VEGF in idiopathic ILD

Simler et al raise an interesting possibility of the prognostic value of plasma VEGF in interstitial lung disease.1 Meyer et al in a previous study did not find any difference in serum VEGF levels in patients with diffuse parenchymal lung disease. It would have been interesting to know the broncho-alveolar lavage (BAL) fluid levels of VEGF in these patients as Meyer et al and Koyama et al have shown reduced BAL fluid VEGF levels in interstitial lung disease. This might simply reflect damage to the alveolar epithelium (a known major source) in this disease or, indeed, VEGF may have an important role in the pathogenesis of interstitial disease. Interestingly, VEGF receptor blockade has been shown to lead to an induction of apoptosis and an emphysema-like histological appearance in rats but with no evidence of fibrosis or inflammatory cells.4

In addition, it is interesting to speculate on the cellular source of the increased plasma levels of VEGF in the more fibrotic patients. Could this be an inflammatory cell source of systemic VEGF corresponding with an inflammatory response that is here associated with a poorer temic VEGF correlating with an inflammatory appearance in rats but with no evidence of fibrosis or inflammatory cells.4


Authors' reply

Dr Medford laudably highlights the interesting findings of other authors regarding levels of vascular endothelial growth factor (VEGF) in idiopathic interstitial pneumonia (IIP). Indeed, our data reflect the findings of Meyer et al who studied 11 patients with IIP.3 We extend their observations in a larger cohort of patients (n=49) and specifically relate plasma VEGF levels to disease progression and extent of fibrosis on HRCT scanning.1 Indeed, HRCT scanning is perhaps the most reliable surrogate for the extent of disease. Like Meyer et al, we observed reduced bronchoalveolar lavage (BAL) fluid levels of VEGF in patients with IIP (91 pg/ml) compared with controls (204 pg/ml). The reduction in the BAL fluid level of VEGF may reflect the absence of angiogenesis in that specific part of the lung, with the plasma VEGF level identifying a secondary phenomenon of compensatory angiogenesis in alternative areas of the lung. Alternatively, VEGF levels appear to be higher in epithelial surface fluid than in the serum, suggesting vectorial intraluminal secretion and the existence of a concentration gradient from air spaces to intravascular spaces.3

Thickett et al4 are correct in their quotation of the normal range for VEGF in plasma (36–76 pg/ml) as measured by the R&D Systems Quantikine ELISA kit. They point out that this range is quoted by the kit manufacturers and is consistent with their own data and, indeed, with other studies.5 We did not use the Quantikine kit in this study but stated clearly that: “The ELISA capture and detection antibodies for assaying IL-8 and VEGF were selected paired reagents optimised for ELISA performance from R&D Systems”. R&D currently sell these paired reagents under the name “DuoSet”. Different ELISA formats for VEGF quantitation using recombinant VEGF165 as standard are available. Capture reagent: (1) rabbit polyclonal anti-VEGF (in house); (2) solubleflt-1 (Sflt-1); (3) Quantikine kit, mouse anti-VEGF; (4) DuoSet, mouse anti-VEGF. Detection reagent: (1) mouse anti-VEGF (Genetech 4.6.1A); (2) rabbit anti-VEGF; (3) mouse anti-VEGF; (4) mouse anti-VEGF. Not surprisingly, each assay reports a different normal range. In our experience the Quantikine kit measures low (with up to a third of samples having undetectable levels) and the DuoSet combination measures high, as reported in the article (648 pg/ml). The other assays report intermediate values.

