241-5.

OCCASIONAL REVIEW

Vascular endothelial growth factor (VEGF) in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS): paradox or paradigm?

A R L Medford, A B Millar

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Acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), remains a devastating condition with a high mortality. It is characterised by alveolar injury and increased pulmonary vascular permeability. Vascular endothelial cell growth factor (VEGF) was identified by its properties to increase permeability and act as a cellular growth factor, hence its potential for a key role in the pathogenesis of ALI/ARDS. This review describes the basic biology of VEGF and its receptors as an essential prerequisite to discussing the available and sometimes paradoxical published data, before considering a paradigm for the role of VEGF in the human lung.

See end of article for authors' affiliations

Correspondence to: Dr A B Millar, Lung Research Group, Department of Clinical Science at North Bristol, University of Bristol Lifeline Centre, Southmead Hospital, Westbury-on-Trym, Bristol BS10 5NB, UK; Ann.Millar@bristol.ac.uk

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A healthy alveolar capillary membrane is essential for the gas exchange function of the human lung. Injury and loss of this tissue contributes to the pathology of many forms of lung disease of which the archetypal example would be the most extreme form of acute lung injury (ALI)—namely, acute respiratory distress syndrome (ARDS).[1] An understanding of the mechanisms involved in the injury and repair of this tissue would have significant impact on the clinical management and treatment of this and many other lung conditions. Vascular endothelial growth factor (VEGF) was originally identified by its properties as both a permogen and a mitogen, key elements in the function of the alveolar capillary membrane, leading to interest in its role in many forms of lung disease, particularly ARDS.[2 3] Intriguingly, in healthy human subjects VEGF protein levels are highly compartmentalised, with the directly oxygenated alveolar levels 500 times higher (2 nM) than plasma levels, despite VEGF production being closely associated with a hypoxia response element.[4 5] These levels in normal alveoli are significant, twice the concentration previously shown to induce permeability and mitogenesis (particularly angiogenesis) in vivo.[3] However, in healthy lung these processes are extremely restricted. These data suggest an important persistent or additional function of VEGF within the human lung that has not yet been characterised, which is normally tightly regulated and which goes awry in ALI/ARDS. Current in vitro work, animal models, and clinical studies are somewhat conflicting as to the role of VEGF in ALI/ARDS. We attempt to resolve these apparent conflicts in the available data by proposing a unifying hypothesis for the role of VEGF in injured lung pertinent to ALI/ARDS—namely, that VEGF protects the alveolar epithelium with a role in repair following lung injury, but causes fluid flux across the exposed endothelium if the alveolar capillary membrane is functionally breached.

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

ARDS, the most extreme form of ALI, was first described in 1967.[1] It is more common than is perhaps appreciated with an estimated incidence of 75 per 100 000 in some studies.[6] It is estimated to account for nearly 16 500 deaths annually in the USA, roughly equal to the number of deaths due to HIV and emphysema, increasing to 74 500 if ALI is considered overall.[7] ARDS continues to have a significant mortality of more than 35% despite recent improvements in ventilator strategies and in sepsis management.[8] A host of conditions, including sepsis, trauma, aspiration, massive blood transfusion and burns, both direct and indirect insults, predispose to ARDS. However, exposure to a given "insult" does not guarantee that ARDS will follow; for example, there is a 40–60% risk of ARDS following Gram negative sepsis.[9] Although the underlying mechanisms and factors governing susceptibility remain unclear, ARDS is characterised by alveolar epithelial injury and increased vascular permeability.[6] Markers of both epithelial and endothelial injury have been correlated with outcome.[6 10–12] An additional factor is the potential to induce damage by mechanical ventilation itself.[13 14] Survival from ARDS requires resolution of these features and renewed integrity of the alveolar capillary membrane.

BIOLOGY OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

To appraise and understand the published evidence in this area, it is essential to have some understanding of the basic biology of VEGF.

Abbreviations: AE, alveolar epithelial; ALI, acute lung injury; AP, activator protein; ARDS, acute respiratory distress syndrome; FLT, fms-like tyrosine kinase; HUVEC, human umbilical venous endothelial cell; LPS, lipopolysaccharide; NRP, neuropilin; VEGF, vascular endothelial growth factor; VEGF-R1, VEGF-R2, vascular endothelial growth factor receptor 1 and 2

VEGF

The superfamily of VEGF proteins consists of at least six members that are structurally and functionally related but with predominantly differing key roles.[15] This review is confined to the importance of VEGF-A, termed VEGF throughout the text. These properties have led to investigation of this molecule in cancer, vascular diseases, chronic inflammatory disorders, and ALI as well as many other lung diseases including asthma, emphysema, pulmonary fibrosis, lung cancer, and pulmonary hypertension. VEGF is a 34–46 kDa glycoprotein that was first isolated from tumour cells but other cellular sources include macrophages, smooth cells, and epithelial cells.[2 16] It is a potent angiogenic factor and critically regulates vasculogenesis such that embryos lacking a single VEGF allele have a lethal phenotype due to abnormal vascular development including that of the lung.[17] It both induces vascular endothelial cell proliferation and promotes survival by induction of anti-apoptotic proteins bcl-2 and A1.[18–21] VEGF increases microvascular permeability 20 000 times more potently than histamine.[2 3] Targets for VEGF bioactivity outside the vascular endothelium include macrophages, type II pneumocytes, and monocytes for which it may be chemotactic.[22–26] It also has a vasodilatory function.[27]

VEGF isoforms

Alternate splicing of the VEGF gene (6p21.3) transcript leads to the generation of several splice variants (isoforms) of differing sizes, the subscript relating to the number of amino acids present ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$).[28–30] $VEGF_{165}$ is the predominant isoform and most biologically active in the physiological state.[31] The longer isoforms are cell associated (exons 6 and 7 have heparin binding activity allowing binding to the extracellular matrix) compared with the shorter diffusible isoforms.[29] Plasmin, the acute phase protein, can also cleave the isoforms to form PL-$VEGF_{110}$ and a recently identified isoform, $VEGF_{165}$b, is inhibitory in function and may not be detected in standard VEGF assays necessitating reappraisal of current VEGF data.[32] The mechanisms by which splicing occurs and is regulated are poorly understood.

VEGF-R1 and VEGF-R2

All VEGF isoforms bind to the tyrosine kinase receptors, VEGF receptor 1 (VEGF-R1) and VEGF receptor 2 (VEGF-R2).[15 31 33] They were initially thought to be largely confined to vascular endothelium but have subsequently been detected elsewhere including activated macrophages, AE2 cells, and neutrophils.[22–25 34–36] Hence, VEGF is capable of having its effect on both sides of the alveolar capillary membrane on both the epithelial and endothelial surfaces. There is evidence that the signal transduction cascades for VEGF-R1 and VEGF-R2 are different and, although VEGF-R1 has greater affinity for VEGF, VEGF-R2 is tyrosine phosphorylated much more efficiently upon ligand binding.[22 31] VEGF-R2 is regarded as the main signalling receptor for VEGF bioactivity (angiogenesis, proliferation and permeability) and can cause proliferation in cells lacking VEGF-R1.[37 38] VEGF-R2 knockout mice fail to develop blood islands or organised blood vessels resulting in early death.[39] VEGF-R2 also has a pro-survival function with anti-apoptotic effects on human umbilical venous endothelial cells (HUVECs).[40] In contrast, VEGF-R1 rarely induces cellular proliferation in cells lacking VEGF-R2.[26 26 41–44] This has led to the suggestion that VEGF-R1 may function mainly as a decoy receptor, although this is still contentious. Nevertheless, VEGF-R1−/− mice die between days 8.5 and 9.5 in utero from excessive proliferation of angioblasts, supporting a negative regulatory role on VEGF by VEGF-R1 at least during early development.[45 46] In addition, targeted deletion of the tyrosine kinase domain but not the VEGF binding domain on VEGF-R1 does not cause death or obvious vascular defects, although it is required for some other functions such as monocyte chemotaxis.[26 42] Alternate splicing leads to a soluble form of VEGF-R1 (*sflt*) which can act as an inhibitor of VEGF activity.[47]

Neuropilins (NRP-1, 2)

By contrast, the neuropilins (NRP-1, NRP-2) are isoform-specific VEGF binding sites of different size and affinity to VEGF-R1 and VEGF-R2.[48 49] They are expressed by endothelial cells in many adult tissues but lack the intracellular component containing tyrosine kinase activity and are regarded as VEGF co-receptors, being unable to signal themselves without the involvement of VEGF-R2. NRP-1 is isoform-specific, recognising exon 7 of VEGF (binding $VEGF_{165}$ but not $VEGF_{121}$), and increases the effect of $VEGF_{165}$ by enhancing its binding to VEGF-R2.[50] This may also partially account for the greater mitogenic potency of $VEGF_{165}$ compared with the $VEGF_{121}$ isoform. Studies also support a role for NRP-1 in vasculogenesis and angiogenesis. NRP-1 knockout and over expressing mice both die prematurely from vascular defects.[51 52] In contrast, NRP-2−/− are viable but do have absent or reduced lymphatic vessels and capillaries.[53 54]

VEGF polymorphism

Several functional human VEGF polymorphisms have been described.[55–57] Significant interindividual variations in plasma VEGF levels and gene expression related to the presence of polymorphism have been reported.[58–60] In one study a CT substitution at position 936 distal to the start of translation in the 3′-untranslated region of the VEGF gene on chromosome 6 was associated with a 75% reduction in plasma levels in both heterozygotes and homozygotes in a Caucasian population.[55] No such changes in plasma levels were detected in another genetic association study, but this may have been due to different racial populations.[61] This polymorphism results in altered binding of the transcription factor activator protein 4 (AP-4), although whether the abolition of the AP-4 binding site is of specific relevance to the reduction in VEGF protein expression remains unclear.[55] The effect of the CT genotype on intrapulmonary levels remains unknown at the current time.

