Early Responses of VEGF during Acute Lung Injury Induced by Seawater Immersion after Open Chest Trauma

Xiao-hong Hu,†,‡ Yun-you Duan, Yi Li, Zhi-qiang Xue

†Academy of Military Medical Sciences, ‡Department of Respiratory Medicine, PLA Naval General Hospital, and §Department of Thoracic Surgery, PLA General Hospital, Beijing, PR China

Abstract

Background: Immersion in seawater after open chest trauma may induce acute lung injury. Higher osmotic pressure is one of the main characteristics of seawater. The effects of vascular endothelial growth factor (VEGF) on endothelial cell permeability and proliferation have been demonstrated in studies. The early responses and effects of the VEGF on acute lung injury induced by seawater immersion after open chest trauma (SWI-ALI) are unknown. Objective: To investigate the levels of VEGF and its receptors in SWI-ALI and further explore whether the levels of VEGFs are connected with the pathogenesis of SWI-ALI. Methods: We put dogs into group ‘seawater’ and group ‘control’. The control group only suffered from open chest trauma, whereas the seawater group was exposed to seawater after trauma. The levels of total protein in plasma and bronchoalveolar lavage fluid were measured to calculate the pulmonary permeability index. 0, 2, 4, 6 and 8 h after open chest trauma, the plasma samples were collected to test the levels of VEGFs with ELISA kit. Western blotting and real-time RT-PCR were used to measure the VEGF levels in lung. Results: Compared with control animals, plasma osmotic pressure, VEGF and sVEGFR-1 significantly increased in plasma, while VEGF and VEGFR-2 significantly increased in seawater-immersion lung tissue. The levels of VEGF in plasma were significantly correlated with plasma osmotic pressure and pulmonary permeability index. Conclusion: Early release of VEGFs increases pulmonary vascular permeability and partially leads to the development of SWI-ALI. VEGFs may have a crucial role in the early onset of SWI-ALI.

Introduction

Acute lung injury (ALI) can result from a direct insult in the lung or an indirect insult from other organs mediated through the systemic circulation [1]. ALI is characterized by enhancement of pulmonary permeability and severe interstitial edema [2]. Seawater is a mixture of various salts and water, which has a salinity of 35 parts per thousand. Compared with the environment within mammals, seawater has a higher osmotic pressure, a lower temperature and an abundance of salts [3]. Seawater immersion after open chest trauma may induce acute lung...
injury (SWI-ALI), and its severe form, acute respiratory distress syndrome (ARDS) [4]. It may further develop multiple organ dysfunction syndrome, a life-threatening complication [5].

Vascular endothelial growth factor (VEGF), a dimeric 46-kD protein, is a vasodilator, endothelial cell-specific, multi-functional cytokine that induces the proliferation of endothelial cells, increases vascular permeability, and plays a pivotal role in the pathogenesis of asthma, emphysema, acute respiratory distress syndrome and lung cancer [6]. In healthy lung tissue, the levels of VEGF protein are 500 times higher than those found in plasma [7]. VEGF is mainly produced by epithelial cells, while endothelial cells are suggested to be its major target [8]. However, with acute systemic inflammatory responses, VEGF may be released by numerous cell types, including alveolar type II epithelial cells, alveolar macrophages, and polymorphonuclear neutrophils [9]. The biological activity of VEGF as both a growth factor and a permeability factor is dependent on its interaction with specific receptors. Two well-defined receptors are: VEGFR-1 (also occurring as a soluble form, sFlt), and VEGFR-2 (KDR) [10]. A wide variety of cells express VEGF receptors, including activated macrophages and alveolar type II epithelial cells [11, 12]. Most of the angiogenic activities of VEGF, as well as its effects on vascular permeability, are mediated by its receptor VEGFR-2, while VEGFR-1, as a biological inhibitor of VEGF, blocks this edema effect [13]. The biological properties of VEGF as a permeability factor have led to interest in its role during SWI-ALI. Improvements in the identification and characterization of possible prognostic parameters will lead to an improvement in patient management [14]. However, the early responses and effects of the VEGF on the SWI-ALI are unknown. In the present study, we investigated the levels of VEGF and its receptors and further explore whether the levels of VEGFs are connected with the pathogenesis of SWI-ALI.

