Vascular endothelial growth factor increases vascular permeability in acute respiratory distress syndrome

Ralucă Solomon1, L. Azamfırei2, Simona Gurzu3, Ruxandra Copotoiu2, Sanda Copotoiu2, L. Jung4, J. Szederesi4, Judit Kovacs4

Objective: investigation of the VEGF expression in the lung tissue of ARDS patients and also the VEGFS plasmatic levels of these patients.

Material and method: there were examined immunohistochemical lung specimens from 10 ARDS patients which were compared to a control group. The VEGF serum levels was determined using ELISA method, at the same time with the tissue sampling from ARDS patients and during the hospital stay in case of the control group of non ARDS patients.

Results: patients who died because of ARDS had VEGF pulmonary expression significantly decreased comparing to non-ARDS patients (p<0.001). The serum VEGF levels of ARDS patients were raised comparing to non-ARDS patients (p<0.001).

Conclusions: A decreased alveolar type II cells, was noticed in ARDS evolution, therefore reducing the VEGF production in the alveolar space and also contributing to the decrease in lung perfusion, but also to the consecutive increase of VEGF plasmatic level.

Keywords: ARDS, Vascular endothelial growth factor

Acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), remains a devastating condition characterised by diffuse injury of the alveolo-capillary wall and alveolar and interstitial oedema consecutive to increased pulmonary vascular permeability. It concerns 1.5-8.3 cases in every 100 000 patients, still having a significant mortality of 30-50% despite improvements in the management of sepsis and lung protective ventilation strategy. There is a heterogeneous group of conditions, both direct or indirect insults, such as, sepsis, trauma, aspiration, massive blood transfusion or burns which predispose to ARDS. The pathogenetic basis of ARDS and factors governing susceptibility are incompletely understood. Markers of both epithelial and endothelial injury have been correlated with outcome, whereas the severity of ARDS depends significantly on the balance between alveolar epithelial and/or vascular endothelial injuries and their repair mechanisms.

Vascular endothelial cell growth factor (VEGF) was identified by its properties to increase permeability and acts as a cellular growth factor, hence its potential for a key role in the pathogenesis of ALI/ARDS. The VEGF family has several members, and each acts through specific receptors. The human VEGF gene family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF), all with multiple and diverse biological functions. The genes for VEGF family members also rely on alternative exon splicing to confer various isoforms for biological and functional specificity.

The most studied molecule of the VEGF family is VEGF-A. Its double effect, both permogen and mitogen induces vascular endothelial cell proliferation and promotes survival by induction of anti-apoptotic proteins bcl-2 and Al. VEGF increases microvascular permeability, but also it also exerts bioactivity outside the vascular endothelium: macrophages, type II pneumocytes, and monocytes for which it may be chemotactic. The biological activity of VEGF is dependent on interaction with specific receptors (VEGF-R1, 2, and 3), which are expressed not only by endothelial cells, but also by activated macrophages and alveolar type II epithelial cells. VEGF acts also on a family of coreceptors: the neuropilins.

The objective of this study was to investigate the VEGF expression in the lung tissue of ARDS patients and also the VEGF plasmatic levels of these patients compared to a control group.
MATERIAL AND METHOD
We realized a prospective study including 10 patients diagnosed with ARDS. These patients were evaluated daily during their stay in the ICU and those included in this study accomplished the ARDS diagnosis criteria as recommended by the international American-European consensus conference. The parameters needed to assess the SOFA score were determined daily and were referred to the score obtained on the first day of setting the ARDS diagnostic.

Lung specimens from ARDS patients were obtained by bronchoscopy or by autopsy in case of the deceased patients. The controls were 10 patients deceased from other causes than ARDS from whom necroptic pulmonary tissue samples were obtained and processed as for the ARDS patients.

All lung samples were standard stained using Haematoxylin Eosine and were examined in order to assess the histopathological diagnosis of ARDS: presence of hyaline membranes, type II pneumocyte proliferation associated with myofibroblast proliferation.

The determination of VEGF expression in the pulmonary tissue was performed using specific monoclonal antibodies VEGF, clones VG1 and JH 121 produced by LabVision firm. A charge-coupled video camera coupled with an optical microscope, was used to view the sections and to digitize images to a PC host computer using a program of assisted quantification. At least 10 fields per section were analysed and the results were given as the ratio (%) of stained surface area over the total field surface area.

The VEGF seric level was determined using the ELISA method, at the same time with the tissue sampling of the ARDS patients and during the hospital stay in case of the control group of non ARDS patients. Plasma probes were acquired for VEGF measurement using the Human VEGF ELISA kit with crossed reactivity for VEGF 165, VEGF 121 and PGDE (BioLife Group).

The statistical analysis of data was made using media and standard deviation through soft SPSS, version 17.0.

RESULTS
The ARDS etiology of the studied patients was mainly extrapulmonary (7 cases out of 10) (Figure 1) and the demographical and clinical data of the ARDS patients appear in Table I.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>52 (31-71)</td>
</tr>
<tr>
<td>Male/Female</td>
<td>6/4</td>
</tr>
<tr>
<td>PaO2/FiO2 (average, extremes)</td>
<td>128 (61-191)</td>
</tr>
<tr>
<td>Period of mechanical ventilation (average days, extremes)</td>
<td>11.37 (4-26)</td>
</tr>
<tr>
<td>ICU stay (average days, extremes)</td>
<td>18.37 (4-39)</td>
</tr>
<tr>
<td>SOFA score (the ARDS diagnostic day)</td>
<td>6 (4-9)</td>
</tr>
<tr>
<td>Mortality</td>
<td>40% (4/10)</td>
</tr>
</tbody>
</table>

The mortality of ARDS patients was of 40% (4 out of 10 patients). PaO2/ FiO2 ratio was lower than 200 for all the ARDS patients according to one of diagnostic criteria, but this ratio will be subsequent modified for the survivors (assessed in the day of extubation) comparing to the non-survivors (assessed in the day of death), with a mean of 282 for survivors versus 128 for the non-survivors (p<0.05) Figure 2.

The mean period of ICU stay was 18 days (with a maximum of 39 days) and the mean ventilation period was 11 days (with a maximum of 26 days). The mean SOFA score acquired in the first day of ICU stay was 6, with similar values for survivors and for the non-survivors.

Patients who died because of ARDS had a VEGF pulmonary expression significantly decreased compared to non-ARDS patients: 8.5 (4.1-9.9) versus 28.7 (9.5-48.6) (p<0.001) (Figure 3).
On the other hand, the seric VEGF levels of ARDS patients were raised (230 pg/ml) compared to non-ARDS patients (131 pg/ml) (p<0,001) (Figure 4).

Figure 4. Distribution of serum VEGF level in ARDS vs non ARDS patients

Alveolar macrophages were immunopositive in both groups.

No significant statistical differences were noted between the two groups with regard to age, gender, period of ARDS conditioned, number of ICU days.

DISCUSSIONS

In the normal lung tissue, VEGF is highly compartmentalised, with levels within the alveolar space 500 times over the plasma figures, despite VEGF production being closely associated with hypoxia response element. In vitro studies have demonstrated an abundance of VEGF especially in the alveolar epithelium (including the A549 cell line and primary human cultured type II pneumocytes) which suggests that the alveolar epithelium is the predominant source. The expression of VEGF in ARDS varies, depending of epithelial and endothelial damage. In the early stages of ARDS plasma VEGF levels rise and intrapulmonary levels fall, with normalisation of both during recovery. A possible explication of this variation is that the alveolar injury is the primary modulator of the alveolar level of VEGF in ARDS. This variation is probably the consequence of VEGF degradation by proteases released from infiltrated neutrophils and other inflammatory cells in the alveolar space.

VEGF release takes place in 3 phases paralleling the development of ARDS. The initial injury of the lung and the proinflammatory cytokines stimulate the production and the release of VEGF from type II pneumocytes, alveolar macrophages and marginal neutrophils.

