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Vascular Endothelial Growth Factor in Bronchoalveolar Lavage Fluid in Sulfur Mustard Exposed Lung Patients

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Abstract

Objectives: To determine the levels of vascular endothelial growth factor isoform consisting of 165 amino acids (VEGF165) in Bronchoalveolar Lavage Fluid from Mustard Exposed Patients.
Methods: Bronchoscopy with Bronchoalveolar Lavage was performed on sulphur mustard exposed patients. A total of 39 patients with documented exposure to Sulfur Mustard during the Iran-Iraq war participated in this study, of which 38 patients were males and one patient was female.
Results: The mean±SD age of patients was 41 ± 6.6 years. The mean time after exposure to sulfur mustard was 19 ±1.7 years. Eighteen patients had concomitant war injuries but they were not related to the respiratory system. While Twenty-two patients had a history of submassive persistent hemoptysis. There was no case with massive hemoptysis. Most of the patients had small airway obstruction (FEV1/FVC% = 78.14 ± 9.76 and FEV1% =82.79±18.23). Twenty-three patients had significant air trapping in the chest. High Resolution Computed Tomography was compatible with BOS. VEGF165 concentrations in BALF were 36.87 ± 34.68 pg/ml. When corrected to total protein of Bronchoalveolar Lavage Fluid (BALF) it was 0.76 ± 0.70 pg/mg. BALF of VEGF did not correlate with hemoptysis or air trapping in chest HRCT. Thus, there was also no correlation between level of VEGF165 in BALF and any of PFT indexes (FVC, FEV1, MMEF or PEF).
Conclusions: Although VEGF is one of the cytokines which has an important role in chronic pulmonary disorders, it seems that it has no essential role in the severity of Mustard Lung Disease.

Keywords: VEGF; BALF; Mustard Gas; Hemoptysis.

Introduction

Sulfur mustard (SM) gas is an alkylating agent that was frequently used by the Iraqi ex-regime during the Iran–Iraq war. This agent can damage the upper and lower respiratory tracts in the acute phase of exposure. Chronically, it can lead to the development of airway obstructive diseases manifested by chronic cough and sometimes hemoptysis.[1]

Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis and vascular permeability. VEGF is a heparin-binding factor that acts specifically on endothelial cells via membrane-spanning tyrosine kinase receptors; many cell types, including neutrophils and macrophages secrete it. VEGF is induced by hypoxemia and tissue inflammation. VEGF is also a specific mitogen for endothelial cells and is important not only for vasculogenesis and angiogenesis, but also for the maintenance of existing blood vessels. VEGF gene expression is induced by environmental stresses such as hypoxia, anemia, myocardial ischemia, and tumor progression to initiate subsequent angiogenesis and neovascularization.[2] It is also highly expressed by lung epithelial cells and may play an important role in the maintenance of pulmonary vascular bed or responses to lung injury.

High expression of VEGF by lung epithelium suggests that VEGF expression may be important in maintaining a normal vascular bed.[3] VEGF can also alter vessel permeability and appears to play an important role in wound healing and in the maintenance of differentiated state of blood vessels in normal vascular beds.[4] Because of the importance of VEGF, many clinical trials are under way for both pro- and anti-angiogenic therapies. We hypothesized that VEGF levels may be altered in Bronchoalveolar Lavage Fluid (BALF) of mustard exposed patients. Therefore, we the VEGF isoform containing 165 amino acids (VEGF165) were measured in BALF of 39 SM injured patients.

Methods

The study population consisted of 39 patients with documented exposure to SM and history of chronic hemoptysis. Bronchiolitis obliterans syndrome (BOS) was diagnosed by chest High Resolution Computed Tomography (HRCT). BALFs from patients with active bacterial or fungal infection were not included. All bronchoscopies were performed at the University of Baqiyatallah.

Before initiating the program and after approval of the experimental protocol by the local human ethics committee,

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all of subjects were completely informed about the project and each subject had signed a statement confirming their consent to participate in the study.

All study protocols were approved by the Research Center of Chemical Injuries, Baqiyatallah Medical Sciences University. The study participants were nonsmokers, and none had a previous history of respiratory disorders. Patients with proven cardiovascular diseases and those with evidence of recent infection or exacerbation of their disease were excluded. Only one of the 39 participants was female. Patients had to meet at least two of the inclusion criteria.