Materials and Methods

Animal Models

For all experiments, we used 16 healthy grown-up dogs, each weighing 15–20 kg. Dogs were purchased from Beijing KeYu animal cultivation center (The SCXF, Beijing, 2002), with no preference for sex. Dogs were housed in a conventional animal facility. Prior to experimental intervention, the dogs were allowed to acclimatize for at least 1 week in the animal facility to recover from transportation. All investigations involving experimental dogs were reviewed and approved by the institutional review board of Beijing Naval General Hospital Animal Care and Use Committee. All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, and the Guide for the Care and Use of Experimental Animals formulated by the National Council on Animal Care.

These animals were randomized into a ‘seawater’ (SW) group and control group, with 8 animals grouping each. The animals in the control group only suffered from open chest trauma, whereas those in the SW group were exposed to seawater after open chest trauma.

The dog model of SWI-ALI was performed as previously described in detail [4]. Briefly, all animals were anesthetized with intramuscular injection of ketamine (20 mg/kg). Anesthesia was continuously maintained with ketamine throughout the experiment. The incision, 0.5 cm in diameter, was made with a sharp instrument in the forth intercostal of the right chest to form the opened pneumothorax in all experimental animals. In group SW, seawater (35 ml/kg) was infused into the pleural cavity. Then the incision skins of all experimental animals were sutured in 10 min. The right carotid artery was cannulated in every animals for measurement of mean arterial blood pressure. The right jugular vein was cannulated in every animal for drawing blood for analysis. The oxygen index (PaO2/FiO2), a ratio of arterial partial pressure of oxygen (PaO2) to fraction of inspired oxygen (FiO2) of $\leq$300 mm Hg (40 Kp) is the standard to prove the success of the SWI-ALI model.

The experimental seawater was made according to the standard of the Third Institute of Oceanography of the State Ocean Bureau: osmotic pressure was 1,250–1,350 mmol/l, pH was 8.20, proportion was 1.05–1.06. Laboratory temperature was 25°C, man-made seawater temperature was 22°C.

Sample Collection

Blood samples were collected at 0, 2, 4, 6 and 8 h after open chest trauma. Bronchoalveolar lavage (BAL) was performed by instilling 15 ml of saline through the endotracheal tube and gently aspirating back at 8 h after trauma. This was repeated twice. Later, 8 h after trauma, the dogs received intracardiac injection of 15 ml of 15% KCl and then were sacrificed. The lungs were removed for immunohistochemistry. BALF and blood samples were centrifuged at 3,000 g for 10 min, and the supernatant was stored at $-20^\circ$C until measurement of protein concentration. Lung samples were stored at $-80^\circ$C until use.

Assessment of Acute Lung Injury

The plasma osmotic pressure (POP) and electrolyte concentration were measured with blood samples collected at 0, 2, 4, 6 and 8 h after trauma. We measured the total protein concentration in BAL fluid and plasma collected at 8 h after trauma. We then calculated the protein concentration ratio of BAL/plasma, known as pulmonary permeability index (PPI).

Enzyme-Linked Immunosorbent Assay

VEGF and sVEGFR1 levels were determined in plasma samples using an ELISA kit (dogs VEGF/sVEGFR1; Sunbio, Beijing, China). An automatic multifunction microplate reader (Multiskan MK3, Thermo, USA) was used to read the value. Assays were performed in duplicate following the manufacturer’s instructions.
pre-treated with 0.25% Triton X-100 for 5 min and blocked for sVEGFR1 in control 0.56