VEGF AND THE ALVEOLAR SPACE

Studies of ARDS/ALI need to consider both sides of the alveolar capillary membrane (fig 1A). Isolated cellular studies of epithelial or microvascular endothelial cells give additional insight to animal models and clinical studies, as discussed below. In vitro studies have demonstrated an abundance of VEGF in lung tissue, especially in alveolar epithelium, including the A549 cell line and primary human cultured type II pneumocytes.[62–65] Indeed, the highest levels of VEGF mRNA are found in animal and human lung, which suggests that the alveolar epithelium is the predominant source.[62 66] Although the embryonic role of VEGF is undoubted, in all species studied to date adult lungs contain higher amounts of VEGF mRNA transcript than the developing lung. Changes in relative isoforms have also been observed with maturity, suggesting an ongoing role.[67] VEGF-R1, NRP-1, and NRP-2 are all expressed in normal lung.[25 68] Primary human type 2 alveolar epithelial (AE2) cells are known to express VEGF-R2, the main functioning VEGF receptor, which would facilitate an autocrine role in the air space for VEGF in addition to its well known paracrine effects on the vascular bed.[15 26]

Studies suggesting a pathological role for VEGF in the alveolar space

The properties of VEGF described previously have led many workers to the hypothesis that VEGF would be solely

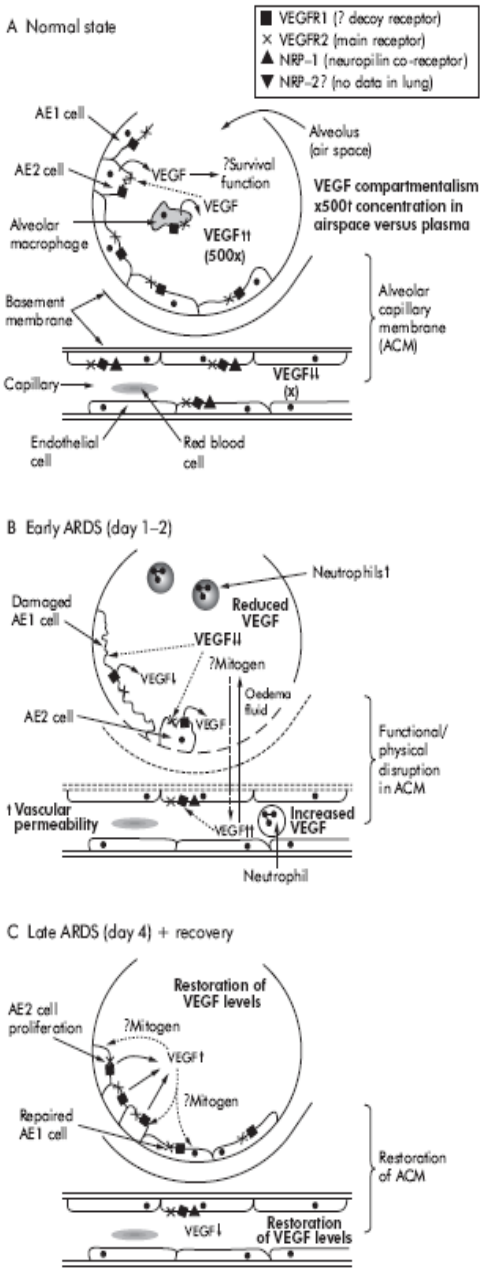

Figure 1 Diagrammatic representation of the human alveolar capillary unit in (A) normal healthy subjects, (B) subjects with lung injury, and (C) during the recovery phase.

pathological in the lung. Certainly, VEGF protein is secreted by alveolar cell-like cell lines in response to a number of pro-inflammatory stimuli potentially involved in ARDS, including lipopolysaccharide (LPS), neutrophil elastase and keratinolytic growth factor.[64] One influential early study of adenoviral $VEGF_{165}$ delivery to murine lung showed non-cardiogenic pulmonary oedema and increased pulmonary capillary permeability on the basis of histology, lung weight wet/dry ratios, increased albumin permeability, and Evans blue dye assay.[69] A subsequent study in $VEGF_{164}$ +/+ mice targeted to respiratory epithelial cells demonstrated pulmonary haemorrhage, endothelial destruction, and alveolar remodelling in an emphysema-like phenotype, although surfactant protein-B production was not affected which suggests that the primary effect may have been vascular disruption.[70] In more specific ALI/ARDS models such as LPS induced murine lung injury, intrapulmonary levels of VEGF increased following injury for 96 hours, mirroring an increase in bronchoalveolar lavage fluid protein and neutrophils with significant VEGF localisation to lung epithelium.[71] In an acid induced murine model of lung injury, high tidal (injurious) volume ventilation strategies increased lung VEGF-R2 (the main signalling receptor for VEGF bioactivity) protein expression, but this was not reduced by a protective ventilatory strategy suggesting that, in this instance, the VEGF response may be secondary to more critical events.[72] These data are consistent with a possible pathological role for VEGF in lung injury.

Studies suggesting a protective role for VEGF in the alveolar space

Increasing recognition of the presence of significant levels of VEGF in normal healthy lungs has led to a reconsideration of its role. Considerable evidence suggests that VEGF acts as an alveolar epithelial mitogen and stimulant. Exogenous VEGF acts as a growth factor on human fetal and neonatal murine pulmonary epithelial cells and is capable of restoring the A549 cell proliferation in an acid exposure model of injury, although this has not been a universal finding.[34 73–75]

These studies would certainly suggest at least a developmental role for VEGF in the lung. A particularly significant contribution to this observation is the work of Compernolle *et al*[73] in which transgenic HIF-2α deficient fetal mice (with consequent fatal respiratory distress syndrome in neonatal mice due to impaired surfactant production by type 2 pneumocytes) had low lung VEGF levels. Intrauterine or postnatal intratracheal delivery of $VEGF_{165}$ to the neonatal mice protected them against developing respiratory distress syndrome and increased surfactant production. In terms of models of lung injury per se, recovery of intrapulmonary VEGF levels to pre-injury levels has been noted following recovery in both hyperoxic rabbit and LPS rat models, and the presence of VEGF in the alveolar space appears to have a protective effect against hyperoxia in interleuking (IL)-13 transgenic mice.[34 76 77] In similar hyperoxic neonatal and adult models the proportion of $VEGF_{189}$ decreases while the proportions of $VEGF_{121}$ and $VEGF_{165}$ increase, all returning to control values in recovery as measured by RT-PCR.[67] In a bacterial model of acute lung injury in rats reduced $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ transcripts were noted.[76] Inhibiting VEGF activity by VEGF-R2 blockade (given subcutaneously) in rats leads to alveolar apoptosis and emphysema.[78] NRP-1 inhibition has also been shown to ameliorate VEGF induced permeability in a lung targeted VEGF overexpressing model.[79] NRP-2 is expressed in normal human lung bronchial epithelium.[68] The role of NRP-2 in an ARDS/ALI context has not been evaluated. An alternative approach of lung targeted ablation of the VEGF gene by adenoviral delivery in the adult mouse led to a persistent emphysema phenotype for at least 8 weeks. Interestingly, no inflammation or proliferation occurred but increased apoptosis was seen in these lungs.[80] These data support the suggestion that VEGF may have a pneumotrophic function and be an autocrine epithelial growth factor in the lung. It is already known to be a survival factor for the vascular bed via induction of anti-apoptotic proteins, but to date this has not been demonstrated in alveolar epithelium.[20] Such an autocrine role has been described on other specialised epithelial cells in the kidney.[81]

In summary, high levels of VEGF exist in normal human lung despite the lack of angiogenesis, oedema, or excess microvascular permeability occurring. Receptors are expressed in the air space, compatible with a biological (but as yet unclarified) physiological role for VEGF in the normal lung.

VEGF AND THE PULMONARY VASCULATURE

Pulmonary hypertension occurs in ARDS as well as endothelial injury and increased microvascular permeability.[6] Studies looking at the effect of VEGF on the endothelial side of the alveolar capillary membrane are limited. This is in contrast to the extensive literature on the systemic vasculature and HUVECs where VEGF has been shown to act as a vasodilator in addition to its angiogenic role.[82] VEGF is known to reduce transendothelial resistance of bovine lung microvascular endothelial cells for less than 60 minutes at concentrations of less than 10 ng/ml and to stimulate endothelial cell chemotaxis maximally at 10 ng/ml.[83] However, most published studies have looked at the vasculature overall and the potential development of pulmonary hypertension, rather than at the microvascular level.

VEGF may have protective effects on the pulmonary vasculature.[84 85] In an ovine model of chronic intrauterine pulmonary hypertension, whole lung VEGF protein expression was downregulated with reduced VEGF expression in the airway epithelium, vascular endothelial and smooth muscle cells on immunohistochemistry.[86] Indeed, transgenic mice overexpressing tumour necrosis factor α (lung or systemic) display features of pulmonary hypertension associated with reduced VEGF and VEGF-R2 mRNA expression.[87] In contrast, VEGF overexpressing transgenic mice develop an abnormal vasculature and lethal phenotype.[88] Blocking VEGF activity in newborn rats with a VEGF-R2 inhibitor decreases arterial density and vascular growth as well as alveolarisation.[89] All these studies certainly support the known role of VEGF in vasculogenesis during embryonic and early life. Perhaps of more relevance to the ALI/ARDS scenario is the observation that, in hypoxic conditions inducing pulmonary hypertension, increased pulmonary arterial VEGF mRNA and protein are detected in both guinea pig and rat species.[90 91] In the rat model these levels correlated with both the degree of vascular remodelling and the mean pulmonary artery pressure.[90] Intervention studies also favour a protective role against pulmonary hypertension. Administration of VEGFR tyrosine kinase inhibitor in newborn rats leads to pulmonary hypertension and abnormal lung structure.[92] Administration of a specific $VEGF_{165}$ inhibitor leads to more significant features of pulmonary hypertension histologically and haemodynamically associated with decreased expression of endothelial nitric oxide synthase.[86] Furthermore, intratracheal adenovirally delivered $VEGF_{165}$ protects against hypoxic pulmonary hypertension in rats, possibly via increased endothelial nitric oxide production.[93] The apparent conflicting observational data may indicate that hypoxia is simply an overriding regulatory factor to VEGF bioactivity even if pulmonary hypertension has developed. The potential role of VEGF in primary pulmonary hypertension is another enormous issue which is not covered here.

HUMAN ALI/ARDS

To date, most observational studies of lung injury in humans show a reduction in intrapulmonary VEGF levels in the early stages of ARDS (fig 1B).[76 94 95] This is consistent with a hyperoxic lung injury model in rabbits where alveolar epithelial expression of VEGF was reduced.[34] Several other investigators have found similar reductions in intrapulmonary VEGF levels in other forms of lung injury, including high altitude pulmonary oedema in adults, bronchopulmonary dysplasia, persistent pulmonary hypertension of the newborn, idiopathic pulmonary fibrosis, and smokers.[96–99] Recovery of intrapulmonary VEGF levels to pre-injury levels has been noted following recovery from both ALI in humans and high altitude pulmonary oedema (fig 1C).[95 96] Potential mechanisms for these observations include physical disruption of the alveolar capillary membrane alone, reduced VEGF production, changes in isoform expression, and increased receptor density or splicing to the soluble form, but human data are limited. Changes in VEGF isoforms have been shown in lung tissue from critically ill patients with sepsis, reduced $VEGF_{121}$ and $VEGF_{165}$ mRNA compared with controls without sepsis (on immunohistochemistry and ELISA), but these patients may not necessarily have had ALI.[100] Intrapulmonary soluble VEGF-R1 has been found to be increased in ARDS.[101] Indeed, in ex vivo experiments, $VEGF_{165}$ induced permeability in human pulmonary arterial endothelial cell monolayers is reduced by nearly 50% in the presence of 20 ng/ml soluble VEGF-R1 (*sflt*).[102] However, in another study homogenates from early and late injured lungs had no difference in VEGF-R2 expression compared with controls, although AE2 cell proliferation and VEGF-R2 expression were noted in injured tissue and these data do not exclude a protective role for VEGF.[94] In contrast to these data, one observational clinical study failed to find a difference in serum VEGF levels in ARDS, although there were significant differences in methodology.[76]

Changes in VEGF could also be related to polymorphism. The VEGF +936 CT polymorphism has recently been investigated in a cohort of controls, ventilated "at risk" patients, and patients with ARDS.[103] The polymorphic T allele occurred significantly more often in the ARDS group than in the other cohorts and was associated with a higher APACHE 3 score suggesting an association with susceptibility and severity, although the intrapulmonary effect is unknown.

