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Vascular Endothelial Growth Factor Bioactivity and Its Receptors in Patients with Acute Respiratory Distress Syndrome

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Background: Pathogenesis of acute respiratory distress syndrome (ARDS) is a controversial issue. Few studies have analyzed the possible role of vascular endothelial growth factor (VEGF) and its receptors in this lesion. **Methods:** We compared the immunohistochemical expression of VEGF, its receptors (VEGFR1, VEGFR2) and CD68, in normal lungs and lungs with ARDS. Fifty necropsy cases and 12 lung biopsies with ARDS were analyzed. In total, eight cases were in the early stage and 54 cases were in late stage of ARDS. In addition, the serum level of VEGF165 was also determined. **Results:** In normal lungs, all antibodies marked the endothelial cells (EC) and pneumocytes (PC), except for CD68, which was expressed in the alveolar macrophages. In early ARDS, the intensity of VEGF165 and VEGFR2 decreased in both EC and PC. VEGF121 was absent in PC but its expression increased in bronchial epithelium. VEGFR1 was expressed in the integral PC. In late ARDS, VEGF165 down-regulation was more significant in PC and EC but its intensity increased in hyaline membranes (HM). In some cases, HM were CD68 positive. The serum level of VEGF165 was up-regulated, while VEGF165 intensity in PC decreased and the HM appeared in alveolar spaces. **Conclusions:** Sporadic positivity of HM for CD68 and decreasing of VEGF165 expression in EC proved that VEGF165 is produced by PC, destroyed macrophages, and extravasated serum.

Key Words: Respiratory distress syndrome, adult; Vascular endothelial growth factor; Pneumocytes; Hyaline membranes

Despite modern treatment methods and clinical studies performed on a high number of patients, the prognosis of acute respiratory distress syndrome (ARDS) still remains unfavorable, with significant mortality rate. In few recent studies, possible pathogenic and prognostic roles of vascular endothelial growth factor (VEGF) and its receptors, VEGF receptor 1 (VEGFR)1 and VEGFR2, were investigated,[1-4] but the results were controversial. Some studies suggested that VEGF is damaging with respect to ARDS outcome, while other experimental studies showed its protective role for alveolar integrity.[5]

VEGF has a great importance in processes as vasculogenesis, angiogenesis, and lung development. In order to keep its functions, the human lung needs to preserve both its epithelial and endothelial layers.[6] Previous studies observed that a low alveolar intensity of VEGF was associated in a human fetus with distress syndrome and increased level of plasmatic VEGF.[7] An *in vitro* study proved that VEGF prevented destruction and apoptosis of pneumocytes and also stimulated proliferation after mechanical or chemical injury.[8] However, another study revealed that blocking VEGF in supernatant with an anti-VEGF antibody, did not influence mechanical stretch-induced apoptosis of pneumocytes and was not essential for their survival.[5]

In normal lung tissue, VEGF is produced mainly by alveolar type II cells (pneumocytes). In acute lung lesions, hypoxia-induced factors (HIF) like HIF-1α and HIF-1β are activated and VEGF expression is increased.[9] Some studies showed that the

activation of macrophages, neutrophils, and other inflammatory cytokines stimulated the VEGF production and its activity,[7,10] and anti-inflammatory treatment could reduce the serum level of VEGF and protected the mice infected with malaria from ARDS.[11] On the other hand, vascular damage in lung with ARDS could be independent of tissue hypoxia and depend on mechanical forces.[3,12]

VEGF-A is the best studied member of the human VEGF gene family, which includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor.[13] The isoforms of VEGF-A, generated through the alternative splicing of pre-mRNA, are different in the number of amino acids and functions, and include VEGF121, VEGF145, VEGF165, VEGF183, VEGF-189, and VEGF206.[9,14] VEGF145 and VEGF183 have less importance in VEGF bioactivity. VEGF189 and VEGF206 remain associated with the cell surface.[15] VEGF121 and VEGF-165 are secreted in the soluble form but 60-70% of VEGF165 could be sequestered in extracellular matrix.[16] VEGF165 is the most predominant and studied isoform of VEGF-A, has a heparin-binding domain and acts as a mitogen for endothelial cells.[3] VEGF121 is the soluble isoform without the heparin-binding domain, in either mitotic activity, in which its role in ARDS has not yet been revealed.[17] There are also antiangiogenic isoforms like VEGF165b which may have a role in repair after a lung injury.