were incubated with designated primary antibodies, with a di-

at 4 °C. The blot was washed with TBS-T containing 0.05% Tween

peroxidase-conjugated goat anti-mouse (1:1,000 dilution) IgG

20 and incubated for 2–3 h at room temperature with horseradish

bodies (mouse monoclonal antibody against VEGF of dog, sc-

for 1 h. PVDF were incubated with the designated primary anti-

were boiled in SDS sample buffer and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with 5% (w/v) nonfat milk in PBS for 1 h. PVDF were incubated with the designated primary antibodies (mouse monoclonal antibody against VEGF of dog, sc-80439; Santa Cruz Biotechnology) at 1:1,000 dilution overnight at 4°C. The blot was washed with TBS-T containing 0.05% Tween 20 and incubated for 2–3 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse (1:3,000 dilution) IgG (ZB-2301; Zhong Shan Biotechnology, Beijing, China). After washing, the blots were visualized with an enhanced chemiluminescence detection kit (ZLI-9032; Zhong Shan Biotechnology). We stripped and reprobed blots with antibody for β-actin as a control. Autoradiographs were quantified by using a densitometer (GS-690; Bio-Rad Laboratories) and normalized to the β-actin control.

Real-Time RT-PCR to Detect mRNA Synthesis of Encoding VEGFs

Quantitative real-time RT-PCR analysis of the mRNA expression of VEGF and VEGFR-2 were performed on RNA isolated from frozen lung tissue. Total RNA of tissue was extracted by using the RNA Extraction Kit (RN0302; Biomed). Total RNA was quantified at 260 and 280 nm, and sample integrity was checked by using 1% agarose gel electrophoresis. Total RNA (2 μg) was converted to cDNA; the reaction volume contained: 5 units of Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega M170A) and 0.5 μg oligo deoxythymidin. The reaction continued for 1 h at 37°C. Reverse transcriptase-generated cDNAs encoding VEGF and GAPDH (used as a control of RNA integrity and internal standard) were amplified by PCR. Amplification of 5 μl of cDNA was performed using 0.2 nM sense and antisense VEGF primers and 2.5 units of Taq polymerase. The oligonucleotide primer sequences are shown in Table 1. Samples were amplified for 40 PCR cycles. A 5-μl aliquot from each PCR reaction was subjected to polyacrylamide gel electrophoresis and visualized by autoradiography. Their authenticity was confirmed by direct nucleotide sequencing. All real-time RT-PCR studies were performed at least 4 times, using lung RNA from different animals.

Statistical Analyses

All data are expressed as mean ± standard deviation and were analyzed with SSPS 10.0 software. Two-way repeated measures analysis of variance (ANOVA) followed by Dunnett's test for multiple time points observation or Student's t test for single time point observation, and correlation among variables was assessed with Pearson's correlation analysis (2-tailed). p values <0.05 were regarded as significant.

Table 1. Reference sequence and primer sequences used for the oligonucleotide

<table>
<thead>
<tr>
<th>Reference sequence</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
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<tbody>
<tr>
<td>VEGF</td>
<td>AF133250</td>
<td>5’-gggctgtgtaatgtgagg-3’</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>DQ269018</td>
<td>5’-ttacagactcagcat-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM001003142.1</td>
<td>5’-aacaatccccgctcccac-3’</td>
</tr>
</tbody>
</table>

Table 2. The changes of VEGFs in plasma with timing (pg/ml) (n = 8, x ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF in control</td>
<td>23.75 ± 1.91</td>
<td>26.02 ± 2.59</td>
<td>26.09 ± 2.39</td>
<td>28.06 ± 3.65</td>
<td>27.28 ± 2.27</td>
</tr>
<tr>
<td>VEGF in SW</td>
<td>23.32 ± 2.03</td>
<td>31.43 ± 7.03</td>
<td>38.91 ± 4.75**</td>
<td>57.89 ± 12.69**</td>
<td>63.46 ± 10.16**</td>
</tr>
<tr>
<td>sVEGF1 in control</td>
<td>0.56 ± 0.07</td>
<td>0.61 ± 0.12</td>
<td>0.69 ± 0.27</td>
<td>0.63 ± 0.16</td>
<td>0.61 ± 0.13***</td>
</tr>
<tr>
<td>sVEGF1 in SW</td>
<td>0.60 ± 0.09</td>
<td>0.81 ± 1.15**</td>
<td>1.04 ± 0.21**</td>
<td>1.61 ± 0.28**</td>
<td>1.92 ± 0.32**</td>
</tr>
</tbody>
</table>

* p < 0.05, compared with the control group; ** p < 0.05, compared with the onset (0 h) in the same group.