All subjects had a complete history and physical examination. Spirometry was performed according to the ATS recommendations by a registered pulmonary function technologist in the Pulmonary Function Laboratory of Baqiyatallah Medical Sciences University. Spirometric data are expressed as percentages of predicted values.

The Flexible Fiberoptic Bronchoscopy (FFB) was performed using the transnasal route. Routine administration of sedatives or anxiolytics was avoided, but IV midazolam was administered during FFB if deemed necessary by the Bronchoscopist to improve patient comfort and tolerance of the procedure. In our institution, most bronchoscopies are performed without premedication other than topical anesthesia.

Initially, white light bronchoscopy (WLB) was used to investigate the tracheobronchial tree. In this phase, normal and abnormal regions were defined. Erythematouse mucosa, inflammation and hyperplasia were considered as nonspecific abnormalities, while regions with irregularity or thickness in the bronchial mucosa were considered as suspicious neoplastic regions (dyspalsia/carcinoma in situ).[5]

During fluorescence bronchoscopy, regions with green light reflects were considered as "Normal Mucosa," while light brown to dark reddish brown reflection lights were considered as "Suspicious Mucosa,"[6,7] and biopsies were taken from these areas. All specimens were sent to an expert pulmonary pathologist in the pathology unit of the hospital.

Bronchial lavage was performed before taking biopsies. For this, the mean bronchoscope was wedged for the lavage in the middle lobe segmental bronchus, and four 60-mL aliquots of sterile physiologic saline solution, warmed to 37 °C were infused. The fluid was immediately recovered by gentle suction after each instillation. The first aliquot, consisting of a bronchial sample, was discarded, whereas the others were pooled for study. The recovered lavage fluid was centrifuged at 4000 rpm for 10 minutes.

The differential cell count for lymphocytes, neutrophils, macrophages, and eosinophils was made under light microscopy by counting approximately 300 cells in random fields. The supernatant was frozen at -70 °C before protein concentrations were measured.

In terms of processing of BALF, the recovered lavage fluid aliquots were pooled and cellular and fluid phases of the pooled lavage fluid were separated by centrifugation. Cytocentrifuge preparations were examined to obtain differential cell counts. BALF specimens were stored until analyzed at -70°C. The total protein concentration was determined via a modified Lowry method.

With regards to measurement of VEGF, a commercial quantitative sandwich enzyme immunoassay (R&D Systems; Minneapolis, MN) was used to measure human VEGF165. This assay predominantly binds the monomeric VEGF165, but will also detect the VEGF isoform containing 121 amino acids. The assay utilizes a monoclonal anti-VEGF capture antibody, a polyclonal anti-VEGF detection antibody conjugated to horseradish peroxidase, and color development with tetramethylbenzidine/hydrogen peroxide. All assays were performed according to the protocol of the manufacturer, and the lower limit of detection was 10 pg/mL. Assays were performed on BALF aliquots that had only been thawed once. Some aliquots were re-assayed after refreezing and re-thawing to assess the effect of freeze-thawing on VEGF concentrations.

The VEGF concentrations obtained after thawing were generally within 10% of the initial values. To minimize other sources of error, such as batch artifact, subject groups were intermingled on enzyme-linked immunosorbent assay plates and technicians were blinded to diagnoses and clinical situations. Protein concentrations were normalized to the albumin concentration.

Data were analyzed on electronic spreadsheets and database-statistics programs for microcomputers (SuperCalc4; Computer Associates; San Jose, CA, and Microsoft Excel, SPSS).

Results

A total of 39 patients entered the study. Thirty eight were male and one patient was female. The mean age of patients was 41 ± 6.6 years, while the mean time after exposure was 19 ±1.7 years. Most of the patients had concomitant war injuries, but they were not related to respiratory system. Most of the patients had small airway obstruction (FEV1/FVC% = 78.14 ± 9.76 and FEV1% = 82.79 ± 18.23). Twenty-three patients had significant air trapping in chest HRCT compatible with BOS. Twenty-two patients had history of persistent submassive hemoptysis. However, there was no case with massive hemoptysis.