A number of possibilities exist as to why these assays read differently. It is unlikely to be due to platelet release as suggested. The greatest difference in VEGF levels that is detected between paired serum (complete platelet release of VEGF) and standard plasma samples (low platelet VEGF release) is at most only three to four fold. Similarly, the difference in VEGF between paired platelet poor and platelet rich plasma samples is of the same order of magnitude. To demonstrate this one has to spin plasma samples at 2700g for 15 min to prepare platelet poor samples. In our experience it is important to use plasma rather than serum samples to optimise VEGF antigen retrieval as each study sample similarly in terms of centrifugation—whether it be 300g for 12 min, 1000g for 15 min, or 2700g for 15 min. This will minimise variation in the study samples due to the “platelet release effect”. There are possible explanations for the VEGF immunoassays reading differently in plasma. It may be a combination of at least two effects—the nature of the epitope detected and the presence of other competing ligands in the sample. Different antibodies raised to VEGF will react variably with available epitopes on the ligand. This can be quite striking with monoclonal antibodies to different epitopes of a ligand when they are used as ELISA capture reagents—antibody A reacts with an epitope on VEGF that is close to or part of the flt-1 receptor binding site and the other antibody B reacts with an epitope well removed from this site, with an identical absolute amount of VEGF in the plasma sample, antibody A would read low or negative and antibody B would read high in relation to the amount of sFlt-1 present in the plasma sample. We have in fact shown that the capture antibody used in the Quantikine VEGF ELISA is, indeed, sensitive to the presence of sFlt-1.3

In addition to this potential variation in the level of free VEGF and VEGF-sFlt-1 complexes in plasma samples, a further confounding species is the amount of placenta growth factor (PLGF). VEGF is a natural homodimer but it does form heterodimers with PLGF and we have detected such complexes.6 Antibodies detecting epitopes that are variably modulated by the binding of PLGF to VEGF will read low or high depending on the PLGF concentration. This focuses on the nature of the R&D Systems monoclonal antibodies to VEGF—one in the Quantikine kit and the other part of the Duoset. Following the recognition of a difference in performance of these two assays, we contacted R&D Systems for information concerning the nature of these antibodies. We were interested to know if the same antibody or different antibodies were used and what information was available on their specificity. The response from R&D Systems was that the capture antibodies were different, so the scenario outlined above is a possible explanation.

It’s in no way to minimise the difficulties in interpreting absolute levels of VEGF in complex media such as plasma. To do this rigorously one ought to quantitate not only free VEGF but also VEGF complexes with sFlt-1, sKDR, and PLGF. To understand how these assays compare against each other and currently this is not possible. It would be simplistic to think that the Quantikine kit values are the “true” VEGF values and the DuoSet assay values artifactual. It might simply reflect the different antibodies. Quantikine values are free VEGF and the DuoSet values total VEGF (free VEGF plus VEGF complexes).
The important observation in our study is not the absolute VEGF plasma values but the relative differences in VEGF levels between patients and control samples over time, where the sampling issues have been fully appreciated and rigorously controlled to allow clinical interpretation of the results. We have emphasised the prognostic value of plasma VEGF in idiopathic pulmonary fibrosis and have shown a significant positive relationship between the HRCT fibrosis score and the plasma concentration of VEGF. A comparison with acute respiratory distress syndrome (ARDS) is not useful and perpetuates the concept that ARDS equates with chronic idiopathic pulmonary fibrosis, which is not the case.

In relation to the point about quantitating local VEGF concentrations in the lung where the influence of epithelial SL-1 might be great, it is to be expected that different assays could lead to a variation in reported VEGF levels for the reasons already discussed.

In conclusion, we have complete confidence in the validity and reproducibility of the VEGF data presented. In a situation of excess VEGF production which could potentially be driving an angiogenic fibrotic pathology in the lung, we suggest it is initially be driving an angiogenic fibrotic pathology in the lung, we suggest it is.

© 2005 British Thoracic Society. All rights reserved.

Raouf M. Kettaneh, Richard J. Blackman, Shoukhrat M. Mitalipov, and James W. Elde

Card 15 gene mutations in sarcoidosis

The last few years substantial progress has been made in unravelling the genetic basis of susceptibility to Crohn’s disease. Three CARD 15 (previously called NOB1) variants, resulting in proteins with modified carboxy terminal regions, have been implicated. 43% of patients with a sarcoid-like disease carry at least one of these CARD 15 mutations (compared with 1% of healthy controls). 1,2 Mutations in CARD 15 have also been identified in affected members of families with Blau syndrome.3,5 This is a rare autosomal dominant disorder, sometimes referred to as familial sarcoidosis, characterised by granuloma formation in joints, skin, and uvea. Card 15 is a microbial sensing protein involved in innate immunity. It recognises conserved structural components of microorganisms (bacterial muramyl dipeptide, MDP and peptidoglycan, PGN) and is part of the danger signal pattern recognition network which forms the front line of protective immunity. Mutations associated with Crohn’s disease render the molecule insensitive to MDP and interfere with the downstream activation of NF-kB. One potential result of this may be the persistence of a pathogen resulting in engagement of other arms of the adaptive immune system and formation of granulomas. Expression of CARD 15 in monocytes (precursors of macrophages and granulomas) further supports its role in granuloma formation. We hypothesised that mutations in CARD 15 may be a unifying defect in the multisystemic granulomatous diseases of Crohn’s disease, sarcoidosis, and Blau syndrome.