Immunohistochemistry

For immunohistochemistry, lung tissue slides (5 μm) were pre-treated with 0.25% Triton X-100 for 5 min and blocked for endogenous peroxidase and biotin with 0.3% H₂O₂. The slides were incubated with designated primary antibodies, with a dilution of 1:100 for VEGF (sc-80439; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), for overnight at 4°C, and then with a secondary antibody for 15 min at 37°C. Detection was done by Avidin Biotin Complex system with 3-3 diaminobenzidine as chromogen from a Histostain-Plus Bulk Kit Invitrogen 2 Generation LAB-SA Detection System (Cat. No. 85-6643; Invitrogen, Carlsbad, Calif., USA) and DAB Kit (Cat. No. 00-2014; Invitrogen). Cell nuclei were counterstained with hematoxylin.

Western Blotting to Determine the VEGF Protein Expression Products

After the sample of lung tissue lysate was available, the protein concentration from homogenized snap-frozen lung samples (8 from each group) was determined by the Bradford method. Equal amounts of protein in each sample were boiled in SDS sample buffer and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with 5% (w/v) nonfat milk in PBS for 1 h. PVDF were incubated with the designated primary antibodies (mouse monoclonal antibody against VEGF of dog, sc-80439; Santa Cruz Biotechnology) at 1:1,000 dilution overnight at 4°C. The blot was washed with TBS-T containing 0.05% Tween 20 and incubated for 2–3 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse (1:3,000 dilution) IgG (ZB-2301; Zhong Shan Biotechnology, Beijing, China). After washing, the blots were visualized with an enhanced chemiluminescence detection kit (ZLI-9032; Zhong Shan Biotechnology). We stripped and reprobed blots with antibody for β-actin as a control.
**Results**

**VEGF Expression Is Up-Regulated in the Early Onset of SWI-ALI**

Detection of Expression of VEGF and sVEGFR₁ in Plasma by ELISA

Expression patterns of VEGF and its soluble receptor (sVEGFR₁) in plasma from both the control and seawater groups are shown in table 2. Plasma levels of the investigated molecules were increased with time after trauma. There is a negligible increase in control animals. A significant increase of the VEGF and sVEGFR₁ levels was found in the seawater group, especially at 4 h after trauma, and achieved their peak at 6–8 h. The increase was significantly greater than that in the control group (p < 0.05).

Detection of Expression of VEGF in Lung Tissue by Immunohistochemistry

Figure 1 provides an overall impression of the changes of VEGF in different groups through the staining intensities in lung tissue. In control animal lungs (a), the alveolus structure retained its integrity, with mild capillary vessel congestion, there was little edema and few inflammatory cells in the alveolar spaces. The VEGF-positive reaction expression spot is less intense. In the SW animal lung tissue (b), there are visible interstitial and intra-alveolar edema and a greater area of VEGF-positive reaction expression. The quantization value suggested that there was a significant difference between the SW and control groups (27,744.75 ± 5,222.84 vs. 12,103.72 ± 551.93, p < 0.05).

**Fig. 1.** Immunohistochemistry for VEGF in the lung. VEGF-positive expression in lung was stained brown. Sections of lung from control dogs (a) and SW dogs (b) are shown with immunohistochemistry. Figure 1 provides an overall impression of the changes of VEGF in different groups through the staining intensities in lung tissue. In control animal lungs (a), the alveolus structure retained its integrity, with mild capillary vessel congestion, there was little edema and few inflammatory cells in the alveolar spaces. The VEGF-positive reaction expression spot is less intense. In the SW animal lung tissue (b), there are visible interstitial and intra-alveolar edema and a greater area of VEGF-positive reaction expression. The quantization value suggested that there was a significant difference between the SW and control groups (27,744.75 ± 5,222.84 vs. 12,103.72 ± 551.93, p < 0.05).
interstitial and intra-alveolar edema and greater area of VEGF-positive reaction expression. Figure 1c shows that the quantization value suggested that there was a significant difference between the SW and control groups (27,744.75 ± 85,222.84 vs. 12,103.72 ± 551.93, p < 0.05).