VEGF activity is mediated by two receptors, VEGFR1 and VEGFR2. In normal lung tissue, both of them are expressed in endothelial cells, pneumocytes, and activated macrophages.[18] Although VEGFR1 has a greater affinity for VEGF, it seems that the main receptor for the bioactivity of VEGF on endothelial cells is VEGFR2, and is more efficiently phosphorylated upon ligand binding and leading to mitogenesis, chemotaxis and cell morphology change.[19] However, for vasculogenesis, in R2 deficient mice, the development of endothelial islands or organized blood vessels was not possible.[20] However, in R1, knock-out mice which died of an excessive proliferation of angioblasts without organization in blood vessels, was observed.[21] This means that VEGFR2 can determine cell proliferation in the absence of VEGFR1, but VEGFR1 has an apoptotic function with an important role in vascular organization.[4] For a normal vasculogenesis process VEGFR1 and VEGFR2 are both indispensible.

The exact roles of VEGFRs in ARDS are still under debate. In the most recent study, VEGFRs were proven to be expressed in both alveolar-capillary membranes of normal and ARDS human lungs, with a significant up-regulation of macrophages, endothelial cells and alveolar epithelium in late ARDS.[1] The authors' explanation was that an increased expression of VEGFRs facilitated an increased number of VEGF binding sites.

In order to determine whether VEGF is protective or damaging in ARDS, we performed a complex clinical and immunohistochemical study. We correlated the immunohistochemical expression of VEGF165, VEGF121 and its receptors in normal lungs and lungs with ARDS, at a plasmatic level of VEGF165, predominant isoform of VEGF-A, and examined patients outcome. These correlations were studied in the early and late ARDS, in survivors and non-survivors.

MATERIALS AND METHODS

Our study included 10 cases of normal lung tissue and 62 cases of ARDS (50 necropsy cases and 12 biopsies obtained by bronchoscopy from patients with clinical ARDS). Transbronchial lung biopsies were chosen when a significant size of the lung parenchyma was obtained. The necropsy cases were used to confirm the results.

ARDS was defined according to the American-European Consensus Conference,[22] which classified the ARDS in two phases: early ARDS (within 3 days from the clinical onset), and late ARDS (after 3 days from the clinical onset).

The 10 normal lung tissues were obtained by autopsy (n = 5) or by open lung biopsy, which were performed in order to remove lung tumors or metastases (n = 5). The patients included 4 females and 6 males, with a median age 61 years.

Plasma samples were acquired for VEGF measurement using the human VEGF enzyme-linked immunosorbent assay kit with cross-reactivity for VEGF165 (BioLife Group, Sarasota, FL, USA) and according to the manufacturer's instructions. The ethical committee of our university granted their approval for these techniques.

All lung tissues were paraffin-embedded and were stained with hematoxylin-eosin. For the immunohistochemical study,

Table 1. Antibodies used for the immunohistochemical study

Antibody	Clone	Dilution	Source
VEGF-A	VG1	1:50	D-line, LabVision, Fremont, CA, USA
VEGF-A	JH121	1:50	D-line, LabVision
VEGFR1 (Flt-1)	Polyclonal	1:25	D-line, LabVision
VEGFR2 (Flk-1, KDR)	Polyclonal	1:50	D-line, LabVision
CD68	KP1	1:75	Dako, Glostrup, Denmark

VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; KDR, kinase-insert domain-containing receptor.

we used the antibodies mentioned in Table 1 and the EnVision system by LabVision (D-Line, Fremont, CA, USA). Heat antigen retrieval was performed in a citrate solution, pH 6 (VEGFR1, VEGFR2, and CD68) or in ethylenediaminetetraacetic acid, pH 9 (both clones of VEGF). The development was performed with diaminodihydrochlorid benzidine solution, which was applied for 3-5 minutes. The nuclei were counterstained with Mayer's hematoxylin.

We chose two clones of anti-VEGF antibody because one (JH-121) was reactive for VEGF121 isoform and the other (VG1) was reactive to both isoforms, VEGF121 and VEGF165. We considered the first clone being anti-VEGF121 and second anti-VEGF165. The goal of this challenge was to observe the differences between expression and functions of the two soluble isoforms of VEGF-A in ARDS.

The intensity of anti-VEGF121, VEGF165 and its receptors was scored in the cytoplasmic area according to the following criteria: score 0, no staining; score 1+, weak diffuse cytoplasmic staining in < 10% of cells; score 2+, moderate cytoplasmic staining in 10-70% of cells; score 3+, strong cytoplasmic staining in > 70% of cells. This type of semi-quantification was independently determined by two pathologists.