To investigate this we recruited a cohort of 29 patients with sarcoidosis from the Oxford Centre for Respiratory Medicine. All had a typical clinical picture of sarcoidosis and either histologically proven disease or characteristic Loéfgering granuloma (defined as an acute onset of disease with erythema nodosum, joint pains, and bilateral hilar lymphadenopathy). The diagnosis of sarcoidosis was also supported by the characteristic appearance of the lungs on a high resolution computed tomographic (CT) scan in all patients. The definition and diagnosis of sarcoidosis adheres to the statement on sarcoidosis adopted by the joint committees of WASOG/ATS/ERS.6 The patients were first diagnosed between the ages of 25 and 40 years and had been followed up for at least 1 year before recruitment. All were white and one third presented with Loéfgering’s syndrome. Written informed consent was obtained for genetic analysis and the study was approved by the local ethics committee.

The entire coding region of CARD 15 (11 exons and flanking intronic sequences) was screened for the presence of mutations. In brief, the CARD 15 gene was amplified from the genomic DNA samples by polymerase chain reaction (PCR) using primers as previously described7 and sequenced on an ABI 377 automated sequencer using a Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems, USA). Sequence data were then aligned using the Sequence Navigator analysis software Version 1.0.1 (Perkin Elmer Applied Biosystems) and compared with the known CARD 15 sequence (EMBL accession number AJ303140). 435 sequence analyses were performed in the 29 patients. We were therefore able to detect new alleles putatively associated with the sarcoidosis phenotype with frequencies as low as 2%. The three mutations associated with Crohn’s disease were specifically examined. The R702W mutation was observed in one patient with sarcoidosis while the G908R and the 1007fs mutations were not found. These results were not different from those reported previously in control populations where the mutations R702W, G908R and 1007fs were present in 4%, 1%, and 2% of 103 European healthy controls.8 Further, these mutations do not differ from data derived from healthy controls recruited in the UK.9,10 The codons 334 and 469 reported to be involved in Blau syndrome were also carefully scrutinised but we did not detect any genetic variation in our sarcoidosis patients. No additional mutations were seen within the rest of the coding region of the gene, suggesting that there were no specific alternative CARD 15 mutations associated with sarcoidosis. Schurmann and colleagues’ recently found no correlation between specific CARD 15 polymorphic alleles and patients with sarcoidosis from families with more than one member affected by the disease. This study provides evidence that CARD 15 is not associated with non-familial sarcoidosis (in patients with a white ethnic background) and that there are no mutations in any part of the coding region of the CARD 15 gene.

There is little doubt that there is a genetic predisposition in sarcoidosis, as indicated by the presence of familial clustering, ethnic susceptibility, and recent evidence of an association with HLA-DRB1.11 Further, in support of this susceptibility gene(s) has recurrently pointed to a locus near the HLA DR region on chromosome 6. This and the exclusion of the NOD2 locus has focused attention on abnormalities in antigen presentation and cytokine/chemokine receptors as a potential basis for the aetiology of sarcoidosis.

