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Imbalance between vascular endothelial growth factor and endostatin in emphysema

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Imbalance between vascular endothelial growth factor and endostatin in emphysema. H. Kanazawa, K. Hirata, J. Yoshikawa. ©ERS Journals Ltd 2003.

ABSTRACT: Vascular endothelial growth factor (VEGF) stimulates endothelial cell proliferation, and endostatin directly antagonises the biological effects of VEGF. The maintenance of pulmonary endothelial cells is also thought to depend upon the local balance of VEGF and endostatin in the lung. Therefore, this study was designed to determine whether there is an imbalance between VEGF and endostatin levels in patients with pulmonary emphysema.

VEGF and endostatin levels were simultaneously measured from 25 emphysema patients and 12 normal control subjects, and their correlation and balance in induced sputum was evaluated.

VEGF levels in induced sputum were significantly lower in emphysema patients (854±307 pg·mL^{-1}) than in normal controls (1,791±1,192 pg·mL^{-1}). In contrast, there was no significant difference in endostatin levels among the two groups. Therefore, the ratio of VEGF to endostatin levels was markedly lower in emphysema patients (4.5±1.8) than in normal controls (8.1±2.6). Moreover, VEGF levels were correlated with endostatin levels in normal controls but not in emphysema patients. In addition, the ratio of VEGF to endostatin levels was correlated with forced expiratory volume in one second (FEV1), FEV1/forced vital capacity and carbon monoxide diffusing capacity of the lung in emphysema patients.

The findings in this study suggest that there is an imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from emphysema patients.

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Vascular endothelial growth factor (VEGF) is a potent and specific angiogenic factor stimulating endothelial cell proliferation [1], and withdrawal of VEGF leads to endothelial cell apoptosis both *in vitro* and *in vivo* [2, 3]. Thus, VEGF is a trophic factor required for the survival of endothelial cells. VEGF is highly abundant in the lungs, but its physiological effects are not fully understood. KASAHARA *et al.* [4] recently reported that the protein levels and messenger ribonucleic acid expression of both VEGF and the VEGF receptor were decreased in lungs from patients with emphysema, and that a decrease in VEGF levels plays a role in the pathogenesis of emphysema. In an earlier study by the current authors, it was also found that VEGF levels in induced sputum were significantly lower in patients with emphysema than in normal controls, and that decreased levels of VEGF were associated with airflow limitation and alveolar destruction in emphysema patients [5].

Angiogenic factors are involved in virtually all aspects of lung development and responses to inflammation, and imbalances in angiogenic factors are increasingly implicated in a wide spectrum of inflammatory diseases [6, 7]. An imbalance in angiogenic factors in the lungs describes a situation in which the expression or activity of one growth factor predominates over another, usually of opposing effect, within the same compartments such as the airways and alveolar septa. Endostatin specifically inhibits endothelial cell growth and migration [8], and directly antagonises the biological effects of VEGF [9]. Therefore, this study was designed to determine whether there is an imbalance between VEGF and endostatin levels in induced sputum from patients with emphysema.

Materials and methods

Subjects

A total of 25 patients with pulmonary emphysema and 12 normal control subjects were included in this study. All normal controls were healthy, life-long nonsmoking volunteers who had no history of lung disease. All patients with emphysema were randomly enrolled from the respiratory outpatient clinic of Osaka City University. They had a history of former smoking (>20 pack-yrs) and an irreversible airflow limitation (reversibility <10% predicted forced expiratory volume in one second (FEV1) after 200 μg inhaled salbutamol). Clinical types of emphysema in chronic obstructive pulmonary disease were classified according to the statement of the American Thoracic Society [10]. Emphysema was defined as abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls. Thus, emphysema was defined in terms of anatomical pathology, based on the high-resolution computed tomographic scans of the chest. Their regular medication consisted of theophylline and an inhaled anticholinergic drug, but none had received oral or inhaled corticosteroids.