Subsequently, the endothelial-alveolar barrier is exposed to high concentration of VEGF, which alters the adherence junction complexes (AIC) leading to microvascular injury and interstitial oedema. As the ARDS lesions develop, there is a distuction of type I and II alveolar epithelium, with a release of protease from neutrophils which leads to the decrease of VEGF concentration in the alveolar compartment. This pulmonary compartment deprivation of VEGF besides the release of VEGF from other organs increases the VEGF seric concentration, as ARDS represents only the pulmonary manifestation of a widespread endothelial injury in multiple organs. During the recovery period of ALI (when this appears!) type I and II alveolar epithelial cells begin to reappear, the VEGF production rises, which may participate in the angiogenesis, an important component of lung repair.

The over-expression of pulmonary VEGF, leads to increased vascular pulmonary permeability and consequently pulmonary oedema. Nevertheless, the VEGF expression in human alveolar epithelial cells also facilitates neovascularization, contributing to endothelial injury repair.

Low VEGF pulmonary levels were associated with the severity of ARDS, whereas high VEGF levels were associated with the recovery from ARDS, signalling the role of VEGF in the pulmonary injury repairing process.

CONCLUSIONS

A decreased alveolar type II cells, induced by apoptosis was noticed in ARDS evolution, therefore reducing the VEGF production in the alveolar space and also contributing to the decrease in lung perfusion, but also to the consecutive increase of VEGF plasmatic level.

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Acute respiratory distress syndrome (ARDS) is the most severe form of acute lung injury (ALI) that remains as a devastating condition. It is characterized by the diffuse injury of the alveolocapillary wall and alveolar and interstitial edema associated with increased pulmonary vascular permeability. The incidence is 1.5-8.3 cases in every 100,000 patients, and there is a significant mortality of 30% to 50% despite improvements in the management of sepsis and lung-protective ventilation strategy. Admission to intensive care units is usually planned for these patients at high risk of mortality and morbidity. Moreover, survival analyses have shown that the group with delayed extubation (ARDS patients usually have a prolonged ventilation) had an eight-fold higher mortality rate after 180 days compared with patients with early extubation. There is a heterogeneous...
group of conditions, both direct or indirect insults, such as sepsis, trauma, aspiration, massive blood transfusion or burns which predispose to ARDS. The pathogenic basis of ARDS and factors governing susceptibility are incompletely understood. Great attention has been recently paid on the potential of determining the possible genetic determinants of more susceptible endothelium, and the onset, severity and outcome of septic syndrome with ALI/ARDS.4

Markers of both epithelial and endothelial injury have been correlated with outcome, whereas the severity of ARDS depends significantly on the balance between alveolar epithelial and/or vascular endothelial injuries and their repair mechanisms.5, 6

Vascular endothelial cell growth factor (VEGF) increase endothelial permeability and acts as a cellular growth factor, hence its potential for a key role in the pathogenesis of ALI/ARDS. The VEGF family has several members, and each acts through specific receptors. The human VEGF gene family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF), all with multiple and diverse biological functions. The VEGF family members’ genes also rely on alternative exon splicing to confer various isoforms for biological and functional specificity.7, 8 The most studied molecule of the VEGF family is VEGF-A. Its dual effects as both perimogen and mitogen induce vascular endothelial cell proliferation and promote survival by induction of antiapoptotic proteins bcl-2 and A1.9, 10 VEGF increases microvascular permeability, but it also exerts bioactivity outside the vascular endothelium: macrophages, type II pneumocytes, and monocytes, for which it may be chemotactic.11 The biological activity of VEGF is dependent on its interaction with specific receptors (VEGF-R1, 2, and 3), which are expressed not only by endothelial cells, but also by activated macrophages and alveolar type II epithelial cells.12 VEGF acts also on a family of co-receptors, the neuropilins.13

The objective of this study was to assess the VEGF levels in lung tissue and plasma from acute respiratory distress syndrome (ARDS) patients compared with controls who died from non-ARDS causes.

Materials and methods

We evaluated prospectively 20 patients according to the international American-European consensus conference criteria for ARDS.14 The study has been approved by the ethics committee, and informed consent was obtained.

The SOFA score was obtained daily for all the patients and was compared with the score obtained on the first day of the ARDS diagnosis.

Plasma and tissue samples were prospectively collected from 20 patients with ARDS without any previous lung diseases before admittance in ICU, within 6 hours after intubation (plasma VEGF and tissue samples) and on the day of extubation (plasma VEGF) or postmortem (lung tissue). Lung specimens from ARDS patients were obtained by bronchoscopy or by autopsy in controls. The control group was comprised of 10 patients who died from causes other than ARDS. Pulmonary tissue samples were retrieved at autopsy and processed similarly to the samples from the ARDS patients.

All lung samples were stained in a standard manner using hematoxylin-eosin and were examined to assess the histopathological diagnosis of ARDS: presence of hyaline membranes and type II pneumocyte proliferation associated with myofibroblast proliferation.

The determination of VEGF expression in the pulmonary tissue was performed using specific monoclonal antibodies VEGF, clones VG1 and JH 121 produced by the LabVision company. A charge-coupled video camera connected to an optical microscope was used to view the sections and to digitize images on a PC host computer, using a program of assisted quantification. For angiogenesis quantification, we chose the most relevant regions, and we realized the digital photo captures at 400x high power fields. The pictures were taken with the optical microscope Nikon 800E that was coupled to a color video camera. The count was made using NIH’s Image software for image processing. We batch-measured the stained surface versus total tissue area ratio. We eliminated the ulcerated regions and also the regions rich in lymphocytes. At least 10 fields per section were analyzed, and the results were given as the ratio (%) of the stained surface area over the total field surface area.
The plasma VEGF level was determined using the ELISA method, at the same as the tissue sampling of the ARDS patients, and during the hospital stay in the case of the control group of non-ARDS patients. Plasma samples were acquired for VEGF measurement using the Human VEGF ELISA kit with crossed reactivity for VEGF 165 (BioLife Group), according to the manufacturer’s instructions. Biomarker levels were compared between survivors, non-survivors and control group.

Statistical analysis

The statistical analysis of the data was conducted using media and the standard deviation through soft GraphPadPrism, version 5. The results are presented as medians (interquartile range) in the text. The data are presented as box-plots indicating the median and the 10th, 25th, 75th and 90th percentiles. The differences between groups were estimated using the Kruskal-Wallis test with post hoc Mann-Whitney analysis. P-values <0.05 were considered statistically significant.

Results

The causes of ARDS are shown in Table I. The demographic and clinical data of the ARDS patients are shown in Table II.

The mortality of ARDS patients was 8 out of 20 patients.

The mean period of ICU stay was 18.3 days (with a maximum of 39.5 days), and the mean ventilation period was 11.4 days (with a maximum of 26.3 days). The mean SOFA score acquired on the first day of ICU stay was 6.4 with similar values for survivors and for the non-survivors.

The PaO2/FiO2 ratio was lower than 200 for all the ARDS patients according to one of the diagnostic criteria, but this ratio would be subsequently modified for the survivors (assessed in the day of extubation) compared with the non-survivors (assessed in the day of death), with a mean of 247.2 (167-342) for survivors versus 115.8 (67-156) the non-survivors (P<0.05)

Patients in the early phase of ARDS (day of intubation) had a VEGF pulmonary expression significantly decreased compared with non-ARDS patients: 8.5 (4.1-10.7) (Figure 1, column A) versus 26.7 (9.5-38.6) (P<0.05) (Figure 1, column C). The lower VEGF expression was observed mainly in patients who died of ARDS: 8.05 (5.4-11.2) (Figure 1, column B). The plasma VEGF
levels of ARDS patients were 234.4 pg/mL (140-323 pg/mL) (Figure 2, column A) in the early phase of ARDS (day of intubation) compared with non-ARDS patients: 141 pg/mL (76-209 pg/mL) (Figure 2, column B) (P<0.05). The level remained high until the day of death (mean: 262.8 pg/mL), (Figure 2, column C) but this level decreased at the day of extubation (mean: 138.5 pg/mL) (Figure 2, column D) for the patients with a favorable evolution, compared with the patients from the control group (P>0.05).

Alveolar macrophages were immunopositive in both groups.

No significant statistical differences were noted between the two groups regarding age, gender, period of ARDS condition, and number of ICU days.