We compared the immunohistochemical expression of anti-VEGF and its receptors in normal lung tissue as well as in early and late ARDS (within 48 hours and 3 days after diagnosis). We determined the correlation among patient condition, survival rate, number of hospitalization days, immunohistochemical expression, and serum level of VEGF165.

We should mention that we identify endothelial cells based on the histological structure of the capillaries. In the cases with difficulty in differentiation between endothelial cells and hyperplastic pneumocytes, the CD34 was used as a marker for endothelial cells. The CD68 was used as a marker for macrophages.

For the statistical analysis, we used GraphPAD in the Stat ver. 3 software (GraphPad Software, San Diego, CA, USA). We used the two-tails unpaired t-test, Chi-squared test, and the contingency tables. A p-value less than 0.05 with 95% confidence interval was considered statistically significant.

RESULTS

Patients' characteristics

A total of 62 ARDS patients included 38 males and 24 females with ages ranging from 24 to 86 years (median age, 63 years). The average interval between the diagnosis of ARDS and patient death was 11 days, regardless of patient age or sex. Causes of ARDS were direct lung injury in 28 cases and indirect lung injury in 34 patients. The direct lung injury refers to chest trauma (n = 15) and bronchopneumonia (n = 13), while the latter included following causes: cardiogenic shock (n = 10), sepsis (n = 8), liver dysfunctions (n = 5), massive transfusion (n = 7), and acute pancreatitis (n = 4).

Out of 12 patients who underwent biopsies, 4 were in the early stage of ARDS (within 3 days), whereas the other 8 were in the late phase (after 3 days). Only 3 of the 12 patients survived, all being in late phase of ARDS (11, 15, and 13 days). Only 4 out of the 50 dead patients were in early stage of ARDS, with the other 46 being in the late phase.

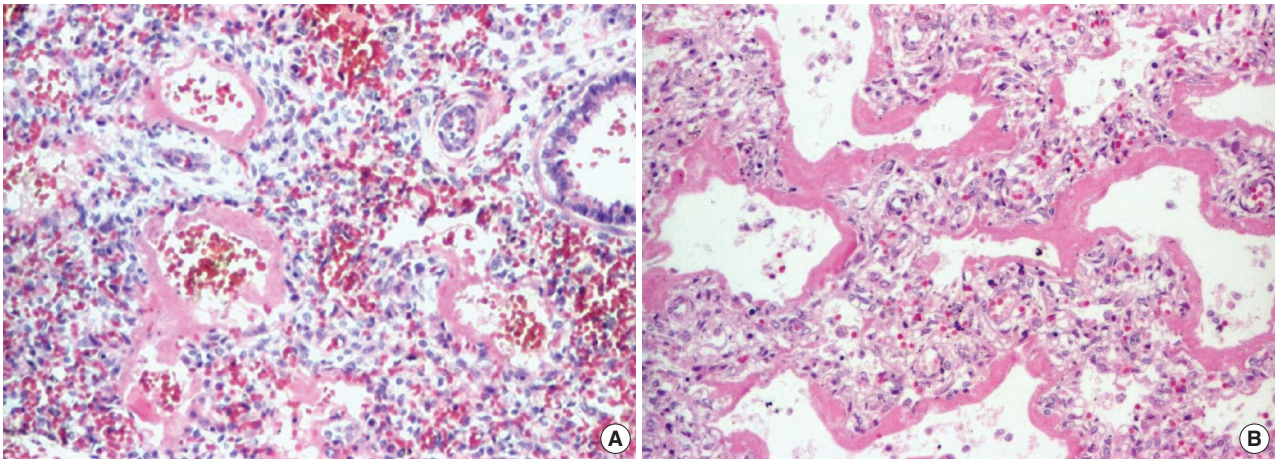

Fig. 1. (A) Light microscopic findings of diffuse alveolar damage in the lung with acute respiratory distress syndrome. (B) High magnification reveals hyaline membranes along the alveolar walls.

Histological and immunohistochemical aspects in normal lung tissues and lungs with ARDS

In normal lungs, VEGF165, VEGF121, and VEGFR1 were expressed in the endothelial cells, pneumocytes and macrophages. VEGFR2 was expressed in the endothelial cells and macrophages and but not in the pneumocytes. CD68 was expressed in the alveolar macrophages. No expression of VEGF and its receptors was observed in the bronchial epithelium (Fig. 1).