CONCLUSIONS

The normal human lung contains significant amounts of the angiogenic factor VEGF without significant angiogenesis—an apparent paradox. Published data conflict. Many studies have suggested that VEGF may contribute to the development of non-cardiogenic pulmonary oedema. Other studies have proposed a more protective role on the alveolar epithelium following injury. In the in vitro and ex vivo studies of changes in VEGF concentrations, differences in the species and methods used may have contributed to apparent differences in findings. Variable animal models, the degree of endothelial injury in the overexpression models, and temporal variation in sample collection in these and in clinical studies also confound the data. Changes in splice variant and soluble receptor expression may also contribute, although published data are limited.

We suggest a paradigm for VEGF in the lung. We speculate that it may function as a pneumotrophic factor behaving in an autocrine fashion with its prime function in facilitating repair following lung injury: protecting and regenerating the epithelial surface yet contributing to the generation of pulmonary oedema across the underlying endothelium if disruption of the alveolar capillary membrane occurs as in ARDS. Functional VEGF polymorphisms may determine in part susceptibility to developing and severity of lung injury, but remain to be further investigated. Anti-VEGF therapy is already under investigation for lung cancer, vascular disease, pulmonary hypertension, and chronic inflammatory diseases.[104 105] In the long term, treatment modulating the VEGF system may be of value in ARDS, but this will require a better understanding of its role in the lung and a better understanding of the regulatory mechanisms influencing VEGF bioactivity including changes in splice variants,

transcription factors, pro-inflammatory cytokines, and soluble receptors. Such a potent molecule is likely to have a physiological role which can be detrimental if activity is excessive, inappropriate, or uncontrolled. The challenge will be to limit the effects of such treatment to those desired, given the pleotropic functions of VEGF in the body.

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Authors' affiliations

A R L Medford, A B Millar, Lung Research Group, Department of Clinical Medicine at North Bristol, University of Bristol, Southmead Hospital, Westbury-on-Trym, Bristol, UK

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ACUTE LUNG INJURY

Vascular endothelial growth factor gene polymorphism and acute respiratory distress syndrome

A R L Medford, L J Keen, J L Bidwell, A B Millar

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See end of article for authors' affiliations

Correspondence to: Dr A B Millar, Lung Research Group, Department of Clinical Science at North Bristol, University of Bristol, Southmead Hospital, Westbury-on-Trym, Bristol BS10 5NB, UK; Ann.Millar@bristol.ac.uk

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Background: Non-cardiogenic pulmonary oedema is a characteristic feature of the acute respiratory distress syndrome (ARDS). The properties of vascular endothelial growth factor (VEGF) as a potent vascular permeagen and mitogen have led to investigation of its potential role in this condition. Lower VEGF plasma levels have been linked to the presence of the T allele in the +936 CT polymorphism. We hypothesised that the presence of the T allele would be associated with the development and severity of ARDS.

Methods: A cohort of 137 normal subjects, 117 ventilated patients with ARDS, and 103 "at risk" of ARDS were genotyped for the VEGF+936 CT polymorphism. The severity of physiological disturbance and mortality was determined in the ventilated cohorts.

Results: The CT and TT genotype frequencies were increased in ARDS patients compared with both normal subjects (OR 2.01, 95% CI 1.13 to 3.58, p=0.02) and those "at risk" (OR 2.05, 95% CI 1.02 to 2.20, p=0.03). In patients with ARDS but not those "at risk", CT and TT genotypes were associated with a higher mean APACHE III score (80.9 (4.3) v 69.3 (2.9), p<0.05).

Conclusion: These data support a role for VEGF in the pathogenesis of ARDS and its associated physiological derangement.

Acute respiratory distress syndrome (ARDS), the most extreme form of acute lung injury, continues to have a significant mortality of at least 35% of patients despite improvements in the management of sepsis and ventilatory support.[1] Death in these patients is usually secondary to the physiological derangement of multiorgan rather than respiratory failure per se.[2]

Non-cardiogenic pulmonary oedema is a characteristic feature of ARDS. The potent effects of vascular endothelial growth factor (VEGF) on vascular endothelium as both a permogen (20 000 times more potent than histamine) and a mitogen have led to investigation of its potential role in the development of ARDS.[3] In the early stages of this condition we have previously reported that plasma VEGF levels rise and intrapulmonary levels fall with normalisation of both during recovery.[4 5] These changes in intrapulmonary VEGF have also been noted by other investigators in ARDS and high altitude pulmonary oedema.[6 7] In normal subjects VEGF is compartmentalised with levels within the alveolar space 500 times that detected in the plasma, suggesting a physiological role which becomes disrupted when injury of the alveolar capillary membrane occurs and ARDS ensues.[8]

VEGF is among those polymorphic genes with a potential role in ARDS, genetic polymorphism being a potential explanation for the low incidence of ARDS within the large numbers of subjects "at risk" of developing this syndrome. A CT substitution at position 936 distal to the start of translation in the 3'-untranslated region of the VEGF gene on chromosome 6p21.3 has been associated with reduced plasma levels in both CT and TT genotypes in normal subjects.[9] However, it is unclear how this relates to intrapulmonary VEGF, the probable major site of production. This substitution results in altered binding of the transcription factor activating enhancer binding protein 4 (AP-4), although whether the abolition of the AP-4 binding site results directly in the reduction in VEGF protein expression remains unknown.[9] The role of this polymorphism has been studied in both COPD and sarcoidosis; no association was found with the former and a negative association with the latter.[10 11] However, no study to date has examined the relationship between this allele and ARDS or any other critical illness.

We hypothesised that the VEGF+936 CT polymorphism would contribute to genetic susceptibility to ARDS. We have examined the association between the VEGF+936 genotype and the development and severity of ARDS, using ventilated "at risk" controls and normal healthy subjects for comparison to ensure any association was with ARDS per se rather than critical illness.

METHODS

Subjects

A total of 137 normal subjects and 220 ventilated patients were prospectively included in this single centre study. ARDS patients fulfilled the 1994 American-European Consensus Conference definition at any time during their intensive care admission (n = 117). "At risk" patients were ventilated and had similar degrees of physiological disturbance to those with ARDS using previously described criteria but did not fulfil the ARDS criteria at any time during their intensive care admission.[12] Patients with trauma were considered to be at risk for ARDS if they were intubated or on mask continuous positive airway pressure (CPAP) and had either two or more of the following: multiple fractures (two or more fractures of femur, tibia, humerus, or stable pelvis); unstable pelvic fracture; pulmonary contusion; or massive transfusion (>15 units in 24 hours). Patients with suspected sepsis were considered to be at risk for ARDS if they had: (1) two or more of the following: temperature ≥39°C or <36°C; white blood cell count $>14\times10^9$/l or $<4\times10^9$/l, a positive blood culture, or a known or strongly suspected source of infection; and (2) two or more of the following: systemic vascular resistance <800 dyne.s/cm^5; unexplained hypotension (systolic blood pressure <90 mm Hg for more than 1 hour); ongoing metabolic acidosis with anion gap >20 mmol/l; inotropic support to maintain systolic blood pressure >90 mm Hg; or a

Table 1 Baseline characteristics of study groups

Group	No	Mean (SE) age (years)	Sex (F:M)
Normal	137	52 (1.7)	66:66
"At risk"	103	64 (1.4)	43:58
ARDS	112	61 (1.4)	44:64

platelet count of $<81\times10^9$/l.[12] All subjects were of North European origin and reflected the general population. The protocol was approved by the North Bristol NHS Trust local research ethics committee and patients or their surrogates gave informed consent.

Clinical data

Murray Lung Injury, Acute Physiology and Chronic Health Evaluation II (APACHE II), Acute Physiology and Chronic Health Evaluation III (APACHE III), and Simplified Acute Physiology (SAPS II) scores were recorded for each ICU patient. The Murray Lung Injury score is an accepted indicator of degree of pulmonary injury and oxygenation whereas APACHE II, APACHE III and SAPS II scores indicate the degree of generalised physiological disturbance. Twenty eight and 60 day mortality were also recorded.

DNA extraction

Genomic DNA was extracted from whole blood using a standard phenol-free high salt method as previously described.[11]

Induced heteroduplex generator (IHG) analysis for VEGF+936 C/T polymorphism

IHG analysis was used to allow simple, rapid and unequivocal genotyping. An IHG reagent was synthesised as a long oligonucleotide before purification. The patient samples and IHG reagents were amplified separately by PCR. PCR mixes (50 µl) contained 0.5 µM each of forward and reverse primers (VEGF forward: 5'-TTTGGGT CCGGAGGGCGAGA-3', VEGF reverse: 5'-TTCCGGGCTCGGTGATTTAGC-3') 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1×*Taq* polymerase buffer (75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% V/V Tween), 0.5 unit *Taq* polymerase (Advanced Biotechnologies), and either diluted IHG reagent or 500 ng genomic DNA.

Following an initial denaturation at 95°C for 5 minutes, 35 cycles of 95°C for 1 minute, annealing at 61°C for 1 minute and 72°C for 1 minute were performed, followed by a final extension at 72°C for 7 minutes. Equal volumes of amplicons from genomic DNA and IHG reagents were mixed, denatured at 95°C for 5 minutes, and allowed to cool slowly using controlled ramping to 37°C over a 30 minute period. Heteroduplexes were resolved by electrophoresis and visualised on a Kodak digital imaging system using a 302 nm UV transilluminator. Ten random samples were directly sequenced to confirm genotyping accuracy.

Statistical analysis

A preliminary power calculation suggested 100 patients would be required in each group to show an odds ratio of 2 in allele or genotype frequency on the basis of previous noted allele frequencies.[9] Allele frequencies were estimated by gene counting. Statistical analysis was performed using Graph Pad Prism version 4 software. χ^2 tables were used to compare the observed number of each genotype with those expected for a population in Hardy-Weinberg equilibrium and to compare genotype frequencies between the patient populations and the control groups. Genotype and allele frequencies were compared using Fisher's exact test. Non-parametric data were normalised by log transformation. Demographic and severity score data were analysed by two factor analysis of variance. When analysis of variance was significant, Bonferroni's correction was applied for multiple group comparisons. For all tests a p value of $\leqslant 0.05$ was considered significant.

RESULTS

Baseline characteristics

Table 1 shows baseline characteristics for normal subjects, ventilated "at risk" patients, and patients with ARDS. The risk factor profiles of the "at risk" and ARDS cohorts are shown in table 2. The "at risk" and ARDS ventilated cohorts were also matched in terms of generalised physiology severity scores (APACHE II, APACHE III and SAPS II), but Murray Lung Injury scores were higher in the ARDS cohort as expected (2.84 (0.06) *v* 1.36 (0.08), p<0.001).

CT, TT genotype and T allele frequencies in patient groups

Table 3 shows the genotype and allele frequencies for the three different groups. For all samples, genotype distribution was in Hardy-Weinberg equilibrium ($\chi^2 = 1.42$, p = 0.23 for normal; $\chi^2 = 0.729$, p = 0.39 for "at risk"; and $\chi^2 = 0.137$, p = 0.71 for ARDS). CT and TT genotypes occurred significantly more frequently in the ARDS group than in the normal group (OR 2.01, 95% CI 1.13 to 3.58, p = 0.02) and in those "at risk" (OR 2.05, 95% CI 1.02 to 2.20, p = 0.03). The polymorphic T allele occurred significantly more frequently in the ARDS group (OR 1.77, 95% CI 1.06 to 2.91, p = 0.04) than in the normal and the ventilated "at risk" groups (OR 1.82, 95% CI 1.04 to 3.18, p = 0.04).