L.P. Ho, A. McMichael

MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford University, Oxford OX3 7DS, UK

L.P. Ho, R.J. Davies

Oxford Centre for Respiratory Medicine, Churchill Hospital, Oxford OX3 7LJ, UK

K. Gaber

Royal Devon and Exeter Hospital, Exeter EX2 5DW, UK

J-P. Hugot, F. Merlin

Fondation Jean Dausset/CEPH, Paris, France

J-P. Hugot

INSERM U458, Hopital Robert Debré, AP-HP, Paris, France
disclosed a white blood cell (WBC) count of 7.4×10^9/L (neutrophils 60.7%, lymphocytes 13.6%), and the level of carcinoembryonic antigen (CEA) was 3.35 ng/mL (reference value <3 ng/mL). Sputum acid-fast stain and mycobacterial cultures were all negative. Bronchoscopic examination showed no endobronchial abnormality. A chest radiograph taken at admission and a computed tomographic scan showed a patchy consolidation over the left basal lung. Magnetic resonance imaging (MRI) and angiography of the chest showed that an aberrant bronchial artery had arisen from the left side of the thoracic aorta and crossed the territory of the consolidation patch, but the venous drainage could not be visualised clearly (fig 1).

The patient underwent lobectomy of the left lower lobe and the patient’s clinical status improved after the diagnosis and surgical intervention. He has remained well during the follow up duration of 1.5 years after the operation. He has not received any anti- mycobacterial agents. No anti-mycobacterial regimen was administered after the diagnosis was made and the patient’s clinical status remained uneventful at the 3 month follow up.

Pulmonary sequestration is a congenital malformation characterised by the presence of lung tissue with abnormal or absent communication with the bronchial tree and arterial blood supply from the systemic circulation. Despite the high frequency of infection, few data are available regarding specific infecting organisms. Acute infections due to bacteria such as *Staphylococcus* and *Pseudomonas aeruginosa* have been described, and some chronic infections due to *Aspergillus* and *Nocardia* have also been described. Infected pulmonary sequestration due to mycobacteria including *M. tuberculosis* and *M. avium-intracellulare* have been reported only recently. In our case, *M. kansasi* was the first organism thought to be responsible for the infection of pulmonary sequestration in the absence of other pulmonary or extrapulmonary involvement. It is assumed that the organism reaches the sequestration through Kohn’s pore without causing evident disease in the normal lung parenchyma. In our case, *M. kansasi* was the first organism thought to be responsible for the infection of pulmonary sequestration in the absence of other pulmonary or extrapulmonary involvement. It is assumed that the organism reaches the sequestration through Kohn’s pore without causing evident disease in the normal lung parenchyma. In our case, *M. kansasi* was the first organism thought to be responsible for the infection of pulmonary sequestration in the absence of other pulmonary or extrapulmonary involvement. It is assumed that the organism reaches the sequestration through Kohn’s pore without causing evident disease in the normal lung parenchyma. In our case, *M. kansasi* was the first organism thought to be responsible for the infection of pulmonary sequestration in the absence of other pulmonary or extrapulmonary involvement. It is assumed that the organism reaches the sequestration through Kohn’s pore without causing evident disease in the normal lung parenchyma. In our case, *M. kansasi* was the first organism thought to be responsible for the infection of pulmonary sequestration in the absence of other pulmonary or extrapulmonary involvement. It is assumed that the organism reaches the sequestration through Kohn’s pore without causing evident disease in the normal lung parenchyma.
Burkholderia infection and survival in CF

We read with interest the paper by Jones et al. indicating the experience of the Manchester Adult CF Unit in the survival of patients with cystic fibrosis (CF) in the first 5 years following chronic infection with the B cepacia complex (Bcc). The authors appear to have shown that 31 patients with B cenocepacia had a worse prognosis than Pseudomonas aeruginosa infected patients. Despite the title of the paper, they had insufficient patients chronically infected with B multivorans to draw any similar conclusions. Although these are not new findings, we congratulate them on their attempt to throw light on a difficult topic within the microbiology of CF. However, there are several anomalies in the study that cause concern. Firstly, they state that the 5 year survival in the B cenocepacia group was 66.6%, yet the figure (which incidentally contains data for 7 years) clearly shows the rate to be only marginally 30%. Also, table 2 states that 19 (of 31) of these patients (61%) died within the study period, a value that fits with neither of the two previous statements. Secondly, although there were significantly more deaths in the B cenocepacia group, these patients apparently did not have increased treatment requirements or diminution in spirometric parameters compared with the matched group. This implies deterioration in respiratory function that was rapid enough to not affect the statistical calculation, but that falls short of the “cepacia” syndrome, which seems unlikely. We wonder whether a different statistical method may have produced more meaningful results. Finally, the authors state in the discussion that the only other studies of outcome of Bcc infection are in CF patients following transplantation. We are surprised that they appear to be unaware of the well conducted study by Ledson et al from our unit, published in this journal in 2002, which showed the outcome for 37 CF patients chronically infected with B cenocepacia, none of whom were listed for transplantation. This study uses a more robust method of statistical analysis to show that B cenocepacia infected patients had an accelerated loss of lung function with a fourfold increased risk of mortality and a trend towards worsening nutrition—results in keeping with those produced by the US CF Foundation.4