**Discussion**

In the normal lung tissue, VEGF is highly compartmentalized, with levels within the alveolar space being 500 times more than those in plasma, even though VEGF production is closely associated with the hypoxia response element. In vitro studies have demonstrated an abundance of VEGF especially in the alveolar epithelium (including the A549 cell line and primary human cultured type II pneumocytes), which suggests that the alveolar epithelium is the predominant source. The expression of VEGF in ARDS varies, depending on epithelial and endothelial damage. Our results showed that in the early stages of ARDS, plasma VEGF levels rose and intrapulmonary levels fell, and during recovery, both levels showed normalization. A possible explanation for this variation is that the alveolar injury is the primary modulator of the alveolar level of VEGF in ARDS. This variation is probably the consequence of VEGF degradation by proteases released from the infiltrated neutrophils and other inflammatory cells in the alveolar space.

VEGF release takes place in 3 phases paralleling the development of ARDS. The initial injury of the lung and the pro-inflammatory cytokines stimulate the production and the release of VEGF from type II pneumocytes, alveolar macrophages and marginal neutrophils.

Subsequently, the endothelial-alveolar barrier is exposed to a high concentration of VEGF, which alters the adherence junction complexes (AJC) leading to microvascular injury and interstitial edema. As the ARDS lesions develop, there is a destruction of type I and II alveolar epithelium, with a release of proteases from neutrophils that leads to the decrease of VEGF concentration in the alveolar compartment. This pulmonary compartment deprivation of VEGF, independent from the release of VEGF from other organs, increases the VEGF plasma concentration, as ARDS represents only the pulmonary manifestation of a widespread endothelial injury in multiple organs.

During the early stage of ARDS, plasma crosses the capillary and alveolar walls, subsequently flooding the alveoli. VEGF is present in higher concentrations than normal in plasma. It is a proven fact that in early stages of ARDS, the level of VEGF decreases.

VEGF signaling is required for the maintenance of adult lung alveolar structures, and the withdrawal of VEGF leads to endothelial cell apoptosis. The increased septal cell death in the lungs is associated with reduced lung expression of VEGF and VEGFR-2. This is consistent with diminished lung perfusion, suggesting a protective mechanism for endothelial cell survival. This was proven by some authors in experimental models who observed that endothelial cell survival was increased through increases in oxidative stress, alveolar enlargement and alveolar cell apoptosis following the blocking of VEGF. In neonatal and preterm mice with respiratory distress syndrome generated by the hypoxia inducible factor (HIF) knockout, the administration of VEGF increased surfactant biosynthesis and improved survival rates.
Likewise, the over-expression of pulmonary IL-13 protected mice from hyperoxic lung injury through a VEGF-dependent pathway, and adenoviral-delivered VEGF has been reported to increase survival from hyperoxic pulmonary hypertension. VEGF protects endothelial cells from apoptotic cell death through the coordinate signaling of phosphatidyl 3-kinase/Akt and inhibition of p38 MAPK. Furthermore, insufficient maintenance of VEGF levels has been reported to inhibit capillary formation in neonatal mice and to lead to regression of newly formed vessels in the retina, heart, and liver.

In our study, the lung tissue expression of VEGF in ARDS patients was low on the day of intubation as well after death, and these results point to the potential importance of this factor. The presence of VEGF has a pro-survival and anti-apoptotic role in endothelial cells. The failure of pulmonary endothelial cell survival induced by VEGF receptor blockade results in lung alveolar septal cell apoptosis and may contribute to the genesis of vascular injury. A vicious circle might be established, because cells undergoing apoptosis display increased oxidative stress, which further contributes to the apoptosis. Our result supports the idea that the protective effect of VEGF depends on the existing reserves of VEGF on lung tissue.

At the same time, the decrease in VEGF may protect lungs from alveolar flooding. In a previous study using an adenovirus-mediated gene transfer, Kaner et al. has shown that VEGF over-expression induces pulmonary edema. Because VEGF increases the permeability, the decreased VEGF production does not exclude its involvement in the early increase of endothelial permeability that may occur concomitantly with extravascular neutrophil migration at the onset of ARDS.

Potential explanations for the reduction in intrapulmonary VEGF levels in early ARDS are manifold and not mutually exclusive. They include increased membrane-bound rather than soluble isoforms, changes in isoform expression and damage to the alveolar-capillary membrane with consequent leakage of intrapulmonary VEGF into the vascular bed. The intrapulmonary VEGF levels are known to be lower also in non-survivors with ARDS.

In patients in the early ARDS stage, intubation induces the secretion of hypoxic inducing factors like HIF-1-α or HIF-2-α, and NO. They all contribute to the release of VEGF and to the genesis of endothelial cells as a consequence. The role of hypoxia in releasing VEGF and angiogenesis initiation was observed in tumors, especially in breast and colorectal carcinomas in which the anti-angiogenic treatment was approved by the US Food and Drug Administration (FDA) in 2004 and 2008, respectively. So, after intubation (including the day of death), the level of VEGF could be slowly increased compared with the level before intubation, although it was still not enough for a protective effect. However, VEGF tissue expression is lower compared with the normal lung.

The ARDS patients who passed on died of MSOF induced by the initial morbidity, thus, they did not live enough to reach the late ARDS stage. During the recovery period of ALI (when it does appear), type I and II alveolar epithelial cells begin to reappear, and the VEGF production rises, which may lead to VEGF stimulating angiogenesis, an important component of lung repair.

The over-expression of pulmonary VEGF leads to increased vascular pulmonary permeability and subsequent pulmonary edema. Nevertheless, the VEGF expression in human alveolar epithelial cells also facilitates neovascularization, contributing to endothelial injury repair.

The three receptors of VEGF-A (VEGF-R1, -R2 and -R3) are present in different types of cells, such as tumor cells, endothelial cells, activated macrophages or alveolar type II cells. In normal lung tissue, VEGF-R1 and -R2 are expressed by alveolar cells, VEGF-R2 mediates endothelial survival in relationship with KDR (Kinase insert domain-containing receptor) and in this way, VEGF plays an important role in the regulation of alveolar-capillary permeability. Although the mechanism remains unclear, in vitro studies have shown that VEGF can increase the rate of alveolar cell proliferation after acidic injury and at the same time, can stimulate surfactant production from alveolocytes.

In our study, the low VEGF pulmonary levels were associated with the severity of ARDS, whereas high VEGF levels were associated with the recovery from ARDS, signaling the role of VEGF in the pulmonary injury repairing process.
Elevated plasma VEGF levels in ARDS probably reflect multiple cellular sources. VEGF has been demonstrated to induce fenestrations in previously non-fenestrated capillaries. These actions are dependent on intracellular third messenger production and the release of nitric oxide. Elevated VEGF levels in ARDS plasma would, therefore, be expected to contribute to abnormal capillary permeability, which is a characteristic feature of ARDS. The elevated VEGF levels in hypotensive patients with ARDS would also support a role for tissue hypoxia in determining plasma levels.

This study has demonstrated that VEGF levels are significantly greater in non-survivors with ARDS than non-ARDS. Furthermore, changes in VEGF appear to be associated with either a good outcome if VEGF levels fall, or a bad outcome if VEGF levels rise. These findings, therefore, need further confirmation before they can be considered useful as a clinical marker of poor outcome in patients with ARDS.

The soluble form of VEGF-R1 is present in the bronchial lavage fluid, and it could be an antagonist for the soluble form of VEGF. Therefore, VEGF165 decreases in plasma in the early stages of ARDS, and there are concurrent VEGF121 and VEGF-R1 increases in bronchial fluid and bronchial epithelium. It is possible that one of these forms of VEGF can stimulate VEGF-R2 to promote endothelial activation.