In early ARDS, the expression of VEGF isoforms and their receptors, VEGFR1 and VEGFR2, was all markedly decreased in the endothelial cells. The VEGF165 expression was down-regulated and sometimes lacked in the pneumocytes. VEGF121 intensity decreased in the pneumocytes, but a strongly positive expression appeared in the bronchial epithelium, where no expression of VEGF121 was observed in the normal lungs. Both VEGFR1 and VEGFR2 were expressed in the pneumocytes, although the latter was patchy in distribution. The alveolar macrophages showed the same immunohistochemical features as those in the normal lungs, i.e., positive for both VEGF isoforms, two VEGFRs, and CD68. In early ARDS, only three cases showed hyaline membrane formation. The hyaline membrane was positive for VEGF165 and CD68, but negative for VEGF121 and both VEGFRs. In these cases, no expression of VEGF165 was observed in the pneumocytes (Fig. 2).

In late ARDS, the immunohistochemical characteristics were different from those of early ARDS lungs. In the survivors (n = 3), the expression of VEGF165 in pneumocytes, which was partially lost in early ARDS, increased to the same degree as in the normal lungs, while was still very weak in the endothelial cells. VEGF121 expression was completely lost in the pneumocytes and endothelial cells, but remained unchanged in the macrophages. VEGFR1 also decreased in the pneumocytes and endothelial cells, whereas VEGFR2 was completely lost, except in the macrophages. Actually, in the pneumocytes and endothelial cells, the down-regulation of VEGF121, VEGFR1, and VEGFR2 appeared more prominent than in the early ARDS lung. In non-survivors, VEGF165 totally lacked in the pneumocytes of both early and late ARDS and was more significantly down-regulated than in survivors. At the same time, the VEGF165 intensity was up-regulated in the hyaline membranes. No difference in VEGFR1 and VEGFR2 expression was observed between survivors and non-survivors. Hyaline membranes, which were observed in all patients who died of late ARDS (n = 51), were strongly positive for VEGF165 and in some cases weakly positive for CD68, although the latter reactivity could be a reflection of the debris of destroyed, CD68-positive macrophages (Figs. 1, 2).

Correlation between immunohistochemical aspects and serum level of VEGF in ARDS

The mean VEGF165 serum level in patients without ARDS or other lung lesions was 141 pg/mL (range, 76 to 209 pg/mL). In patients with early and late ARDS, the mean VEGF165 serum levels increased significantly to 233.4 pg/mL (range, 140 to 323 pg/mL) and 262.8 pg/mL (range, 205 to 311 pg/mL), respectively ($p < 0.05$). However, between the early and late ARDS patients, the serum VEGF levels did not show a significant difference ($p > 0.05$). In patients who survived, VEGF levels were higher than in non-survivors but decreased to 138.5 pg/mL (range, 92 to 154 pg/mL), in late ARDS, after extubation.

In both early and late phases of ARDS, a significant indirect correlation between immunohistochemical intensity of VEGF-165 in endothelial cells and the serum level of VEGF was observed ($p < 0.05$). In all cases with no expression of VEGF165 in endothelial cells, plasma levels were higher than 200 pg/mL.

DISCUSSION

Regulation of the activity of VEGF and its receptors may be an important factor in the pathophysiology of ARDS, having clinical applications in individualized treatment, and possibly improving the prognosis. We consider that clinical and histological criteria are necessary in order to realize a proper classification of ARDS and to identify those patients whose outcome could be improved by an anti- or pro-angiogenic treatment. Taking into consideration that all survivors in this study were in the late phase of ARDS, but also 46 of the 50 deceased patients were in this phase, we can affirm that the time from the onset of ARDS is not enough to predict patient outcomes.

In ARDS, the number of integral pneumocytes seems to be a decisive factor for patient survival.[23] The appearence of the hyaline membranes predicts the unfavorable prognosis, independent of the time from the onset of ARDS.