CT, TT genotypes and mortality (28 and 60 day)

Table 4 shows 28 and 60 day mortality according to disease group and genotype. There were no significant differences in mortality between the "at risk" and ARDS cohorts as a whole (28 and 60 day mortality rates 27.2% *v* 36.6%, 30.1% *v* 38.4%, respectively). Twenty eight and 60 day mortality rates did not

Table 2 Risk factor profiles for ventilated cohorts

Risk factor	"At risk"	ARDS	p value
Sepsis (combined)	72 (69.9)	99 (84.6)	0.01*
Sepsis (chest)	37 (35.9)	61 (52.1)	0.21
Sepsis (abdomen)	22 (21.4)	30 (25.6)	1.0
Sepsis (unknown site)	10 (9.7)	6 (5.1)	0.11
Sepsis (nervous system)	3 (2.9)	2 (1.7)	0.65
Massive transfusion	22 (21.4)	7 (6.0)	0.001*
Acute pancreatitis	5 (4.9)	9 (7.7)	0.42
Inhalational injury	4 (3.9)	2 (1.7)	0.42
Total	103	117	N/A

Values are given as number (%).
*Statistically significant (Fisher's exact test).

Table 3 Genotype and allele frequencies

Group	No	Genotype frequencies (%) CT, TT (polymorphic)	CC (normal)	Allele frequencies (%) T (polymorphic)	C (normal)
Normal	137	27 (19.7)	110 (80.3)	30 (10.9)	244 (89.1)
"At risk"	103	20 (19.4)	83 (80.6)	22 (10.7)	184 (89.3)
ARDS	112	37 (33.0)*	75 (67.0)	40 (17.9) †	184 (82.1)

*OR 2.01, 95% CI 1.13 to 3.58, p=0.02 (ARDS v N); OR 2.05, 95% CI 1.02 to 2.20, p=0.03 (ARDS v AR).
†OR 1.77, 95% CI 1.06 to 2.91, p=0.04 (ARDS v N); OR 1.82, 95% CI 1.04 to 3.18, p=0.04 (ARDS v AR).

differ between CT/TT and CC genotypes in "at risk" (OR 1.19, 95% CI 0.41 to 3.48, p = 0.78 and OR 1.32, 95% CI 0.47 to 3.72, p = 0.59, respectively) or ARDS cohorts (OR 1.81, 95% CI 0.80 to 4.05, p = 0.21 and OR 1.90, 95% CI 0.85 to 4.23, p = 0.15, respectively).

CT, TT genotypes and physiological scores

Table 5 shows the ICU severity scores according to disease group and genotype. There was no association between genotypes and Lung Injury score, APACHE II or SAPS II scores. However, ARDS patients with CT or TT genotypes had significantly higher APACHE III scores than those with CC genotypes (80.9 (4.3) *v* 69.3 (2.9), p<0.05).

DISCUSSION

This study suggests an association between a specific allele (the VEGF+936 T allele) and susceptibility to ARDS and its associated physiological disturbance (APACHE III score). This supports previously published data suggesting a role for VEGF in ARDS.[4 5]

We attempted to minimise genetic confounding by rigorous phenotypic classification using an accepted definition of ARDS and "at risk" subjects. A ventilated "at risk" cohort was used for comparison to exclude the possibility of a false association with critical illness. In addition, prospective recruitment to each cohort was undertaken over the same time period reducing the possibility of recruitment bias as a cause of chance variation in genotype frequencies. The cohorts were all of North European origin to remove the possibility of altered genotype frequencies in different ethnicities. A higher VEGF+936 CT genotype frequency has been reported in a Japanese cohort than would be expected in a normal white population.[14]

There are some limitations to this study. In any genetic epidemiological study the cohort size is key and our sample size is modest compared with other similar studies, although consistent with our initial power calculation. Furthermore, the higher proportion of patients with sepsis and lower proportion of transfusion-related injury in the ARDS cohort and age differences limit the strength of our conclusions. However, the polymorphism under study is associated with a functional effect on the gene product and our results are biologically plausible.

In assessing the relationship between the VEGF polymorphism and other ARDS parameters, only the APACHE III physiological score was associated. This system uses statistical modelling techniques to weight and select the variables and multiple logistic regression to estimate risk of death, unlike the other scoring systems which use a more subjective method with weights and variables selected by expert opinion. There is some evidence to suggest that the APACHE III is a superior prognostic model.[13] However, further larger studies are required to confirm the absolute specificity of this association.

So why is there an apparent relation between the T allele, ARDS susceptibility, and severity of physiological dysfunction? Untested hypotheses include reduced VEGF expression via cellular gene enhancer effects or changes in VEGF isoform or receptor expression. These hypotheses would assume VEGF to have a protective role in recovery from lung injury and hence a more important effect of this polymorphism in the lung. It is possible the VEGF+936 CT polymorphism might be in linkage disequilibrium with another functional polymorphism, although this has yet to be demonstrated.

In vitro studies have confirmed that VEGF is abundant in the lung, especially in alveolar epithelium including A549 cells and preliminary data in primary human cultured type 2 alveolar epithelial cells (AE2 cells), suggesting it is the predominant pulmonary source of VEGF.[16–19] Several lines of in vitro evidence point to a possible role for VEGF in repair and recovery following injury. Exogenous VEGF has been shown to act as a growth factor on human fetal pulmonary epithelial cells and is capable of restoring the ability of A549 cells to express VEGF in an acid exposure cellular model of injury, raising the possibility of an autocrine function in the lung.[20 21] This has been described in specialised epithelial cells in other tissues.[22]

Animal models have contributed conflicting evidence for the role of VEGF in the lung. Some studies suggest a possible role in mediating lung injury. Adenoviral delivery of $VEGF_{165}$ to the lung causes non-cardiogenic pulmonary oedema. High tidal volume ventilation strategies in an acid induced murine model of lung injury increase VEGF receptor 2 (VEGFR2) expression although intrapulmonary VEGF expression per se was unchanged.[23 24] However, other studies suggest a role in recovery from lung injury and a possible survival function for

Table 4 28 and 60 day mortality

Group	N	Genotype	28 day mortality (%)	60 day mortality (%)
"At risk"	103	All	28/103 (27.2)	31/103 (30.1)
	20	CT, TT	6/20 (30.0)	7/20 (35.0)
	83	CC	22/83 (26.5)	24/83 (28.9)
ARDS	112	All	41/112 (36.6)	43/112 (38.4)
	37	CT, TT	17/37 (45.9)†	18/37 (48.6)†
	75	CC	24/75 (32.0)†	25/75 (33.3)†

*OR 1.81, 95% CI 0.80 to 4.05, p=0.21.
†OR 1.90, 95% CI 0.85 to 4.23, p=0.15.

Table 5 ICU severity scores

Group	Genotype	ICU severity score: Murray Lung Injury	SAPS II	APACHE II	APACHE III†
"At risk"	All	1.36 (0.08)	42.2 (1.31)	17.0 (0.78)	67.9 (2.34)
	CT, TT	1.21 (0.20)	39.9 (2.85)	18.0 (1.42)	62.2 (4.87)
	CC	1.40 (0.09)	42.8 (1.47)	17.0 (0.78)	69.3 (2.65)
ARDS	All	2.84 (0.06)	45.7 (1.32)	19.0 (0.75)	73.0 (2.47)
	CT, TT	2.74 (0.11)	46.8 (2.63)	19.0 (1.30)	80.9† (4.33)
	CC	2.90 (0.07)	45.2 (1.51)	18.5 (0.92)	69.3† (2.92)

Severity scores are expressed as mean (SE) values.
Data for APACHE II were $\log_{10}$ transformed to normalise before statistical analysis.
†p<0.05 (Bonferroni) ARDS CT, TT v ARDS CC (two way ANOVA, p=0.02).

alveolar epithelium. Chronic VEGFR2 blockade in rats leads to alveolar apoptosis and emphysema and delivery of $VEGF_{165}$ to fetal mice protects against respiratory distress syndrome in hypoxia inducible transcription factor-2α (HIF-2α) knockout fetal mice.[25 26] In addition, VEGF partially mediates the protective effects of interleukin-13 (IL-13) in a murine hyperoxic model of lung injury.[27] In a lipopolysaccharide (LPS) induced murine model of lung injury, intrapulmonary levels of VEGF increased following injury for 96 hours, mirroring the increase in bronchoalveolar lavage fluid protein and neutrophils with significant VEGF localisation to lung epithelium.[28]

The role of VEGF in the normal human lung remains uncertain but evidence from clinical studies suggests a possible protective role in resolution from lung injury. Intrapulmonary levels are low in the early stages of ARDS and these increase with recovery in both ARDS and normal subjects exposed to high altitude pulmonary oedema.[4 5 7] However, VEGF levels may simply reflect damage to the alveolar epithelium as described in normal smokers and patients with idiopathic pulmonary fibrosis (IPF).[29] The reported functional effect of CT and TT genotypes in normal subjects has been a reduction in plasma levels.[9] We have previously detected raised plasma levels in the early stages of ARDS which normalised in recovery but increased in non-survivors, in contrast to bronchoalveolar lavage (BAL) fluid levels as described above.[4] There are no previous reports of the effects of the CT and TT genotypes on intra-alveolar VEGF and how these alleles might affect intrapulmonary production and resultant plasma levels if the lung is the main source of VEGF. In the presence of a normal alveolar epithelial membrane, we hypothesised that plasma levels would reflect alveolar levels and hence the T allele would predispose to ARDS. We did not obtain matched BAL fluid and plasma data at constant time points in relation to the onset of ARDS/"at risk" patients which would have answered this question. The effect of these genotypes on resident lung cells such as the alveolar epithelium is required to enable the mechanism by which they may influence ARDS pathogenesis.

In conclusion, individuals with CT and TT genotypes are more susceptible to ARDS than normal subjects and ventilated "at risk" subjects and have a higher APACHE III score. Our data therefore suggest a potential role for VEGF gene polymorphism in the development of ARDS in humans. The possible role of other functional VEGF polymorphisms needs to be appraised.[12–14] Intrapulmonary delivery of VEGF may have a potential therapeutic role, either in reducing the risk of ARDS in an "at risk" group or reducing severity of disease in those with established disease, and genotyping may help target such therapy.

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Authors' affiliations
A R L Medford, A B Millar, Lung Research Group, Division of Medicine, University of Bristol, Southmead Hospital, Bristol BS10 5NB, UK
L J Keen, J L Bidwell, Department of Pathology and Microbiology, Homeopathic Hospital Site, University of Bristol, Bristol BS6 6JU, UK

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Expression of Functional Toll-Like Receptor-2 and -4 on Alveolar Epithelial Cells

Lynne Armstrong, Andrew R. L. Medford, Kay M. Uppington, John Robertson, Ian R. Witherden, Teresa D. Tetley, and Ann B. Millar

Lung Research Group, and Academic Renal Unit, Department of Clinical Science North Bristol, University of Bristol, Southmead Hospital, Westbury on Trym, Bristol; Department of Respiratory Medicine, Bristol Royal Infirmary, Bristol; and Respiratory Medicine, National Heart and Lung Institute, Imperial College School of Medicine, Charing Cross Hospital, London, United Kingdom

The recognition of potentially harmful microorganisms involves the specific recognition of pathogen-associated molecular patterns (PAMPs) and the family of Toll-like receptors (TLRs) is known to play a central role in this process. TLR-4 is the major recognition receptor for lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, whereas TLR-2 responds to bacterial products from gram-positive organisms. Although resident alveolar macrophages are the first line of defense against microbial attack, it is now understood that the alveolar epithelium also plays a pivotal role in the innate immunity of the lung. The purpose of the current study was to determine whether human primary type II alveolar epithelial cells (ATII) express functional TLR-2 and TLR-4 and how they may be regulated by inflammatory mediators. We have used reverse transcriptase–polymerase chain reaction and flow cytometry to determine basal and inducible expression on ATII. We have used highly purified preparations of the gram-positive bacterial product lipoteichoic acid (LTA) and LPS to look at the functional consequences of TLR-2 and TLR-4 ligation, respectively, in terms of interleukin-8 release. We have shown that human primary ATII cells express mRNA and protein for both TLR-2 and TLR-4, which can be modulated by incubation with LPS and tumor necrosis factor. Furthermore, we have demonstrated that these receptors are functional. This suggests that ATII have the potential to contribute significantly to the host defense of the human alveolus against bacteria.

