ACUTE LUNG INJURY

Vascular endothelial growth factor gene polymorphism and acute respiratory distress syndrome

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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 permogen 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℃ or <36℃; 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

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Table 1 Baseline characteristics of study groups

Group	No	Mean (SE) age (years)	Sex (F:M)
Normal	137	52 (1.7)	66:64
"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.[13]

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℃ for 5 minutes, 35 cycles of 95℃ for 1 minute, annealing at 61℃ for 1 minute and 72℃ for 1 minute were performed, followed by a final extension at 72℃ for 7 minutes. Equal volumes of amplicons from genomic DNA and IHG reagents were mixed, denatured at 95℃ for 5 minutes, and allowed to cool slowly using controlled ramping to 37℃ 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).

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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.[15] 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.

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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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The authors have no competing interests.

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LUNG ALERT

Prostanoid DP receptor polymorphisms may cause changes in susceptibility to asthma

▲ Oguma T, Palmer LJ, Birben E, *et al*. Role of prostanoid DP receptor variants in susceptibility to asthma. *N Engl J Med* 2004;**351**:1752–63

Prostaglandins have been implicated in the pathophysiology of asthma. The prostaglandin receptor is expressed on the surface of airway eosinophils and mast cells. This group has studied the gene for the prostanoid DP receptor (PTGDR) and its relationship with the asthma phenotype.

Screening revealed four new single nucleotide polymorphisms (SNPs) in addition to the two previously reported SNPs in the areas of the gene promoter region that bind transcription factors. The frequencies of the four most common SNPs were determined by restriction fragment length polymorphism (RFLP) analysis in patients with mild to moderate asthma and controls. Genotypic analysis showed an increased risk of asthma associated with the T-549C SNP (odds ratio (OR) >2.0; $p<0.05$) for white and black ethnic groups. The T allele of the C-441T SNP was significantly more common in white subjects with asthma (OR >1.8; $p \leqslant 0.02$); this association did not reach significance in the black population. Black patients carrying both the C-441T and T-549C alleles were more likely to have asthma (OR 24.36; $p = 0.01$). Patients who had at least one copy of the haplotype with low transcriptional efficiency were under-represented in the asthma group (OR 0.32 for blacks, 0.55 for whites; $p<0.05$). There was no association between any of the SNPs and total IgE.

Certain genetic variants that impair the expression of PTGDR seem to reduce susceptibility to asthma. This study supports the hypothesis that PTGDR is one of a number of genes that determine susceptibility to developing the asthma phenotype.

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