Endothelial activation refers to a specific change in endothelial phenotype, characterized most notably by an increase in endothelial-leukocyte interactions and permeability, which is pivotal to inflammatory responses in both physiologic and pathologic settings. VEGF has been well characterized as an endothelial survival factor, and it prevents microvascular apoptotic cell loss. VEGF survival signals in endothelial cells (ECs) are mediated by VEGFR-2 through the phosphatidylinositol 3-kinase/Akt signal transduction pathway. A constitutively active Akt is sufficient to promote survival of serum-starved endothelial cells in transient transfection experiments. In contrast, the over-expression of a dominant-negative form of Akt blocks the survival effect of VEGF. These findings identify the Flk-1/KDR receptor and the PI3-kinase/Akt signal transduction pathway as important elements in the processes leading to endothelial activation and cell survival induced by VEGF.

VEGF also promotes the expression of anti-apoptotic proteins Bel-2 and A1 in vascular ECs and can specifically block extrinsic signal-induced apoptosis of ECs, mediated by TNF receptors and Fas. Pro-survival signaling by VEGF may, therefore, alter the threshold level of ECs susceptibility to intrinsic and extrinsic inducers of apoptosis.

On the other hand, VEGF recruits lactosylceramide (a member of the glycosphingolipid family, which has been implicated in the inflammatory process and in the vascular lesion formation) to induce endothelial cellular adhesion molecules (CAMs) expression. CAMs play an essential role in tethering circulating leukocytes to the vascular endothelium at sites of inflammation. They are also instrumental in enabling leukocytes to transmigrate from blood vessels into adjacent inflamed tissues. In the absence of signals to stimulate expression of CAMs, the adherent forces between leukocytes and the vascular endothelium are below the threshold level required to tether leukocytes. Several cytokines, including tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), potentiate increase the expression of many CAMs and, thus, increase the adhesiveness between leukocytes and the endothelium. The CAM-inducing activity of these cytokines is crucial to the regulation of the inflammatory processes.

Conclusions

The initial phase of ARDS is associated with a decrease in VEGF in the lung and with an increase in the plasma, suggesting that VEGF in the alveolar space may reflect the development of, and the recovery from, ARDS in a manner opposite to that in plasma. This down-regulation may represent a protective mechanism aimed at limiting endothelial permeability and may participate at the decrease in capillary number that is observed during early ARDS. Persistent elevation of plasma VEGF over time predicts a poor outcome, and increased production of VEGF may contribute to the resolution of inflammation in the lung.

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Vascular endothelial growth factor and related molecules in acute lung injury

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1Thoracic Surgery Research Laboratories, Toronto General Research Institute, University Health Network, Toronto M5G 2C4; Departments of 2Critical Care Medicine and 3Cardiology and Division of Molecular and Cell Biology Research, St. Michael’s Hospital, Toronto M5B 1W8; and 4Institute of Medical Science, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Mura, Marco, Claudia C. dos Santos, Duncan Stewart, and Mingyao Liu. Vascular endothelial growth factor and related molecules in acute lung injury. J Appl Physiol 97: 1605–1617, 2004. doi:10.1152/japplphysiol.00202.2004.—VEGFs and their receptors have been implicated in the regulation of vascular permeability in many organ systems, including the lung. Increased permeability and interstitial and pulmonary edema are prominent features of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Extrapolating data from other organ systems and animal experiments have suggested that overexpression of VEGF functions primarily as proinjurious molecules in the lung. Recent data, from animal models as well as from patients with ARDS, have shown decreased levels of VEGF in the lung. The role of VEGF and related molecules in ALI/ARDS is, therefore, controversial: what has become clear is that there are many unique features in the regulation of pulmonary vascular permeability and in VEGF expression in the lung. In this review, we explore a growing body of literature looking at the expression and function of VEGF and related molecules in different models of ALI and in patients with ALI/ARDS. Novel evidence points to a potential role of VEGF in promoting repair of the alveolar-capillary membrane during recovery from ALI/ARDS. Understanding the role of VEGF in this disease process is crucial for developing new therapeutic strategies for ALI/ARDS.

Acute respiratory distress syndrome; pulmonary edema; angiopoietins; hypoxia; hyperoxia

ACUTE LUNG INJURY (ALI) AND ITS MOST SEVERE MANIFESTATION, acute respiratory distress syndrome (ARDS), are clinically defined as severe dysfunction of gas exchange and chest radiographic abnormalities following a predisposing injury, in the absence of heart failure (14). ARDS and ALI may occur following various inciting events, including serious illness, such as sepsis, trauma, or organ transplantation. Overwhelming intrapulmonary inflammation, endothelial and epithelial injury, and consequent reparative responses are key components of the evolving ALI and progression to ARDS (14, 91).

The hallmarks of ALI are increased capillary permeability, interstitial and alveolar edema, influx of circulating inflammatory cells, and formation of hyaline membranes. Increased permeability leads to pulmonary edema, a life-threatening condition resulting from an imbalance between forces driving fluid into the air spaces and biological mechanisms for its removal. The severity and outcome of ALI depend on the balance between alveolar epithelial and/or vascular endothelial injuries and their repair mechanisms. The importance of endothelial injury and increased vascular permeability to the formation of pulmonary edema in this disorder is well established (14, 91).

VEGF plays an important role in several organs by directly regulating vascular permeability to water and proteins. For example, in the brain, VEGF is responsible for hypoxia-induced vascular leakage and edema formation; inhibition of VEGF activity by a neutralizing antibody can block the hypoxia-induced increase in vascular permeability (117). The role of VEGF in the control of pulmonary permeability is, however, controversial. Systemic expression of VEGF has been shown to cause widespread multiorgan capillary leakage in an animal model, suggesting that the overexpression of VEGF plays a pivotal role in the development of pulmonary edema (67). However, recent animal studies and clinical data support a protective role for VEGF in ALI and ARDS patients (30, 133). Understanding the relationship between VEGF and pulmonary permeability in ALI/ARDS may lead to the development of novel therapeutic interventions for this syndrome.

In the lung, the regulation of pulmonary permeability and the expression of VEGF and VEGF-related molecules have many unique features. Consequently, it is not appropriate to simply extrapolate knowledge from other organ systems and apply them to the lung. Therefore, we have undertaken a systematic review of the literature, focusing on features pertaining to VEGF regulation and function in the lung and in particular its potential role in the pathophysiology of ALI/ARDS. In addition to reviewing the current state of knowledge, the objective of this paper is to further discuss controversial observations related to the role of VEGF, its related factors,
and their receptors in ALI/ARDS, offering alternative perspectives on this intricate system.

**BIOLOGY OF VEGF AND RELATED MOLECULES**

The VEGF family has several members, and each acts through specific receptors. The biology of VEGFs has recently been the focus of many excellent reviews (9, 10, 43). Interactions between VEGFs and angiopoietins (Ang) is very important in the regulation of angiogenesis and vascular permeability (83). For the purpose of this review, we will focus on the role of VEGF and related molecules that have been implicated to play in the lung. The characteristics and properties of VEGFs and Ang and the potential interplay between VEGF and related molecules in the lung are summarized in Table 1 and illustrated in Fig. 1.

**VEGFs**

The human VEGF gene family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF), all with multiple and diverse biological functions (43). The genes for VEGF family members also rely on alternative exon splicing to confer various isoforms for biological and functional specificity (109, 113).

The most studied molecule of the VEGF family is VEGF-A. The gene encoding human VEGF-A is organized into eight exons. Multiple protein isoforms are generated through alternative splicing of the pre-mRNA (136). Human VEGF-A isoforms include 121, 145, 165, 183, 189, and 206 amino acids (VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206, respectively). VEGF121 is a soluble isofrom, whereas VEGF189, VEGF206, and 60–70% of VEGF165 are found in association with cells or sequestered in the extracellular matrix (43). Most VEGF-A isoforms contain a heparin-binding domain. VEGF189 and VEGF206 bind to heparin with high affinity (60). The absence of heparin-binding domain in VEGF121 results in a loss of mitogenic activity (73), as demonstrated by the observation that transgenic mice expressing exclusively VEGF120 (mouse VEGF is one amino acid shorter) die shortly after delivery, due to severe angiogenic defects (22). VEGF145 and VEGF183 are less frequent splicing variants (109, 113). Due to its bioavailability and biological potency, VEGF165 is the predominant isofrom of VEGF-A, and from hereon the abbreviation VEGF refers to VEGF165, except if otherwise specified.