The appropriate recognition of potentially harmful microorganisms is particularly pertinent to the lower respiratory tract, because the lung is a primary site for the introduction and deposition of pathogenic microorganisms and a large quantity of airborne particles. Because much of the particulate matter poses no significant threat to the host, mechanisms are in place to permit activation of the immune response only when there is a genuine risk of infection. This involves the specific recognition of pathogen-associated molecular patterns (PAMPs) and the family of Toll-like receptors (TLRs) is known to play a central role in this process. These type I transmembrane proteins share significant similarities with the interleukin (IL)-1 receptor (IL-1R) and all contain the highly homologous toll/IL-1R cytoplasmic domain. The homology present in the toll/IL-1R is contrasted by significant variation in the leucine-rich repeats of the extra-cellular domains, which permits discrimination of a variety of PAMPs, including lipopolysaccharide (LPS), bacterial lipoprotein, peptidoglycans, and lipoteichoic acid (LTA) (1). Of the ten TLRs so far identified, TLR-2 and TLR-4 have been the most extensively studied. The identification of a functional mutation in the *tlr4* gene, responsible for LPS hyporesponsiveness in C3H/HeJ mice (2), first suggested that TLR-4 was the major recognition receptor for the gram-negative bacterial component LPS (3), and subsequent studies have now confirmed this. By contrast, TLR-2 responds to bacterial lipoproteins from gram-positive organisms (4), as well as yeasts (5) and mycobacteria (6).

Although resident alveolar macrophages are the first line of defense against microbial attack, and are known to express TLR-2 and TLR-4 (7, 8), it has become increasingly apparent that the lung epithelium also plays a pivotal role in innate immunity. Indeed A549 carcinoma cells, which are a type II–like line, have been shown to express functional TLR-4, predominantly in the intracellular compartment (9). In terms of primary cells, human alveolar type II epithelial cells (ATII) have recently been described to express mRNA and protein for TLR-2 (8), although its functional responses were not explored.

The purpose of the current study was to determine whether human primary ATII express functional TLR-2 and TLR-4 and whether their expression can be modulated *in vitro* in response to inflammatory mediators. We have used reverse transcriptase (RT)-polymerase chain reaction (PCR) and flow cytometry to determine basal expression on the ATII cells. We have also looked at induction of TLR-2 and TLR-4 expression by tumor necrosis factor (TNF)-α, which is implicated in the pulmonary inflammatory response and has been shown previously to upregulate TLR-2 and TLR-4 expression on renal epithelial cells (10). We have also used highly purified preparations of LTA and LPS to look at the functional consequences (TLR-2 and TLR-4 expression, and IL-8 release) of TLR-2 and TLR-4 ligation, respectively.

Materials and Methods

Subjects

Macroscopically normal lung tissue sections (~ 15 × 5 × 5 cm) were donated by eight patients undergoing lobar resection for malignancy (three female, five male). The median age was 67 yr. Seven donors were ex-smokers and one donor had no smoking history. Ethical approval was obtained from North Bristol NHS and United Bristol Healthcare Trusts.

Isolation and Purification of ATII

ATII cells were purified according to the method of Witherden and colleagues (11). Sections were perfused with 0.9% saline to remove alveolar macrophages and digested with 0.25% trypsin (Sigma, Poole, UK). The digested tissue was chopped into pieces ~ 1–2 mm^2 in size, in the presence of newborn calf serum (Invitrogen, Paisley, UK). DNase I was then added to the suspension at 250 μg/ml in 7 ml Hanks' balanced salt solution (HBSS). The suspension was shaken vigorously for 5 min

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Address correspondence to: Dr. Lynne Armstrong, Lung Research Group, University of Bristol, Department of Clinical Science North Bristol, Southmead Hospital, Westbury on Trym, Bristol BS10 5NB, UK. E-mail: lynne.armstrong@bris.ac.uk

Abbreviations: aquaporin, AQP; alveolar type II epithelial cells, ATII; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; Hanks' balanced salt solution, HBSS; interferon, IFN; immunoglobulin, IgG; interleukin, IL; lipoteichoic acid, LTA; lipopolysaccharide, LPS; pathogen-associated molecular patterns, PAMPs; phosphate-buffered saline, PBS; phycoerythrin, Pe; repurified lipopolysaccharide, rLPS; reverse transcriptase polymerase chain reaction, RT-PCR; surfactant protein C, SP-C; Toll-like receptor, TLR; tumor necrosis factor-α, TNF-α.

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before filtering through a large mesh (~ 500 μm), followed by a 40-μm mesh (Fahrenheit, Milton Keynes, UK). The filtered suspension was centrifuged at 300 × *g* for 10 min at 4°C and the pellet resuspended in 15 ml HBSS/15 ml DCCM (React Scientific, Troon, UK) containing 100 μg/ml DNase I. The suspension was incubated in a T-75 flask for 2 h at 37°C to allow any residual contaminating macrophages to adhere. The supernatant containing nonadherent cells was then centrifuged at 300 × *g* for 10 min at 4°C and the pellet resuspended in complete medium (DCCM-1, 10% newborn calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and incubated for 2 h at 37°C to allow contaminating fibroblasts to adhere. The nonadherent cells were centrifuged at 300 × *g* for 10 min at 4°C and resuspended at 1×10^5 ATII cells/ml in complete media and put into 60-mm dishes (Greiner Cell Star, Stonehouse, UK) precoated with Vitrogen 100 (Cohesion Technologies, Palo Alto, CA). The ATII cells were subsequently adhered at 37°C for 24 h. The medium and any remaining contaminating cells were then removed and fresh complete medium added. The cells were then incubated at 37°C, and after 16 h the medium was removed and the cells washed with HBSS. Fresh complete medium was then added and the cells incubated for a further 24 h to establish confluent monolayers with type II morphology (ATII). ATII cell phenotype was confirmed by positive staining for alkaline phosphatase and mRNA transcripts for surfactant protein-C (SP-C) and aquaporin (AQP)3. Morphologic characteristics were confirmed by electron microscopy. ATI cell phenotype was excluded by negative staining for aquaporin 5.

RNA Extraction

Total cellular RNA was extracted from 5×10^5 ATII after culture with stimuli stated below. Cells were washed in sterile PBS and cellular RNA extracted using RNAbee (AMS Biotechnology, Abingdon, UK) according to manufacturer's instructions. Cellular RNA concentration was measured using a GeneQuant II (Amersham Biosciences, Little Chalfont, UK).

Semiquantitative RT-PCR

RT-PCR was performed in a 20-μl one-step reaction using Reverse-IT RTase blend (ABgene, Epsom, UK) with 200 ng of total RNA as a template. RT was performed at 47°C for 30 min followed by 94°C for 2 min to inactivate the RT enzyme. For TLR-2 and TLR-4 amplification, PCR was performed with 30 cycles of 94°C for 20 s, 54°C for 45 s, and 72°C for 1 min. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene, PCR was performed with 17 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. For SP-C and AQP3 amplification the conditions were 54°C for 30 cycles and 58°C for 32 cycles, respectively (for specific primer sequences *see* Table 1). Products were electrophoresed through a 1.5% agarose gel and visualized using ethidium bromide staining. mRNA quantity was determined by digital imaging densitometry (Geldoc 1000 with Quantity One software; Bio-Rad, Hemel Hempstead, UK).

Flow Cytometry

Flow cytometry was performed on 1×10^5 ATII that had been removed from tissue culture plates by nonenzymatic digestion (Cell dissociation solution; Sigma). Cells were incubated on ice for 2 min with 10 μg of human IgG before a 30-min incubation with 5 μl of monoclonal anti-TLR-2 antibody, anti-TLR-4 antibody (clone TL2.1 and HTA125, respectively; Cambridge Bioscience, Cambridge, UK), or mouse IgG1 control (Dako, Ely, UK). The primary antibodies were washed off in PBS/0.5% bovine serum albumin, 0.1% azide and the cells incubated for a further 30 min on ice with 2.5 μl of rabbit anti-mouse IgG1-pe (Dako). The secondary antibodies were washed off in PBS/0.5% bovine serum albumin, 0.1% azide and labeled cells were acquired on an EpicsXL flow cytometer (Beckman Coulter, High Wycombe, UK). TLR-2 and TLR-4 expression was analyzed using Expo 32 software (Beckman-Coulter).

Preparation of Purified LPS and LTA

The LTA was a kind gift of Dr. Siegfried Morath, University of Konstanz. The preparation, from *Staphylococcus aureus*, had been repurified by phenolic extraction to remove any residual endotoxin activity and therefore does not stimulate TLR-4 signaling (12, 13). LPS from *Escherichia coli* (serotype 0001:B4; Sigma) was repurified by phenolic extraction according to the method of Hirschfeld and coworkers (14), which has been shown to remove any TLR-2 stimulatory activity.

IL-8 Enzyme-Linked Immunosorbent Assay

ATII cells were initially cultured for 48 h in purified monolayers. Following this, the medium was replaced with fresh medium + 10% fetal bovine serum for a further 20 h with or without repurified LPS (rLPS) (10 μg/ml), highly purified LTA (1 μg/ml), or TNF-α (10 ng/ml). Supernatants were stored at −80°C until analysis. IL-8 levels were determined in diluted supernatants using the Pelikine IL-8 kit (sensitivity, 1–3 pg/ml; Mast Diagnostics, Bootle, UK), according to manufacturer's instructions.

Statistical Analysis

The mRNA and flow cytometry data were normally distributed as determined by the Ryan Joiner normality test. Comparisons between multiple groups were performed using one-way ANOVA, with Tukey's multiple comparison test (mct) to compare individual group differences (GraphPad Prism). A P value of < 0.05 was regarded as significant.

Results

Assessment of ATII Phenotype

Morphologic characteristics were confirmed by electron microscopy showing the existence of lamellar bodies and tight junctions in keeping with ATII phenotype. The cells were also positive for alkaline phosphatase expression. To confirm that the cells purified from the lung were indeed of the ATII phenotype, we also performed RT-PCR on RNA lysates. The presence of mRNA transcripts for SP-C and AQP3 confirmed type II characteristics, whereas the presence of the ATI cell phenotype in the cultures was excluded by negative expression of AQP5 (Figure 1A).