In this study, we may suggest that the progress of ARDS is accompanied by changes in the expression of VEGF and its receptors in the following steps, independent of the onset time (early or late ARDS):

(1) The destruction of some pneumocytes and VEGF165 ex-

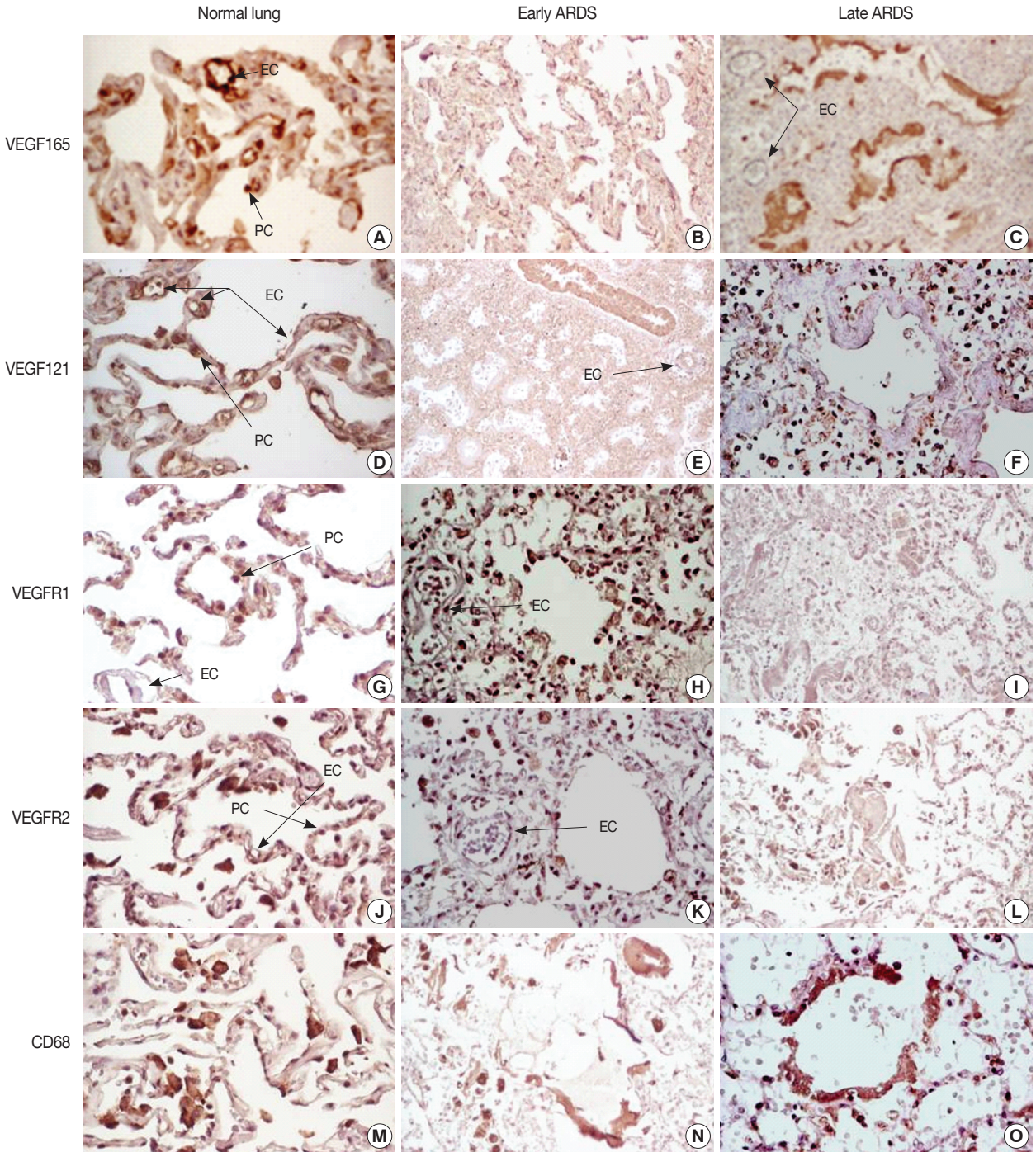

Fig. 2. (A-C) Vascular endothelial growth factor (VEGF)165 expression is decreased in pneumocytes (PC) and endothelial cells (EC) in both early and late acute respiratory distress syndrome (ARDS). The hyaline membranes are strongly positive for VEGF165 while integral PC and EC are negative. (D-F) The intensity of VEGF121 expression decreases in both EC and PC in ARDS lung and disappears in hyaline membranes, but newly appears in bronchial epithelium in early ARDS. (G-I) VEGF receptor (VEGFR)1 marks integral PC in both early and late ARDS, but its intensity decreases in EC and no expression in hyaline membranes is observed. (J-L) VEGFR2 intensity decreases in EC and is completely lost in PC. It is not expressed in hyaline membranes, but is observed in macrophages. (M, O) CD68 marks alveolar macrophages in normal lungs as well as ARDS and sometimes is observed in hyaline membranes, without any differences between early and late ARDS.

travasation were followed by a decrease of its immunohistochemical expression in the endothelial cells and pneumocytes. In this stage, integral pneumocytes expressed both VEGF165 and VEGFR1, but few expressed VEGFR2.