The major site for VEGF-B expression is the heart (103). VEGF-B forms heterodimers with VEGF and may, therefore, modulate its signaling (103). A study on VEGF-B knockout mice suggested that VEGF-B may have a role in the development of chronic hypoxic pulmonary hypertension in mice by contributing to pulmonary vascular remodeling (142).

VEGF-C and VEGF-D induce growth of the lymphatic vasculature in vivo (63). They can also induce capillary endothelial cell (EC) migration and proliferation in culture (19, 95) and act as vascular permeability factors at higher concentrations (68, 115). VEGF-D shares 61% identity with VEGF-C (111). VEGF-C and VEGF-D mRNA are most abundant in the heart, lung, skeletal muscle, colon, and small intestine (95, 104). Furthermore, VEGF-C is highly expressed by activated macrophages (124).

**Table 1. Characteristics and properties of VEGF and related molecules in the lung and their potential role in acute lung injury.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sources</th>
<th>Receptor</th>
<th>Stimulating Factor</th>
<th>Potential Functions</th>
</tr>
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<tbody>
<tr>
<td>VEGF-A</td>
<td>Alveolar type II cells</td>
<td>VEGFR-1</td>
<td>Hypoxia</td>
<td>EC proliferation</td>
</tr>
<tr>
<td></td>
<td>Airway epithelial cells</td>
<td>VEGFR-2</td>
<td>Mechanical stretch</td>
<td>↑ Vascular permeability</td>
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<td></td>
<td>Mesenchymal cells</td>
<td>NRP-1</td>
<td>ROS</td>
<td>Angiogenesis</td>
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<tr>
<td></td>
<td>Macrophages</td>
<td></td>
<td>Glucose deprivation</td>
<td>Vasculogenesis</td>
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<td></td>
<td>Neutrophils</td>
<td></td>
<td>TGF-β1</td>
<td>Antiapototic for ECs</td>
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<td>IL-6</td>
<td>Migration of monocytes</td>
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<td>IFN-γ</td>
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<td>IL-13</td>
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<td></td>
<td>Endothelin</td>
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<tr>
<td>VEGF-B</td>
<td>(Heart)</td>
<td>VEGFR-1</td>
<td></td>
<td>Pulmonary vascular remodeling</td>
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<td></td>
<td></td>
<td>NRP-1</td>
<td></td>
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<tr>
<td>VEGF-C/D</td>
<td>Lung</td>
<td>VEGFR-3</td>
<td>Proinflammatory cytokines</td>
<td>Lymphangiogenesis</td>
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<td></td>
<td>Macrophages</td>
<td>VEGFR-2</td>
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<td>VEGF-E</td>
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<tr>
<td>PIGF</td>
<td>Alveolar type II cells</td>
<td>VEGFR-1</td>
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<td>NRP-1</td>
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<tr>
<td>Ang-1</td>
<td>Airway epithelial cells</td>
<td>Tie-2</td>
<td>Hypoxia</td>
<td></td>
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<tr>
<td></td>
<td>Interstitial cells</td>
<td></td>
<td>VEGF-A</td>
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<td>ECs (at sites of active vascular remodeling)</td>
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<td>Ang-2</td>
<td>Airway epithelial cells</td>
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<td></td>
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<td>Ang-3/4</td>
<td>Airway epithelial cells</td>
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<td></td>
<td>Interstitial cells</td>
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</tbody>
</table>

PIGF, placenta growth factor; Ang, angiopoietin; EC, endothelial cells; NRP, neuropilin; ROS, reactive oxygen species; TGF, transform growth factor; HIF, hypoxia-induced factor; ↑, increase; ↓, decrease.
Decreased VEGF concentration in lung tissue and vascular injury during ARDS

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ABSTRACT: Endothelial injury is an important prognostic factor in acute respiratory distress syndrome (ARDS). Decreased production of vascular endothelial growth factor (VEGF) in ARDS may favour vascular lesions, since VEGF promotes endothelial survival by inhibiting apoptosis.

This study sought to document low VEGF levels in lung tissue from ARDS patients, to determine whether the cause was injury to alveolar type II cells (the main pulmonary source of VEGF) and to evaluate the vascular consequences. Lung specimens were obtained by open biopsy or autopsy from 29 patients with severe ARDS (two survivors) and five controls.

As compared with controls, homogenates of lung tissue from ARDS patients contained less VEGF (median (interquartile range) ARDS 8.2 (4.7–12.2) versus controls 28.4 (9.9–47.1) ng g⁻¹ protein). Increased immunostaining with surfactant protein B was seen in ARDS lungs. Extensive cellular apoptosis (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling staining), including endothelial and alveolar type II cells, was demonstrated, and vascular bed density (CD31 immunostaining) decreased in ARDS lungs as compared with controls. VEGF levels were negatively correlated to apoptotic endothelial cell counts.

In conclusion, decreased vascular endothelial growth factor levels in lung tissue may participate in the decrease in lung perfusion in acute respiratory distress syndrome.

KEYWORDS: Alveolar type II cells, endothelial apoptosis, lung tissue

The detailed morphological description of acute respiratory distress syndrome (ARDS) by BACHOFEN and WEIBEL [1] includes a decrease in lung capillary density. Conceivably, the resulting decrease in lung perfusion may lead to an increase in alveolar dead space, which has been recently shown to be a major prognostic factor during ARDS [2]. The pathophysiology of vascular lesions in ARDS is a challenging issue that has received little attention [3]. Recently, HAMACHER et al. [4] have demonstrated that bronchoalveolar lavage fluids of ARDS patients exhibit an ex vivo pro-apoptotic activity against pulmonary microvascular endothelial cells [4]. Conversely, a decrease in survival factors (anti-apoptotic) of the endothelium may be an alternative hypothesis. Low levels of vascular endothelial growth factor (VEGF) have been found in the lungs of patients with early ARDS [5, 6]. This VEGF decrease may contribute to the genesis of vascular injury, inasmuch as VEGF has been demonstrated to be a major survival factor for endothelium [7]. VEGF (or VEGF-A) is a highly conserved, dimeric, heparin-binding glycoprotein (molecular weight 46 kDa). At least four different VEGF transcripts resulting from alternate splicing of a single gene have been identified in human cells. VEGF121 and VEGF165 are secreted in a soluble form, whereas VEGF189 and VEGF206 remain cell-surface associated or are primarily deposited in the extracellular matrix. VEGF seems to specifically affect endothelial cell growth, survival and permeability. In the lung, VEGF is expressed primarily by epithelial cells and macrophages. VEGF is also produced by and stored in both human platelets and polymorphonuclear neutrophils. The biological activity of VEGF is dependent on interaction with specific receptors (VEGF-R1, 2, and 3), which are expressed not only by endothelial cells, but also by activated macrophages and alveolar type II epithelial cells. Endothelial survival is mediated via VEGF-R2/kinase insert domain-containing receptor (KDR) [8]. The aim of this study was two-fold: to determine whether the decrease in lung VEGF is related to epithelial injury, and whether it may be associated with increased endothelial apoptosis and decreased capillary density.
Kit (Qbiogen Inc., Carlsbad, CA, USA), according to the manufacturer’s instructions. Nuclei of apoptotic cells appeared brown and granular. To quantify the extent of microvascular endothelial apoptosis, co-staining with an anti-human mouse monoclonal antibody to CD31 (WM-59; PharMingen, San Diego, CA, USA) was used, which recognises the endothelial surface marker platelet/endothelial cell adhesion molecule 1. TUNEL sections were incubated with 2% normal goat serum (in PBS with 0.05% Tween 20), and, subsequently, with the primary anti-CD31 antibody (1:100,000 dilution, in PBS with 1% BSA) overnight at 4°C. Mouse nonimmune IgG1 (Sigma) was used as the negative control. Biotinylated goat anti-mouse antibodies (Sigma) were applied for 30 min at room temperature, followed by avidin-biotin peroxidase (Sigma) complexes for 30 min (room temperature). True Blue Peroxidase substrate was used as the final chromogen (KPL Laboratories, Gaithersburg, MD, USA). No counterstaining was used, to avoid possible interference with the specific dark-blue immunostaining of the endothelial cell surface. Double staining was considered positive when specific cells displayed a brown nucleus with a surrounding blue-to-black membrane-immuneactive pattern. Positive cells were counted in 10 randomly selected areas (final magnification 400 × ).