The patients were divided into 2 major groups; a) patients with hemoptysis, and b) patients without hemoptysis. Then the FVC%, FEV1%, PEF%, Age, the amount of Lymphocytes, monocytes, WBC and total cell (in BALF) were compared as well as protein concentration and VEGF concentration in BALF among the two groups. The mean rate for pulmonary indexes in the studied patients was below normal (less than 80%) and no difference was observed between the two groups. Table 1 shows the mean rate reported in the two groups.

Table 1: The mean rate of our variables in the two groups.

Variables	Hemoptysis	N	Mean
FVC%	No	13	71.31
	Yes	22	73.23
FEV1%	No	13	64.15
	Yes	22	70.32
PEF%	No	13	57.62
	Yes	22	66.45
Age	No	17	40.59
	Yes	22	41.55
Lymphocytes	No	14	60.36
	Yes	15	61.33
Monocytes	No	14	0.21
	Yes	14	0
WBC	No	14	421.07
	Yes	17	16.59
Total cell	No	14	3556.79
	Yes	17	353.24
Protein concentration	No	14	22.189
	Yes	17	8.271
VEGF conc. in BALF	No	17	27.988
	Yes	21	38.148

N: number of patients, Yes: patients with hemoptysis, No: patients without hemoptysis.

To have statistically viable results, the data was compared with an independent sample *t* test and we found that for protein concentration in BALF, there was a significant difference between the two groups (p=0.034). In other variables, no statistically significant differences were observed. (Table 2)

Table 2: *t* test for equality of Means

Variable	Significance (2 tailed)
FVC%	0.831
FEV1%	0.544
PEF%	0.408
Age	0.595
Lymphocytes	0.906
Monocytes	0.82
Total cell	0.138
WBC	0.311
Protein concentration	0.034
VEGF concentration in BALF	0.290

Also, air trapping in chest HRCT was not related to VEGF in BALF. This shows that the level of VEGF in BALF concentration does not have any correlation with hemoptysis, and that protein concentrations in BALF for patients without hemoptysis was significantly higher than in patients with hemoptysis. The results of bronchial biopsy showed epithelial cell metaplasia in three cases and the remaining patients had mild to moderate inflammation.

Discussion

Alternative splicing of the VEGF gene yields four isoforms of 121, 165, 189, and 206 amino acids, and other less frequent splice variants. VEGF-165, a 45-kiloDalton (kD) homodimeric glycoprotein, is the dominant form and is in part, secreted and, in part, matrix bound. The actions of VEGF-165 involve the activation of proteinase cascades, including that leading to plasmin generation, so the consequent plasmin-mediated release of matrix-bound VEGF isoforms provides an amplification mechanism.[8,9]

Hemoptysis is an often alarming presenting symptom and VEGF is a major regulator of both normal and abnormal angiogenesis, including many inflammatory diseases. Lo DK et al. investigated clinical significance of the serum VEGF level in patients with hemoptysis. They showed that regardless of the etiology, the serum VEGF may contribute to abnormal neovascularization in patients with hemoptysis. Therefore, it is suggested that serum VEGF measurements may help predict a massive hemoptysis.[10] It has been shown that in patients with hemoptysis, serum VEGF levels were significantly higher than in patients without hemoptysis. VEGF levels also decrease significantly in parallel with the alleviation of hemoptysis. VEGF is one of the predictive serum markers for the likelihood of developing hemoptysis.[11] However, results of from our study were not in consistence with previous report regarding hemoptysis.

In our study, VEGF level not in serum, but in BALF was measured, and we did not find any correlation between BALF VEGF levels and the presence of hemoptysis. It is important to note therefore, that of the total 39 patients, 22 had hemoptysis in which most of them were in submassive category. Although VEGF is one of the cytokines which has an important role in chronic pulmonary disorders, it seems that its level in BALF plays no essential role in the severity of Mustard Lung Disease. Also, biopsy via bronchoscopy cannot diagnose granuloma. Open lung biopsy could be a valuable method for further evaluation of angiogenesis evidence in this setting. However, we suggest further studies analyzing angiogenesis in these patients. Complementary studies with a larger sample size are also recommended to generalize findings to target population.

Conclusion

Although VEGF plays an important role in chronic pulmonary disorders, the results obtained from this study suggest that the concentration of VEGF in BALF was not related to hemoptysis severity and it would seem that it plays no major role in the severity of Mustard Lung Disease.

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