(2) The increase of VEGF121 in the bronchial epithelium and of VEGFR2 in the macrophages: During this step, VEGF165 and VEGFR1 expression was maintained in the pneumocytes and endothelial cells, but their intensity was down-regulated. The serum level of VEGF165 was up-regulated, probably as a result of activated macrophages by inflammatory mediators, which strongly expressed VEGFR2. Reduction in bioactive VEGF in the lung tissue with ARDS, contrasted with increased plasma levels, was confirmed by other previous studies,[24] but the responsible mechanism still remains unclear. After this phase, lung functions may be recovered, the VEGF serum levels decrease, and patients could be extubated. However, in other cases, the third step of ARDS was observed.

(3) The destruction of surfactant and many pneumocytes, followed by the appearance of hyaline membranes and an important decrease of lung perfusion: In the lung areas without hyaline membranes, a weak or lack of VEGF165 expression was observed in integral pneumocytes, however VEGFR1 expression was preserved in pneumocytes. On the other hand, hyaline membranes expressed only VEGF165, and was negative for VEGF121, VEGFR1, and VEGFR2. This aspect could be explained through the structure of VEGF165, which is partially soluble and partially cell-associated.[16] However, further confirmation is necessary before we admit this assumption. The serum level of VEGF165 was significantly up-regulated and the lung endothelial cells were negative for both isoforms and both receptors of VEGF. The decreased VEGF expression may result in a decrease in the lung capillary density and contribute to the weak lung perfusion, with increasing serum levels.[8]

Important aspects regarding the pathogenesis and prognosis of ARDS were revealed by the immunohistochemical findings of hyaline membranes. First, we observed that some membranes were CD68 positive and included destroyed macrophages marked by CD68 and VEGFR2. This aspect, correlated with their strong intensity for VEGF165 and extremely high VEGF165 serum level proved that VEGF originated partially from destroyed macrophages and partially from the extravasation of serum protein. This supposition was sustained by a recent experimental study which revealed high levels of circulating VEGF-165 and that its production in the spleen compared with the low intensity in the lung tissue of mice with malaria and ARDS.[11] In an unpublished study, we observed that in patients with ARDS, after a surgical intervention is performed on myocardium, the high intensity of VEGF appeared in the myocardium and kidneys, which was contrary to the low intensity in the lung.

We conclude that the genesis of hyaline membranes correlate with VEGF165 and the alveolar macrophage (marked by VEGFR2 and CD68) activity, but it is independent of VEGF121 and VEGFR1. On the other hand, VEGFR1 positivity of integral pneumocytes, regardless of phases of ARDS phase, proved that this receptor may be essential for their survival. Other authors suggested that VEGFR1 activity is important only in early ARDS because after 4 days from the onset of the ARDS the level of its soluble form decreased in lung epithelial lining fluid,[2] and higher VEGF levels in this fluid may predict a favorable outcome.[25] We tried, but failed to find studies about VEGFR1 immunohistochemical expression in early and late ARDS.

Our findings are not in accordance with those studies which proposed that VEGF activity depended mainly on VEGFR2,[19] which mediates the endothelial survival in the relationship with kinase-insert domain-containing receptor, and playing an important role in the regulation of the permeability in the alveolar-capillary membrane.[18] Increased VEGF165 expression in late ARDS in the integral pneumocytes of survivors correlated with the *in vitro* studies which showed that VEGF increased at the rate of alveolar cell proliferation after injury and also stimulated surfactant production from pneumocytes.[26,27]

Some studies showed an increased VEGF intensity in the lung with ARDS,[1] but VEGF was only densitometrically quantified and the expression in different cells of lung tissue was not separately analysed.

We did not find any previous studies explaining the pathogenesis of ARDS and hyaline membranes in relation to the changing expression of VEGF and its receptors, or CD68 by immunohistochemistry. Further studies are necessary in order to confirm our results, in a larger number of ARDS and other lung disease cases.

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