Detection of VEGF mRNA Synthesis of Encoding VEGF Proteins in Lung Tissue by RT-PCR

The mRNA synthesis encoding VEGF and VEGFR-2 protein in lung tissue were significantly increased in the SW group (fig. 2, table 3). VEGF mRNA: SW versus control, 5.04 ± 0.29 vs. 0.25 ± 0.04, p < 0.05. VEGFR-2 mRNA: SW versus control, 5.08 ± 0.20 vs. 5.08 ± 0.20, p < 0.05.

Detection of VEGF Protein in Lung Tissue by Western Blotting

VEGF protein production was determined in lung tissue (fig. 3, table 3). The optical densities of blot bands were quantified with densitometry and normalized to that of actin as controls. From the image in figure 3, we found that the optical density of blot bands of VEGF protein in lung tissue in SW animals is more concentrated than that in control animals. The quantization value suggested that there was a significant difference between the SW group and control group (0.2375 ± 0.036 vs. 0.1649 ± 0.031, p < 0.05).

Correlation of VEGF with POP and PPI

We assessed the correlations among the level of VEGF protein in plasma, the POP and PPI with Pearson’s correlation analysis. POP was significantly positively correlated with PPI, with a Pearson’s correlation coefficient of

Table 3. VEGF mRNA and VEGF protein in the lung tissues (n = 8, X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>VEGF mRNA (PCR)</th>
<th>VEGFR-2 mRNA (PCR)</th>
<th>VEGF (Western)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.25 ± 0.04</td>
<td>0.64 ± 0.02</td>
<td>0.1649 ± 0.031</td>
</tr>
<tr>
<td>SW</td>
<td>5.04 ± 0.29*</td>
<td>5.08 ± 0.20*</td>
<td>0.2375 ± 0.036*</td>
</tr>
</tbody>
</table>

* p < 0.05, compared with the control group.
VEGF protein levels in plasma were significantly positively correlated with PPI and POP (VEGF with PPI correlation coefficient = 0.596, p = 0.015; VEGF with POP correlation coefficient = 0.917, p = 0.000).

Discussion

Our experiment was the first attempt to investigate the levels of VEGF and its receptors in ALI induced by seawater immersion after open chest trauma. We chose dogs to duplicate SWI-ALI as they are a robust model that does not die quickly after trauma and seawater immersion. Furthermore, large animals provide a model that is close to the clinical situation in humans.

We observed a significant increase of VEGF and sVEGFR2 in plasma of SWI-ALI. Increased VEGF levels in plasma from our results agree with some studies in ARDS patients [15] or animal models of ALI induced by other etiologies [16, 17]. The mechanism of increased VEGF levels in plasma may be associated with the following 2 factors: one is that alveolar macrophages and neutrophils are both potential sources of VEGF in ALI, and the activated alveolar macrophages and neutrophils produce plentiful VEGF [9, 15, 18]; the other factor is that a widespread destruction of the alveolar epithelial membrane in ALI leads to increased VEGF through a transepithelial VEGF gradient [19]. We also found that the levels of VEGF and VEGFR-2 increased in the lung tissue of SWI-ALI. The results seem contrary to other studies. The down-regulation of VEGF has been noted in ARDS patients [16] or animal models of ALI induced by other etiologies [19]. We also found that the levels of VEGF and VEGFR-2 increased in the lung tissue of SWI-ALI. The results seem contrary to other studies. The down-regulation of VEGF has been noted in ARDS patients [16] or animal models of ALI induced by other etiologies [19].

VEGF Early Response in Acute Lung Injury

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Conclusion

This study has shown for the first time that increasing VEGF levels in intrapulmonary and plasma are associated with the early onset of SWI-ALI and elevated VEGF in plasma would be expected to contribute to abnormal capillary permeability. We speculate that significant and dynamic changes of the VEGF levels represent a marker of acute lung injury. VEGF may have diverse effects on different stages. We only collected plasma and lung tissue on the early onset of SWI-ALI and thus are unable to evaluate the levels and effects of VEGF on other phases of SWI-ALI. Therefore, further investigation is required to address the levels and effects of VEGF on other phases and to expound the intricate role of the VEGF system in SWI-ALI.

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References