Human Primary ATII Express TLR-2 and TLR-4

We identified transcripts for TLR-2 and TLR-4 mRNA in total RNA lysates from ATII after 2 d in culture (Figure 1B). The mean expression of TLR-2 at baseline was 1.70 ± 0.20 as a ratio of GAPDH expression. TLR-4 expression was significantly lower than TLR-2 expression in the same samples at 0.43 ± 0.08 relative to GAPDH ($P = 0.045$) (Figure 2). We confirmed the existence of protein for both TLR-2 and TLR-4 by flow cytometry using specific antibodies. As a ratio of isotypic control antibody, TLR-2 expression was 0.91 ± 0.10, compared with 1.42 ± 0.15 for TLR-4 (Figure 3). This suggests that, in contrast to TLR-4, TLR-2 protein levels are not detectable on the surface of unstimulated ATII.

TABLE 1. Primer sequences for RT-PCR

	Forward Primer	Reverse Primer	Base Pairs
TLR-2	GCCAAAGTCTTGATTGATTGG	TTGAAGTTCTCCAGCTCCTG	318
TLR-4	TGGATACGTTTCCTTATAAG	GAAATGGAGGCACCCCTTC	469
SP-C	AGCAAAGAGGTCCTGATGGA	CTAGTGAGAGCCTCAAGACT	405
AQP3	CCTTTGGCTTTGCTGTCACTC	ACGGGGTTGTTGTAAGGGTCA	373
AQP5	GGTGTGCTCCGTGGCCTTCCT	CTTCCGCTCTTCCCGCTGCTC	760
GAPDH	GCCAAAAGGGTCATCATCTC	GTAGAGGCAGGGATGATGTT	287

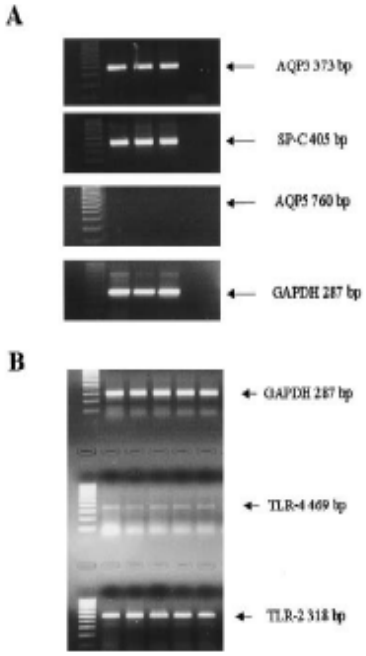

Figure 1. (*A*) Aquaporin and SP-C mRNA expression in ATII cultures derived from three separate lung isolations. (*B*) Basal levels of TLR mRNA expression in ATII cultures derived from five separate lung isolations.

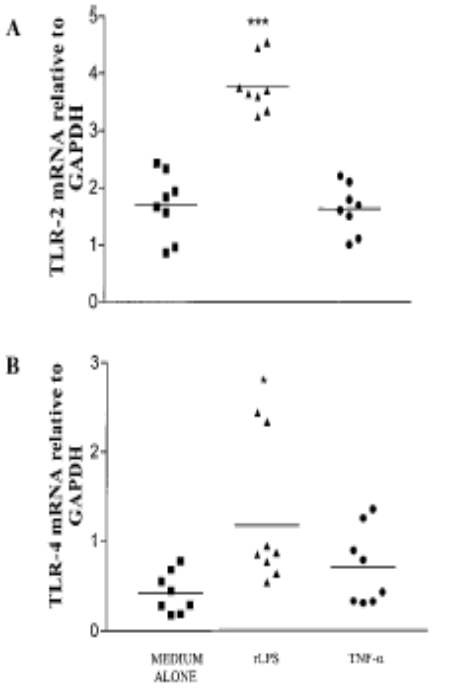

Figure 2. (*A*) TLR-2 mRNA expression induced by rLPS in ATII cells from eight separate experiments. $P < 0.0001$, one-way ANOVA; ***$P < 0.005$ relative to medium alone (Tukey's mct). (*B*) TLR-4 mRNA expression induced by rLPS in ATII cells from eight separate experiments. $P < 0.05$, one-way ANOVA; *$P < 0.05$ relative to medium alone.

Expression of TLR-2 and TLR-4 mRNA and Surface Protein can be Modified by Inflammatory Mediators

Culture for 2 h with rLPS led to a 2.2-fold increase in the expression of TLR-2 mRNA compared with medium alone (3.78 ± 0.17 versus 1.71 ± 0.20, $P = 0.0001$) (Figure 2A). Similarly, there was a 2.7-fold increase in the expression of TLR-4 mRNA in response to rLPS (1.18 ± 0.27 versus 0.43 ± 0.08, $P = 0.02$) (Figure 2B). Culture with TNF-α had no measurable effect on the expression of either TLR-2 or TLR-4. 20 h cultures of ATII showed no significant increase in TLR-2 surface expression after culture with rLPS (1.19 ± 0.18 versus 0.91 ± 0.10), and LTA also had no significant effect on TLR-2 surface levels. Interestingly, TNF-α increased TLR-2 surface expression 1.8-fold (1.63 ± 0.13, $P < 0.05$) (Figure 3A). By contrast, TLR-4 surface expression was significantly upregulated after culture with rLPS (1.89 ± 0.18 versus 1.42 ± 0.15, $P < 0.01$), whereas LTA and TNF had no effect on surface expression of TLR-4 protein (Figure 3B).

TLR-2 and TLR-4 Generate a Functional Response in Human Primary ATII Cells

We cultured the cells with rLPS and LTA to determine whether TLRs on ATII play a functional role in the inflammatory response to bacterial products. Using TNF-α stimulation as a positive control, we determined expression of IL-8 in our ATII culture supernatants of 4,135 ± 467 pg/ml ($P < 0.01$ relative to a medium alone value of 1,547 ± 88 pg/ml), which is in accordance with other studies (15) (Figure 4). We detected 2,854 ± 306 pg/ml IL-8 in response to 10 μg/ml rLPS ($P < 0.01$ relative to medium alone) and 2,097 ± 118 pg/ml IL-8 in response to 1 μg/ml LTA ($P < 0.001$ relative to medium alone). This suggests that human ATII are able to generate an inflammatory response via TLRs after ligation.

Discussion

This study has demonstrated for the first time the existence of functionally active TLR-2 and TLR-4 on human primary ATII cells. These cells have recently been suggested to play an important role in the innate immunity of the human lung, and their ability to recognize PAMPs has been highlighted by a recent study that localized TLR-2 mRNA and protein to ATII by *in situ* hybridization and immunohistochemistry on human lung sections (8). There has been no equivalent study looking at the expression of TLR-4 on these cells, although TLR-4 expression has been demonstrated in the type II–like epithelial cell line A549 and in bronchial epithelium (9, 16). In a recent study by Guillot and coworkers, they were unable to localize TLR-4 to the surface of A549 cells or the bronchial epithelial cell line BEAS-2B; instead, they described a perinuclear location in association with the Golgi apparatus. However, they were able to show that intracellular TLR-4 was functionally responsive to LPS, possibly through internalization of the ligand, as has previously been described for intestinal epithelial cells (17). By contrast, we have been able to demonstrate the presence of TLR-4 on the cell surface by flow cytometry, although at a lower level than we have observed on alveolar macrophages or monocytes (unpublished observations). A549 cells, which are an adenocarcinoma line originally isolated in 1972 from a single parent cell (18), are known to differ from primary ATII cells in many respects, including IL-8 and nitric oxide production (15, 19). Many generations later they often poorly reflect the phenotype of the parent primary cell, which may explain the discrepancy with our data. Intestinal epithelial cells also do not appear to express surface TLR-4, which is possibly a modification to prevent inappropriate activation by gram-negative commensal gut organisms (20). By

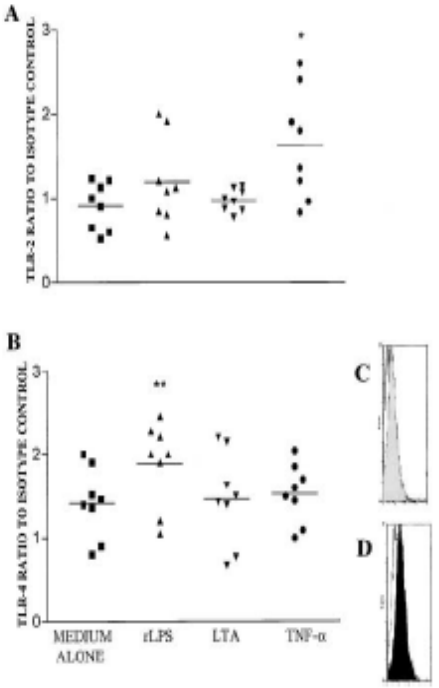

Figure 3. (*A*) TLR-2 surface expression induced by TNF-α, determined by flow cytometry from 8 separate experiments. $P < 0.05$, one-way ANOVA; $^{*}P < 0.05$ relative to medium alone (Tukey's mct). (*B*) TLR-4 surface expression induced by rLPS, determined by flow cytometry from eight separate experiments. $P = 0.19$, one-way ANOVA; $^{**}P < 0.01$ relative to medium alone (Tukey's mct). (*C*) Representative basal TLR-4 surface staining (*light gray*) relative to isotype control (*white*). (*D*) Representative TLR-2 surface staining in response to rLPS (*black*) relative to basal levels (*white*).

contrast, epithelial cells of the human bladder, which occupy a sterile environment, similar to that found in the alveolus, are known to express a significant level of functional TLR-4 on the cell surface (21). Although the upper airways of the lung are often exposed to large amounts of particulate matter, much of it containing traces of LPS, the alveolar compartment is predominantly sterile. Therefore it may be more appropriate for ATII to have TLR-4 expression more comparable to bladder rather

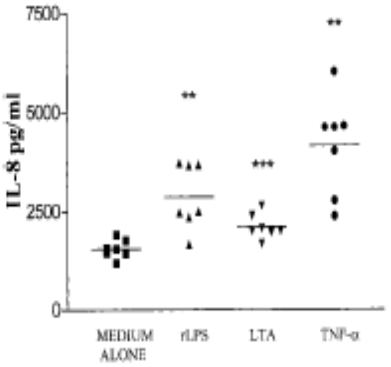

Figure 4. IL-8 production induced by rLPS, LTA, and TNF-α in ATII cells from eight separate experiments. $P < 0.0001$, one-way ANOVA; $^{**}P < 0.01$, $^{***}P < 0.005$ relative to medium alone (Tukey's mct).

than intestinal epithelium. Although we have successfully demonstrated functional responses to LPS and LTA, these responses depend on higher concentrations and are of a lesser magnitude to those reported in monocytes and macrophages. This could be explained by the observation in our laboratory that TLR-2 and TLR-4 surface expression on ATII is ~ 5-fold lower in both cases than that detected on alveolar macrophages (unpublished observations). Another potential explanation is the lack of CD14, which is a co-receptor for LPS signaling, on the surface of lung epithelial cells (16). Indeed, epithelial cells only respond to LPS when there is a source of exogenous sCD14, such as fetal bovine serum (16). It is also possible that other TLR downstream signaling components are implicated in this altered response, and this would warrant further investigation.