**Statistical analysis**

Results are presented as medians (interquartile range) in the text. Data are presented as box-plots indicating the median and the 10th, 25th, 75th and 90th percentiles. Differences between groups were estimated using the Kruskal-Wallis test with post hoc Mann-Whitney analysis. To assess the possible influence of VEGF on pulmonary microcirculation, the relationships linking VEGF levels to endothelial quantification and apoptosis were assessed by Spearman’s rank correlation. p-Values ≤0.05 were considered statistically significant.

**RESULTS**

**Patient characteristics**

A total of 29 ARDS patients were studied (20 males and nine females; median age 64 yrs (50–73)). Their median acute physiology and chronic health evaluation II score at admission was 16.9 (13.6–23.6). Open lung biopsy was performed as a diagnostic procedure in 15 patients and immediately after death in 14 patients.

At the time of lung tissue sampling, the arterial oxygen tension/inspiratory oxygen fraction ratio was <200 mmHg in 22 patients and 200–300 mmHg in seven patients. The cause of ARDS was direct lung injury in 17 patients (related to infection in 11 and to inhalation in six) and indirect lung injury in 12 patients (nonpulmonary sepsis in seven, nonseptic shock in four, and pancreatitis in one). The six patients who died within 5 days after ARDS onset were classified as having acute ARDS, whereas the remaining 23 patients were classified as having late ARDS; histological findings confirmed the classification in every case. Survival was only 7% (two out of 29) in this selected population.

The control group was composed of five patients (all males; median age 65 years (54–70)).

**Vascular endothelial growth factor expression in lungs from acute respiratory distress syndrome and control patients**

VEGF levels in the lung homogenates were lower in the ARDS patients than in the controls: 8.2 (4.7–12.2) versus 28.4 (9.9–47.1) ng·g⁻¹ protein, respectively (p=0.03). There was no difference between the early and late ARDS subgroups (fig. 1). Results were similar when VEGF concentrations were normalised per gram of lung weight. In the controls, immunostaining for VEGF (fig. 2) labelled the bronchiolar cells and alveolar macrophages and strongly labelled some alveolar type II epithelial cells. Alveolar type I epithelial cells were negative. In the ARDS patients, staining was highly heterogeneous. Bronchiolar and alveolar epithelial cells were negative in some areas and positive in others, even within a tissue section. In early ARDS tissue samples, inflammatory cells were also positive.

**Vascular endothelial growth factor receptor 2 expression in lungs from acute respiratory distress syndrome and control patients**

There was no significant difference between VEGF-R2 concentrations measured by ELISA in lung homogenates from ARDS and control patients (116 (78–157) versus 156 (131–191) ng·g⁻¹ protein). No difference was found between early and late ARDS patients.

Immunostaining for VEGF-R2 was noted in endothelial cells in the control group; however, not all endothelial cells were
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

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.

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). 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 per agonist 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.

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. These levels in normal alveoli are significant, twice the concentration in healthy human subjects VEGF protein levels are normally 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. It is more common than is perhaps appreciated with an estimated incidence of 75 per 100 000 in some studies. 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. ARDS continues to have a significant mortality of more than 35% despite recent improvements in ventilator strategies and in sepsis management. 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. Although the underlying mechanisms and factors governing susceptibility remain unclear, ARDS is characterised by alveolar epithelial injury and increased vascular permeability. Markers of both epithelial and endothelial injury have been correlated with outcome.

An additional factor is the potential to induce damage by mechanical ventilation itself. Survival from ARDS requires resolution of these features and renewed integrity of the alveolar capillary membrane.

BIOLGY 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. 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. 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. It both induces vascular endothelial cell proliferation and promotes survival by induction of anti-apoptotic proteins bcl-2 and A1. VEGF increases microvascular permeability 20 000 times more potently than histamine. Targets for VEGF bioactivity outside the vascular endothelium include macrophages, type II pneumocytes, and monocytes for which it may be chemotactic. It also has a vasodilatory function.

VEGF isotypes

Alternate splicing of the VEGF gene (6p21.3) transcript leads to the generation of several splice variants (isotypes) of differing sizes, the subscript relating to the number of amino acids present (VEGF121), VEGF145, VEGF164, VEGF165, VEGF183, VEGF189 and VEGF206). VEGF165 is the predominant isotype and most biologically active in the physiological state. The longer isotypes are cell associated (exons 6 and 7 have heparin binding activity allowing binding to the extracellular matrix) compared with the shorter diffusible isotypes. Plasmin, the acute phase protein, can also cleave the isotypes to form PL-VEGF and a recently identified isoform, VEGF165. In one study a CT polymorphism have been reported.

VEGF polymorphism

Several functional human VEGF polymorphisms have been described. Significant interindividual variations in plasma VEGF levels and gene expression related to the presence of polymorphism have been reported. 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. No such changes in plasma levels were detected in a separate genetic association study, but this may have been due to different racial populations. 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. 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. Indeed, the highest levels of VEGF mRNA are found in animal and human lung, which suggests that the alveolar epithelium is the predominant source. Although the embryonic role of VEGF is undoubtedly important, in all species studied to date adult lungs contain higher amounts of VEGF mRNA transcript than the developing lung. Changes in relative isofoms have also been observed with maturity, suggesting an ongoing role. VEGF-R1, NRP-1, and NRP-2 are all expressed in normal lung. Primary human type II 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.

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
PERIOPERATIVE TISSUE OXYGENATION (STO2) IS RELATED TO LONG TERM SURGICAL OUTCOMES
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INTRODUCTION. There is increasing evidence to suggest perioperative complications are predictive of long term survival and that reducing them may improve survival rates. Goal directed therapy has been shown to reduce mortality and morbidity perioperatively, with those unable to increase oxygen delivery peroperatively having demonstrably worse outcomes. The advent of non invasive tissue oxygenation monitors using near infrared spectroscopy has allowed further study of oxygen flux during goal directed therapy.
OBJECTIVES. To observe changes in tissue oxygenation during an 8 h oxygen delivery targeted post surgical optimisation program and provide long term mortality followup of a surgical cohort of high risk patients.
METHODS. 40 patients undergoing high risk surgery and postoperative optimisation (targeting of oxygen delivery index of >600 mmHg/minute/m²) on the intensive care unit at a London teaching hospital were enrolled. Each patient underwent a protocolised haemodynamic optimisation protocol as per our standard unit policy for 8 h with consecutive recordings of tissue oxygenation at the thenar eminence using an inspectra 325 monitor. Additional variables relating to global and tissue perfusion were measured concurrently. Patients were followed up for survival status at 3.5 years using routinely available information held within our hospital records.
RESULTS. In hospital mortality was 17.5% (N = 7), whilst at 3.5 years this had increased to 50% (N = 20). There was no significant difference between APII scores 11 (4) versus 9.5 (4), Age 64.5 ± 16.23 versus 65.8 ± 4.35 or operation type for survivors and non-survivors at 3.5 years respectively. Significant differences between groups were found however for admission and mid optimisation protocol (4 h) HR and STO2 (see Table 1), but not for DO2, Lactate, or Base excess. results are shown as mean and sd or median and IQR where not normally distributed.

STO2 DIFFERENCES FOR SURVIVORS AND NON-SURVIVORS
Optimisation time point Occur all Survivors Non-Survivors Significance (Mann-Whitney)
Admission 72.5 (29) 79.5 (26) 66.5 (29) p = 0.009
4 h 69 (24) 75.5 (15) 58.5 (20) p = 0.007
End of optimisation 79 (13) 79.5 (10) 77.5 (25) p = 0.009

There were no significant differences in measured variables for 30 day mortality.
CONCLUSIONS. There appears to be a statistical and clinical difference in HR and tissue oxygenation between the long term survivors of high risk surgery who underwent monitored postoperative goal directed optimisation.

REFERENCES.

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Research Grant no 136/IDEI/National Authority of Scientific Research, Romania.