However, we do agree with the authors that further work needs to be done to assess the effect of infection by other genomovars (including B multivorans) on morbidity and mortality in patients with CF.

M J Ledson, M J Walshaw
Adult CF Unit, The Cardiacathoracic Centre, Liverpool L14 3PE, UK; MWalshaw@doctors.org.uk

References


Authors’ reply

We thank Drs Ledson and Walshaw for their interest in our recent paper, although we are not in agreement with some of their comments. They feel that there are anomalies for fig 1; they need to inspect it again more carefully. The survival for 1 year (12 months) and 5 years (60 months) is given in the table as 80% and 66%, as is represented in the figure. The timescale on the x axis in fig 1 is given in months. The data in the figure are not limited to 5 years as it contains survival data for patients throughout the entire study period. For some patients, data were available and are presented for over a decade rather than the 7 years suggested by Ledson and Walshaw.

As is clearly stated in the paper, the data for spirometry, body mass index and treatment requirements were, however, limited to 5 years from onset of infection. We collected annual spirometry figures from time of acquisition of infection. We did not demonstrate a significant difference in the decline in FEV1 or FVC between the two groups of patients infected with Burkholderia cenocepacia and Pseudomonas aeruginosa, respectively, but we are unable to exclude the possibility that there may have been a large fall in spirometric parameters in the last few months before death in patients infected with B cenocepacia. If the rate of decline in spirometric data is linear, the use of linear regression—as suggested by Drs Ledson and Walshaw—would not significantly alter the findings. We note that they reported a linear rate of decline in their previous study.5 We also observe that the study by Ledson et al did not match the patients for spirometry at baseline. The effect of the B cepacia complex (Bcc) on lung function and spirometry is complex and, as mentioned in our discussion, other studies have also shown a decreased survival among patients with Bcc without any demonstrable difference in lung function decline.6,7

Drs Ledson and Walshaw have looked at survival at their own centre. Although a relatively recent paper, they used the redundant term of B cepacia in their title rather than the Burkholderia cepacia complex which is the current nomenclature. It is accepted that infection with the ET12 B cenocepacia strain confers a clinical disadvantage, and many authors have reported their own experiences of a poor clinical outcome following infection over the past two decades. Our study was the first to compare survival between P aeruginosa infected patients and those with different Bcc genomovars (other than the papers discussed in our article that look at this after transplantation). It presents data to show that adults with cystic fibrosis (CF) infected with some Bcc genomovars have the same outcome as those infected with P aeruginosa. Our study has been supported by another recently published paper from the Belfast CF Centre.8

A M Jones, M E Dodd, A K Webb
Manchester Adult Cystic Fibrosis Centre, South Manchester University Hospitals NHS Trust, Wythenshawe Hospital, Manchester M23 9LT, UK

J R W Govan, V Barcus, C J Doherty
Medical Microbiology, University of Edinburgh, Edinburgh EH8 9AG, UK

J Morris
Department of Medical Statistics, South Manchester University Hospitals NHS Trust, Wythenshawe Hospital, Manchester M23 9LT, UK

Correspondence to: Dr A M Jones, Manchester Adult Cystic Fibrosis Centre, South Manchester University Hospitals NHS Trust, Wythenshawe Hospital, Southmoor Road, Manchester M23 9LT, UK; andmarkj@hotmail.com

References


CORRECTION

CARD 15 GENE MUTATIONS IN SARCOIDOSIS

The order of the authors of this letter which appeared on pages 354–355 of the April issue of Thorax was published incorrectly. The correct order is: L-P Ho, F Merlin, K Gaber, R J O Davies, A J McMichael, and J-P Hugot. The publishers apologise for the error.