Although this study is the first to describe TLR-2 function on human primary alveolar epithelial cells, our data are supported by a previous study by Hertz and coworkers (22), who investigated TLR-2 expression and function on human primary bronchial epithelial cells. Using immunohistochemistry, they detected TLR-2 *in situ* on these cells, which was at a lower level than that observed in monocytes. They also demonstrated that lipoprotein was able to induce IL-8 release by bronchial epithelial cells and reduce bacterial growth in co-culture by a TLR-2–dependent mechanism. In contrast, TLR-2 has also been explored in intestinal epithelial cell lines, and as seen with TLR-4, expression and responses to LTA are diminished (23). Oral epithelial cells have also been shown to express low levels of TLR-2, which can be increased after priming with interferon (IFN)-γ (24).

We have explored the regulation of TLR-2 and TLR-4 mRNA and protein in ATII and have been able to demonstrate that the expression of TLR-4 protein is moderately elevated on ATII when compared with the equivalent TLR-2 expression, whereas the reverse is true for mRNA. Although this could be a limitation of the flow cytometry detection, it could suggest that the expression of TLR-4 protein may be under tighter transcriptional regulation. Our mRNA studies have demonstrated that both TLR-2 and TLR-4 mRNA are LPS-inducible, whereas TNF-α had no effect. We chose to look at responses to TNF-α, because this cytokine is implicated in a number of inflammatory lung conditions and a previous study had demonstrated a TNF-dependent increase in TLR-2 and TLR-4 mRNA, albeit in murine renal epithelial cells (10). Our inability to detect a TNF-α response at the mRNA level may reflect species or cell-type specificity for this pathway. In terms of translation to protein, TLR-4 surface expression reflected the mRNA findings, but TLR-2 surface expression was lower, unaffected by LPS, and upregulated by TNF-α. These data suggest that TLR-4 protein levels may be determined at the transcriptional level in ATII, but that post-transcriptional mechanisms may be important for TLR-2 regulation. Studies of myeloid cells have demonstrated that the human form of TLR-2 is not inducible by LPS or TNF-α, although the murine *tlr2* is LPS-responsive (25). One explanation for this species difference is that murine *tlr2* promoter has only 10% homology with the human promoter, compared with 70% homology between the human and mouse TLR-4 promoter. Murine *tlr2* contains binding sites for the transcription factors ets and SP-1, which are known to be inducible by LPS (26). Studies of TLR-4 regulation in mouse macrophages have shown that it is regulated both transcriptionally and post-transcriptionally. LPS can affect mRNA stability, leading to a decrease in TLR-4 mRNA expression (7). However, this is counterbalanced by an increase in the transcription rate of TLR-4 in response to LPS. There has been little research into the regulation of TLRs on nonmyeloid cells. However, it is known that there are tissue-restricted transcription factors in myeloid cells (27) (PU.1 and IFN consensus-sequence binding protein), which suggests that conclusions about

epithelial regulation of TLR-2 and TLR-4 cannot easily be drawn from these studies. TLR-4 mRNA and protein expression has been shown to be unresponsive to LPS in the BEAS-2B bronchial epithelial cell line (9), but because bronchial epithelium would normally be exposed to larger amounts of endotoxin than alveolar epithelium it may be expected that their TLR-4 expression may be lower in comparison. In addition, TLR-2 and TLR-4 mRNA have been shown to be upregulated by IFN-γ in oral epithelial cells (24) suggesting that interferon response elements, rather than nuclear factor-κB binding may be important in the initiation of transcription in epithelium.

There has been much debate about the specificity of TLR-2 and TLR-4 for the recognition of gram-positive and gram-negative PAMPs, and this is in part due to the impurities present in the commercial preparations (28). In this study, we chose to use highly purified LTA and LPS, which have been demonstrated to be highly specific ligands for TLR-2 and TLR-4, respectively (14, 29). We found that both ligands were able to induce IL-8 release from ATII cells, suggesting functional activity for both TLR-4 and TLR-2. The response to LPS was greater than that seen for LTA, and this may reflect the lower TLR-2 protein expression detected on the surface of ATII cells. There is now increasing evidence from studies of intestinal epithelial cells and A549 cells that the TLR-4 is located intracellularly and that LPS needs to be internalized for responses to occur (9, 17, 30). Although we have not excluded this as a possible mechanism for LPS responses in ATII, the existence of TLR-4 on the cell surface of these cells supports the formation of a cell surface complex for the initiation of LPS signaling.

In summary, we have demonstrated the existence of TLR-2 and TLR-4 mRNA and protein in human primary ATII, which can be regulated differentially by bacterial products and inflammatory mediators. This suggests that ATII play an important role in the innate immunity of the alveolus, with potential implications for our understanding of the pulmonary inflammatory response.

Conflict of Interest Statement: L.A. has no declared conflicts of interest; A.R.L.M. has no declared conflicts of interest; K.M.U. has no declared conflicts of interest; J.R. has no declared conflicts of interest; I.R.W. has no declared conflicts of interest; T.D.T. has no declared conflicts of interest; and A.B.M. has no declared conflicts of interest.

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APPENDIX 2: ABSTRACTS

Oral presentations

1. Medford ARL, Armstrong L, Millar AB. The effect of vascular endothelial growth factor (VEGF) on primary human cultured type 2 alveolar epithelial (ATII) cell proliferation. *Eur Resp J* 2004 24(Suppl 48): 29-30s [S318].

2. Medford ARL, Armstrong L, Gillespie KM, Millar AB. The effect of proinflammatory cytokines on primary human cultured alveolar epithelial cell expression of vascular endothelial growth factor (VEGF) isoforms. *Thorax* 2003 58(Suppl III): iii18-19 [S58].

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Poster presentations

1. Medford ARL, Thickett DR, Keen LJ, Armstrong L, Hunter KJ, Millar AB. Relationship between vascular endothelial growth factor plasma and bronchoalveolar lavage levels and +936 C/T polymorphism in acute respiratory distress syndrome. *Am J Resp Crit Care Med* 2003 167(7): A664.

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10. Armstrong L, Medford ARL, Thorley A, TD Tetley, Millar AB. Primary human alveolar type II cells constitutively produce vascular endothelial growth factor. *Am J Resp Crit Care Med* 2002 165(8): A372.

APPENDIX 3:
TRAVEL AWARDS AND FELLOWSHIPS

Schering Plough Travel Fellowship [British Thoracic Society]	2001
Myre Sim Award [Royal College of Physicians of Edinburgh]	2002
Pfizer Academic Travel Award	2002
Allen and Hanbury's Travel Fellowship [British Lung Foundation]	2002
Schering Plough Travel Fellowship [British Thoracic Society]	2003
GlaxoSmithKline Sparrow Travel Fellowship	2003

APPENDIX 4:
PRIMER SEQUENCES FOR RT-PCR

NB: All programmes use 35 cycles

Product	Forward primer	Reverse primer	Base Pairs	Annealing temp
VEGF (exon 4-8): $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$	GAGATGAGCTT CCTACAGCAC	TCACCGCCTCG GCTTGTCACAT	90, 222 and 294	55
VEGF (exon 4-9): $VEGF_{165}b$	GAGATGAGCTT CCTACAGCAC	TTAAGCTTTCAG TCTTTCCTGGTG AGAGATCTGCA	222	62
VEGF (exon 7-3'UTR): $VEGF_{165}b$, $VEGF_{165-206}$	GTAAGCTTGTA CAAGATCCGCA GACG	ATGGATCCGTA TCAGTCTTTCCT	150, 200	62
VEGFR1	GTCCACAGAAG AGGATGAAGGT GTCTA	CACAGTCCGCC ACGTAGGTGAT T	413	60
VEGFR2	GCATCTCATCT GTTACAG	CTTCATCAATCT TTACCCC	332	55
NRP-1	AAAAGCCCACG GTCATAG	TCTCATCCACA GCAATCC	509	55
SP-C	AGCAAAGAGGT CCTGATGGA	CTAGTGAGAGC CTCAAGACT	405	55
AQP3	CCTTTGGCTTTG CTGTCACTC	ACGGGGTTGTT GTAAGGGTCA	373	55
AQP5	GGTGTGCTCCG TGGCCTTCCT	CTTCCGCTCTTC CCGCTGCTC	760	55
B_2M	GCATCATGGAG GTTTGAAGATG	TAAGTTGCCAG CCCTCCTAGAG	233	55
GAPDH	GCCAAAAGGGT CATCATCTC	GTAGAGGCAGG GATGATGTT	286	55

Table App4.1: Primer sequences for RT-PCR

APPENDIX 5: ELISA STANDARD CURVES

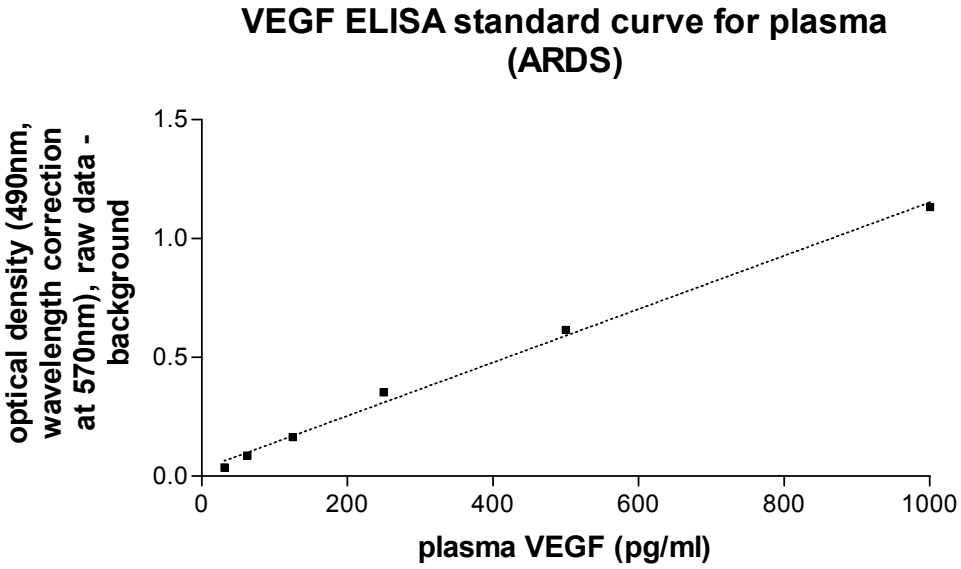


Figure App5.1: ELISA standard curve for ARDS plasma samples (linear regression r^2 = 0.996, p < 0.0001). Dotted line denotes goodness of fit.

Standard (pg/ml)	Optical density (mean)	Optical density (sd)
31.2	0.037	0.001
62.5	0.087	0.012
125	0.164	0.003
250	0.353	0.006
500	0.615	0.014
1000	1.133	0.052

Table App 5.1: Absolute values for ARDS plasma ELISA standard curve.