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SIGNIFICANCE OF VEGF RECEPTORS IN THE ACUTE RESPIRATORY DISTRESS SYNDROME
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INTRODUCTION. Vascular endothelial growth factor (VEGF) is produced by many types of cells, including the alveolar ones. Its activity depends on the interaction with kinase receptors VEGF-R1 and -R2. Many studies showed that in normal lung tissue both VEGF and its receptors were immunohistochemically expressed by the alveolocytes, macrophages, epithelial and endothelial cells. Their exact role in pathogenesis of Acute Respiratory Distress Syndrome (ARDS) is not yet known.

OBJECTIVE. To investigate the expression of VEGF-A, VEGF-R1 and -R2 in the lung of ARDS patients.

METHODS. Lung specimens were obtained from bronchoscopic biopsy from living ARDS patients in the early phase or by open biopsy in the proliferative phase during ARDS evolution in 20 patients. Patients with fibrosis were excluded. For the immunohistochemical technique, we used the following antibodies, provided by LabVision: VEGF—clone VGI and polyclonal antibodies Fi-l/VEGFRI and Fi-l/CD34/VEGFR2. A charged-couple video camera connected to an optical microscope, was used to view the sections and to digitize images on a PC host computer, using a program of assisted quantification.

RESULTS. For the specimens obtained from living ARDS patients, the early ARDS phase was characterized by both VEGF and VEGFR1-endothelial and alveolar expression: 9.5 (5.3–11.8) and 26.7 (9.5–38.6). Moving along the time frame, VEGF expression decreased in the lung tissue: 6.05 (4.4–9.21) (< 0.05), while alveolocytes were still marked by VEGFR1, but to a lesser extent: 23.5 (15.7–36.2) (p < 0.05). For the specimens obtained from autopsy, in the proliferative phase the hyaline membranes strongly expressed VEGF, being negative for VEGFR1. However, the alveolocytes in lung areas without hyaline membranes were positive for VEGFR1 but the number of alveolar cells positive for VEGFR1 was smaller. VEGF-R2 marked only bronchial epithelium in both early and proliferative phase with no expression on alveolocytes or hyaline membranes.

CONCLUSIONS. The expression of VEGF-A and VEGF-R1-R2 is significantly increased in the acute phase of ARDS, but decreases in the chronic phase. The expression of VEGFR1 is enhanced in the alveolocytes but not in the hyaline membranes. Further researches are needed to find better models and to determine if suitable for clinical use.

REFERENCES.

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EVALUATION OF NONBRONCHOSCOPIC LAVAGE BY AIRWAY EXCHANGE CATHETER AS A DIAGNOSTIC INSTRUMENT FOR MONITORING CHANGES IN THE ALEVOLAR MILIEU
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INTRODUCTION. Bronchoscopic bronchoalveolar lavage (B-BAL) is today the gold standard for sampling of inflammatory markers in the distal airways. Nonbronchoscopic bronchoalveolar lavage (N-BAL) by ordinary suction catheter has been investigated as a more easily accessible method for alveolar sampling in the setting of acute respiratory distress syndrome (ARDS). The results, however, were disappointing, probably due to more proximal sampling by the N-BAL.

OBJECTIVES. To investigate wether N-BAL, by a catheter with physical properties similar to those of the bronchoscope is comparable to B-BAL.

METHODS. B-BAL and N-BAL by Cook’s airway exchange catheter was performed with 3 x 30 ml normal saline on opposite sides 15 min apart at nine different occasions on 5 anesthetized and intubated pigs. The volume of the recovered lavage was noted, after which the fluid was analyzed for albumin, total cell count, viability and differential cell count. Statistical analysis was performed using Wilcoxon's rank-sum test.

RESULTS. N-BAL yielded significantly higher albumin content than B-BAL (20.1 ± 8.7 vs. 11.7 ± 3.4 mg/L, p = 0.027). In all other measurements there were no significant differences between N-BAL and B-BAL (recovered volume 52.1 ± 19.2 vs. 67.9 ± 4.6 mL, total cell count 40.1 ± 28.4 vs. 40.7 ± 17.3 x 10⁶ cells, viability 49.6 ± 20.4 vs. 62.6 ± 13.1% and percent macrophages 70.5 ± 15.5 vs. 77.1 ± 6.6%.

CONCLUSIONS. N-BAL by airway exchange catheter does not differ significantly in recovered volume and cell count when compared to B-BAL. N-BAL for alveolar sampling deserves further investigation.

REFERENCES.
Inhibition of VEGF receptors causes lung cell apoptosis and emphysema

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Pulmonary emphysema, a significant global health problem, is characterized by a loss of alveolar structures. Because VEGF is a trophic factor required for the survival of endothelial cells and is abundantly expressed in the lung, we hypothesized that chronic blockade of VEGF receptors could induce alveolar cell apoptosis and emphysema. Chronic treatment of rats with the VEGF receptor blocker SU5416 led to enlargement of the air spaces, indicative of emphysema. The VEGF receptor inhibitor SU5416 induced alveolar septal cell apoptosis but did not inhibit lung cell proliferation. Viewed by angiography, SU5416-treated rat lungs showed a pruning of the pulmonary arterial tree, although we observed no lung infiltration by inflammatory cells or fibrosis. SU5416 treatment led to a decrease in lung expression of VEGF receptor 2 (VEGFR-2), phosphorylated VEGFR-2, and Akt-1 in the complex with VEGFR-2. Treatment with the caspase inhibitor Z-Asp-CH₂-DCB prevented SU5416-induced septal cell apoptosis and emphysema development. These findings suggest that VEGF receptor signaling is required for maintenance of the alveolar structures and, further, that alveolar septal cell apoptosis contributes to the pathogenesis of emphysema.


Introduction

Emphysema is estimated to affect 1.65 million people in the US (1). Emphysema is defined as abnormal permanent enlargement of the airspaces distal to terminal bronchioles. The pathogenesis of pulmonary emphysema, which is characterized by the disappearance of alveolar septa, remains enigmatic, although the protease-antiprotease imbalance hypothesis of inflammation is widely supported (2). Briefly, the concept is that activated alveolar macrophages release elastase, which destroys the lung tissue, overwhelming local antiprotease activities (3). However, because many cigarette smokers and patients with severe inflammatory lung parenchyma diseases (like pneumonia and adult respiratory distress syndrome) do not develop significant emphysema, this hypothesis may not fully explain the loss of lung tissue in cigarette smoking-induced emphysema.

Preceding the discovery of the association between panacinar emphysema and a hereditary deficiency of α1-antitrypsin (4), A.A. Liebow (5), based on histological examination of emphysema lungs, pointed out that the alveolar septa in centrilobular emphysema appear to be remarkably thin and almost avascular. He also considered that a reduction in the blood supply of the small precapillary blood vessels might induce the disappearance of alveolar septa. We revisit this early vascular hypothesis of emphysema development and propose that the disappearance of lung tissue in emphysema may involve the progressive loss of capillary endothelial and epithelial cells through the process of programmed cell death, apoptosis (6). One known survival factor for endothelial cells is VEGF, initially characterized as a factor that increases endothelial permeability (7) and induces endothelial cell growth (8). It is not only essential for the normal development of blood vessels in the embryo (9, 10), but it is also required for survival of endothelial cells. Withdrawal of VEGF leads to endothelial cell apoptosis in vitro (11, 12) and in vivo (13). VEGF binds to two tyrosine kinase receptors present on endothelial cells: VEGF receptor 1 (VEGFR-1; Flt-1) and VEGFR-2 (KDR/Flk-1). Although VEGF is highly abundant in the lung, its normal biological activity in the lung is not well understood. We postulate that VEGF signaling may be required for the maintenance of adult lung alveolar structures. We have recently reported increased septal cell death in human emphysematous
lungs, which was associated with reduced lung expression of VEGF and VEGFR-2 [KDR/FK-1] (9). Here we report that chronic treatment of rats with the VEGF receptor blocker SU5416 causes alveolar cell apoptosis-dependent emphysema.

Methods

Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center. Male Sprague-Dawley rats were purchased from a commercial vendor and kept for 7 days in the Animal Care Facility of the University of Colorado Health Sciences Center. In the first set of experiments, rats then were divided into three groups: (a) control group (n = 6), (b) SU5416 [3,4-dimethylpyrrol-5-yl)methylenyl]-indolin 2-one] group (n = 6), and (c) SU5416+caspase inhibitor group (n = 6). SU5416 (provided by SUGEN Inc.) and SU5416+caspase inhibitor groups were injected subcutaneously three times per week for 3 weeks. SU5416 (20 mg/kg) was suspended in diluent (0.5% carboxymethylcellulose sodium, 0.9% sodium chloride, 0.4% polysorbate 80, 0.9% benzyl alcohol in deionized water). The control group received only the diluent.

I. EXPERIMENTAL PROCEDURES

Pharmacological experiments. In the first set of experiments, rats then were divided into three groups: (a) control group (n = 6), (b) SU5416 [3,4-dimethylpyrrol-5-yl)methylenyl]-indolin 2-one] group (n = 6), and (c) SU5416+caspase inhibitor group (n = 6). SU5416 (provided by SUGEN Inc.) and SU5416+caspase inhibitor groups were injected subcutaneously three times per week for 3 weeks. SU5416 (20 mg/kg) was suspended in diluent (0.5% carboxymethylcellulose sodium, 0.9% sodium chloride, 0.4% polysorbate 80, 0.9% benzyl alcohol in deionized water). The control group received only the diluent. SU5416+caspase inhibitor group was injected intraperitoneally with 1 mg of Z-Asp-CH2-DCB (Alexis Corp., San Diego, California, USA), dissolved in 50 μl of PBS solution containing 20% DMSO, every day for 3 weeks. Control and SU5416-alone groups were injected only with 50 μl of PBS solution containing 20% DMSO. To study the time course of the effect of SU5416, rats (n = 6) were treated for 3, 7, and 21 days.

Tissue processing. After completion of the treatment period, the chest was opened and the cardiac and pulmonary block was quickly isolated and excised, and the ratio of right ventricular weight/left ventricle plus septum weight was obtained. The right main bronchus was cross-clamped and the left lung was filled with 0.5% low melting agarose in 10% formalin at a constant pressure of 25 cm H2O, allowing for homogenous expansion of lung parenchyma (14). After that, the lungs were fixed in 10% formalin for 48 hours. The paraffin-embedded tissues were sectioned and prepared for histological analysis. Lung sections were taken from the same lung regions in both the treated and control groups, representative of upper and lower lobes of the left lung.

Morphological assessment. After fixation, 5-μm sections were stained with hematoxylin and eosin. The mean linear intercept, as a measure of interalveolar wall distance, was determined by light microscopy at a total magnification of ×100. The mean linear intercept was obtained by dividing the total length of a line drawn across the lung section by the total number of intercepts encountered in 72 lines per each rat lung, as described by Thurlbeck (15).

Angiograms. Arteriograms were performed in lungs that were infused with barium sulfate (16). Immediately after sacrifice, PBS was infused through a main pulmonary artery catheter to flush the pulmonary circulation free of blood. A barium sulfate-gelatin mixture was heated to 65°C, and infused into the main pulmonary artery at a perfusion pressure of 74 mmHg. This pressure was maintained for at least 5 minutes to ensure penetration of the barium mixture. Lung tissue was subsequently fixed for angiograms. The quantitation of the peripheral pulmonary arteries filled with barium was performed as described recently (17).

Immunohistochemistry. Anti-caspase 3 polyclonal antibody CM1 (1:250 dilution; kindly provided by Anu Srinivasan, Idun Pharmaceuticals, La Jolla, California, USA) (18), proliferating cell nuclear antigen (PCNA; 1:50 dilution; sc-56 from Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), and anti-macrophage antibody (1:50 dilution for immunohistochemistry; Chemicon International, Temecula, California, USA) were used. Immunolocalization was performed on paraffin-embedded, formalin-fixed rat lungs. Briefly, after paraffin removal in xylene, the sections were rehydrated and submitted to microwave treatment (800 W/15 min) in 10 mM citric acid monohydrate solution. After quenching of endogenous peroxidase with 3% H2O2 for 20 minutes, the sections were exposed to CM1 polyclonal antibody, which recognizes active caspase-3, or to PCNA monoclonal antibody for 30 minutes. After incubation with the primary antibody, immunodetection was performed using biotinylated anti-rabbit or anti-mouse IgG. Peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, California, USA), with diaminobenzidine (DAB) as the substrate, completed the immunostaining. Negative controls for nonspecific binding included normal rabbit or mouse serum.

TUNEL. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was performed with TACS 2 TdT DAB kit (Trevengen, Gaithersburg, Maryland, USA), following the manufacturer’s instructions. Briefly, after deparaffinization and rehydration, sections were digested with proteinase K at a concentration of 20 μg/ml for 15 minutes. Endogenous peroxidase activity was quenched with 2% H2O2 for 5 minutes. The sections were immersed in terminal deoxynucleotidyl transferase (TdT) buffer. TdT, 1 mM Mn2+, and biotinylated dNTP in TdT buffer were then added to cover the sections and incubated in a humid atmosphere at 37°C for 60 minutes. The slides were washed with PBS and incubated with streptavidin–horseradish peroxidase for 10 minutes. After rinsing with PBS, the slides were immersed in DAB solution. The slides were counterstained for 3 minutes with 1% methyl green.

In situ ligation of labeled DNA fragments. A 200-bp double-stranded DNA fragment was prepared by polymerase chain reaction with Taq polymerase using primers 5′-CAGTCAAGAAGATTTTGCATG-3′ and 5′-GTC-CCACACCCCTTTAGGAAG-3′ complementary to human adenocarcinoma DNA. Deparaffinized 5-μm sections were subjected to Taq polymerase–based DNA in situ ligation assay using the DNA fragments labeled with...
RELATIONSHIP BETWEEN LUNG TISSUE EXPRESSION AND PLASMATIC LEVEL OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN PATIENTS WITH ACUTE RESPIRATORY DISTRESS SYNDROME

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Abstract

PURPOSE: To investigate the vascular endothelial growth factor (VEGF) expression in the lung tissue of acute respiratory distress syndrome (ARDS) patients and also the VEGF plasmatic levels of these patients.

METHODS: We realized a prospective study including 10 patients diagnosed with ARDS. Lung specimens from ARDS patients were obtained by bronchoscopy or by autopsy in case of the deceased patients. The controls were 10 patients deceased from other causes than ARDS from whom necroptic pulmonary tissue samples. The determination of VEGF expression in the pulmonary tissue was performed using specific monoclonal antibodies VEGF, clones VG1 and JH 121. The controls were 10 patients deceased from other causes than ARDS from whom necroptic pulmonary tissue samples.

RESULTS: The ARDS etiology of the studied patients was mainly extrapulmonary (7 cases out of 10). Patients who died because of ARDS had a VEGF pulmonary expression significantly decreased compared to non-ARDS patients: 8.5 (4.1–9.9) versus 28.7 (9.5–48.6) (p < 0.001). (Fig. 1). The seric VEGF levels of ARDS patients were raised (230 pg/ml) compared to non-ARDS patients (131 pg/ml) (p < 0.001). Alveolar macrophages were immunopositive in both groups. No significant statistical differences were noted between the two groups with regard to age, gender, period of ARDS condition, number of ICU days.

CONCLUSION: A decreased alveolar type II cells, induced by apoptosis was noticed in ARDS evolution, therefore reducing the VEGF production in the alveolar space and also contributing to the decrease in lung perfusion, but also to the consecutive increase of VEGF plasmatic level.

CLINICAL IMPLICATIONS: The over-expression of pulmonary VEGF, leads to increased vascular pulmonary permeability and consequently pulmonary oedema. Nevertheless, the VEGF expression in human alveolar epithelial cells also facilitates neovascularization, contributing to endothelial injury repair. Low VEGF pulmonary levels were associated with the severity of ARDS, whereas high VEGF levels were associated with the recovery from ARDS, signalling the role of VEGF in the pulmonary injury repairing process.

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