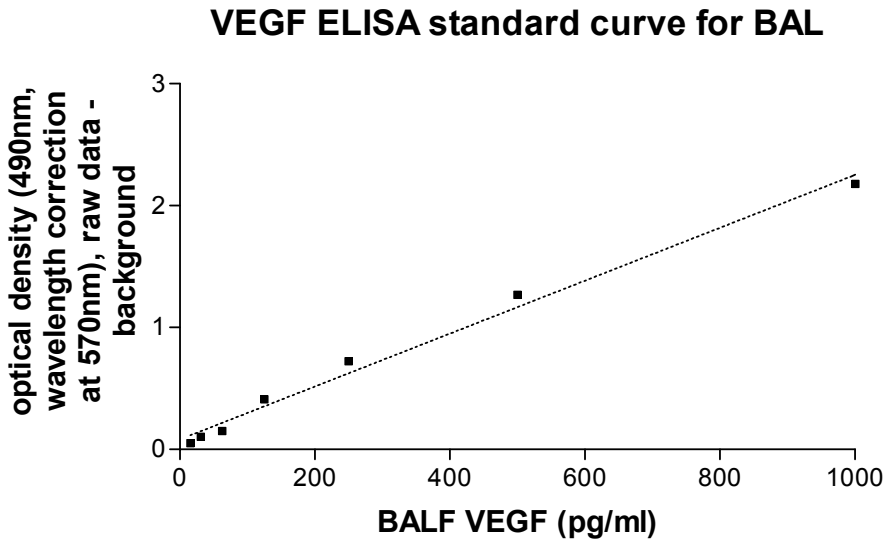


Figure App5.2: ELISA standard curve for BAL samples (linear regression $r^2 = 0.99$, $p < 0.0001$). Dotted line denotes goodness of fit.

Standard (pg/ml)	Optical density (mean)	Optical density (sd)
15.6	0.051	0.004
31.2	0.105	0.004
62.5	0.152	0.028
125	0.411	0.005
250	0.723	0.065
500	1.268	0.025
1000	2.178	0.034

Table App 5.2: Absolute values for BAL ELISA standard curve.

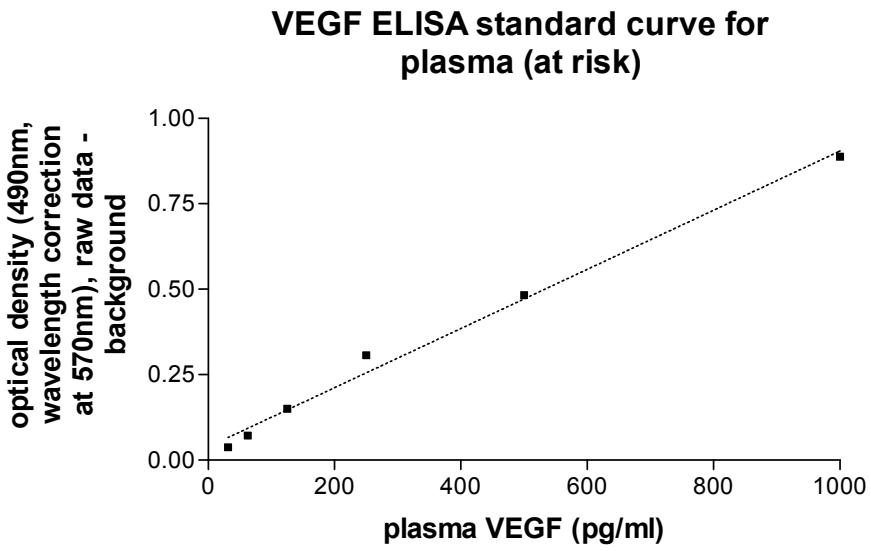


Figure App5.3: ELISA standard curve for "at risk" plasma samples (linear regression $r^2 = 0.99$, $p < 0.0001$). Dotted line denotes goodness of fit.

Standard (pg/ml)	Optical density (mean)	Optical density (sd)
31.2	0.038	0.018
62.5	0.072	0.025
125	0.15	0.009
250	0.307	0.073
500	0.483	0.085
1000	0.888	0.173

Table App 5.3: Absolute values for "at risk" plasma ELISA standard curve.

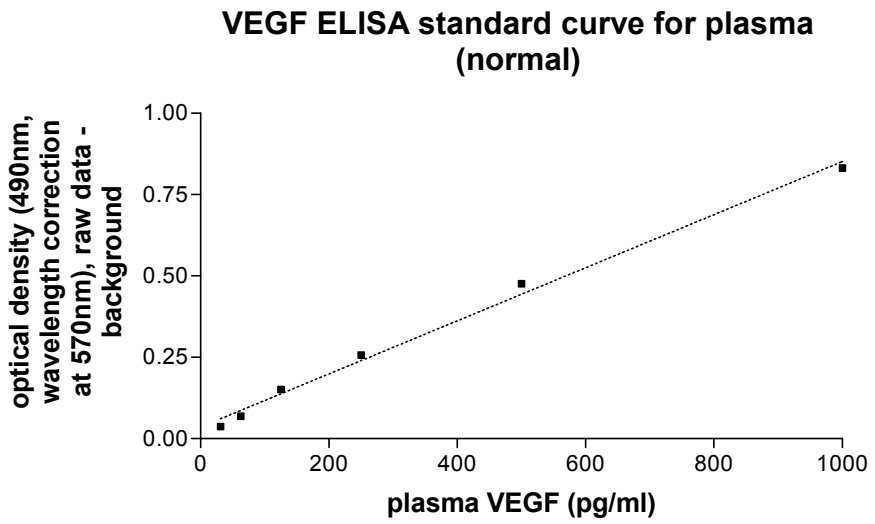


Figure App5.4: ELISA standard curve for normal plasma normal samples (linear regression $r^2 = 0.99$, $p < 0.0001$). Dotted line denotes goodness of fit.

Standard (pg/ml)	Optical density (mean)	Optical density (sd)
31.2	0.037	0.011
62.5	0.069	0.022
125	0.151	0.001
250	0.257	0.035
500	0.476	0.031
1000	0.832	0.108

Table App 5.4: Absolute values for normal plasma ELISA standard curve.

APPENDIX 6: SOLUTIONS

(Sigma unless stated)

1% Agarose gel

1g agarose

100ml 1 x TBE buffer

ALP buffer

127.1g magnesium chloride

15.6g aminomethylpropanol

Made up to 1 litre with distilled water

pH 8.9

ALP stain

10mg napthol-AS-bisphosphate

40µl dimethyl sulphoxide (DMSO)

Made up to 10ml with ALP buffer

10mg fast red violet

Shake well

Leave for 5 minutes then filter

Make up just before use

4.5M ammonium acetate

129.8g ammonium acetate

Made up to 100ml with distilled water

Complete ATII culture medium

10% NCS in DCCM-1

2mM glutamine

100 U/ml penicillin

100 µg/ml streptomycin

0.02% fungazone

Digest buffer (FFPE)

0.1g proteinase K

10ml 10% SDS

4ml 0.5M EDTA

2ml 1M Tris, pH 7.4

Made up to 100ml with distilled water

0.5M EDTA

18.6g EDTA

Made up to 100ml with distilled water

3% hydrogen peroxide (H_2O_2)/methanol

500µl H_2O_2

4.5ml absolute methanol (BDH, Poole, UK)

LB (Luria-Bertani) medium and plates

1% tryptone

0.5% yeast extract

1% NaCl

pH 7.0

Add 15g/L agar prior to above prior to autoclaving for plates

Add 100µg/ml ampicillin after cooling to 55^{o}C and invert and store at 4^{o}C

10x ligation buffer (cloning)

60mM Tris-HCl, pH 7.5

60mM magnesium chloride ($MgCl_2$)

50mM NaCl

1mg/ml bovine serum albumin

70mM β-mercaptoethanol

1mM adenosine triphosphate (ATP)

20mM dithiothreitol

10mM spermidine

Nuclei lysis buffer

4.67g sodium chloride

0.149g EDTA (BDH, Poole, UK)

0.142g TrisHCl

0.134g Trizma base

pH 8.2

Phosphate buffered saline (PBS)

40g sodium chloride (NaCl)

5.8g di-sodium hydrogen phosphate (Na_2HPO_4)

1g potassium di-hydrogen phosphate (KH_2PO_4)

1g potassium chloride (KCl)

Made up to 5 litres with distilled water

pH 7.0

Red cell lysis buffer

7.7g (0.144M) ammonium chloride (BDH, Poole, UK)

1ml 1M sodium hydrogen carbonate (Fisons, Loughborough, UK)

Made up to 1 litre with distilled water

RQ1 10x DNase reaction buffer (Promega)

400mM Tris-HCl, pH 8.0

100mM magnesium sulphate ($MgSO_4$)

10mM calcium chloride

RQ1 DNase stop solution (Promega)

20mM EGTA (pH 8.0)

0.1% saponin/PBS

2g saponin

Made up to 2 litres with PBS

pH 7.3

0.15M saline

8.77g sodium chloride

Made up to 1 litre in distilled water

10% SDS

10g SDS

Made up to 100ml with distilled water

SOC medium

20g tryptone

5g yeast extract

0.5g NaCl

Made up to 950ml with distilled water

10ml of 250mM KCl added (1.86g KCl in 100ml distilled water)

pH adjusted to 7.0 and volume increased to 980ml with distilled water

10ml of 1M $MgCl_2$ (20.33g $MgCl_2.6H_2O$ in 100ml distilled water)

10ml of 2M glucose (36g glucose in 100ml distilled water)

3M sodium acetate

24.6g Anhydrous sodium acetate

Made up to 100ml with distilled water

pH 6.0 (adjusted with glacial acetic acid)

10 x TBE buffer

14.8g EDTA

111.4 g Boric acid

217.8g Trizma base

Made up to 2 litres with distilled water.

Used at 1:10 diluted with distilled water (ie 1 x TBE buffer).

TE buffer

1ml IM Tris, pH 7.5

20µl 0.5M EDTA, pH 8.0

Made up to 100mls with distilled water

Tris (1M)

121.1 g Trizma base

Made up to 1 litre with distilled water

0.01M tri-sodium citrate buffer

4.4g tri-sodium citrate

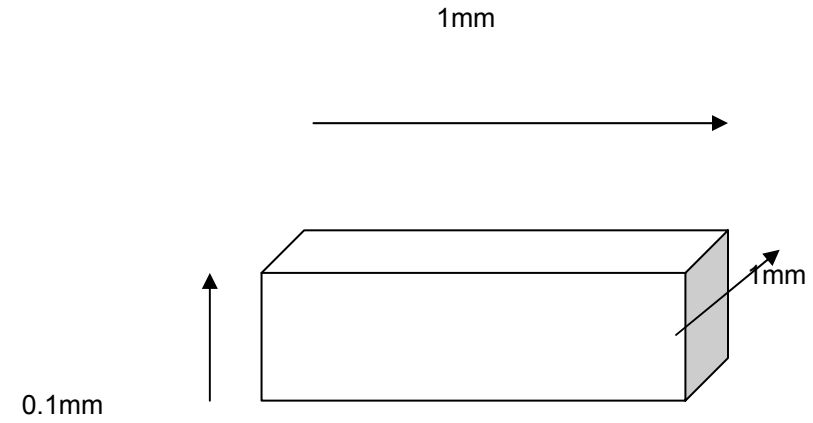
Made up to 1.5 litres with distilled water

pH 6.0

APPENDIX 7: MISCELLANEOUS

Using the Neubauer Haemocytometer

The volume of one of the central nine squares equates to $0.1mm^3$ (1mm x 1mm x 0.1mm depth) ($10^{-4}cm^3$), see Figure App7.1 below. 1ml has a volume of $1cm^3$. Therefore, the number of cells in one of the central nine squares (subdivided into 25 squares) x 10^4 will give the number of cells per ml.



N= the number of cells in $0.1mm^3$ (10^{-4} cm^3)

Total number of cells/ml = N x 10^4

Total number of cells in solution = N x 10^4 x volume of original solution (ml)

Figure App7.1: Principles of the Neubauer haemocytometer.