No patients received medication during the 12-h period preceding the spirometric study and sputum induction. All patients with emphysema were not current smokers and were clinically stable, and none had a history of respiratory infection for, at least, the 4-week period preceding the study. All subjects gave their written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Measurements

Spirometry was performed using a Chestac-25 F system (Chest Co., Tokyo, Japan) by the same technician using the same spirometer. The carbon monoxide diffusing capacity of the lung (*D*L,CO) was measured by the single-breath carbon monoxide method at least twice. Spirometry was performed before inhalation of 200 μg salbutamol *via* a metered-dose inhaler. Following salbutamol, all subjects were instructed to wash their mouth thoroughly with water. They then inhaled 3% saline at room temperature, nebulised by an ultrasonic nebuliser (NE-U12; Omron Co., Tokyo, Japan) at maximum output. The dose of saline used in sputum induction was equal for all subjects. They were encouraged to cough deeply at 3-min intervals thereafter. The sputum sample diluted with phosphate buffer solution containing dithiothreitol (final concentration 1 mM) was then centrifuged at 400×*g* for 10 min and the cell pellet was resuspended. Slides were made by using a cytospin (Cytospin 3; Shandon, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain for differential cell counts. The differential cell counts were measured by at least two chest physicians on separate occasions. The supernatant was stored at -70°C for subsequent assay for VEGF and endostatin. The concentration of VEGF was measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D System Inc., Minneapolis, MN, USA) and the endostatin concentration was also measured using an ELISA kit (Cytimmune Science Inc., College Park, MD, USA). The concentrations of VEGF and endostatin were assayed in at least triplicate and reproducibility of the assay was confirmed by repeat measurements in the same subjects on separate days. All subjects produced an adequate specimen of sputum; a sample was considered adequate if the patient was able to expectorate at least 2 mL of sputum and if, on differential cell counting, the slides contained <10% squamous cells.

Statistical analysis

All values are presented as mean±SD. The Mann-Whitney U-test was used for intergroup comparisons. The significance of correlations was evaluated by determining Spearman's rank correlation coefficients. A p-value <0.05 was considered significant.

Results

Clinical characteristics of the 25 emphysema patients and 12 age-matched normal control subjects are shown in table 1. All patients with emphysema had significant obstructive changes in pulmonary function and decreased *D*L,CO. Moreover, the absolute neutrophil count per unit volume of induced sputum and percentage of neutrophils in induced sputum was significantly higher in emphysema patients ($32.6\pm17.3\times10^4\cdot mL^{-1}$, 38.7±6.1%) than in normal controls ($14.7\pm6.7\times10^4\cdot mL^{-1}$, 21.5±4.0%; both p<0.0001).

Table 1. – Clinical characteristics of the study subjects

	Controls	Emphysema
Subjects n (M:F)	12 (10:2)	25 (20:5)
Age yrs	62.8±4.6	61.9±5.3
FEV1 % pred	90.2±2.7	48.7±13.1**
FEV1/FVC %	81.8±3.1	42.0±8.5**
*D*L,CO %	ND	39.3±6.6
Sputum		
Total cell count $\times10^4\cdot mL^{-1}$	68.7±29.5	83.4±38.6
Neutrophil count $\times10^4\cdot mL^{-1}$	14.7±6.7	32.6±17.3**
Neutrophils %	21.5±4.0	38.7±6.1**

Data are presented as mean±SD. M: male; F: female; FEV1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; *D*L,CO: carbon monoxide diffusing capacity of the lungs; ND: no data. **: p<0.01.

The concentration of VEGF in induced sputum was significantly lower in emphysema patients (854±307 $pg\cdot mL^{-1}$) than in normal controls (1,791±1,192 $pg\cdot mL^{-1}$, p=0.022) (fig. 1a). In contrast, there was no significant difference in the concentration of endostatin in induced sputum between emphysema patients (203±70 $pg\cdot mL^{-1}$) and normal controls (214±120 $pg\cdot mL^{-1}$) (fig. 1b). Therefore, the ratio of VEGF to endostatin levels was markedly lower in emphysema patients (4.5±1.8) than in normal controls (8.1±2.6; p=0.0003) (fig. 2). VEGF levels were significantly correlated with endostatin levels in normal controls (r=0.916, p=0.0024) (fig. 3a). However, no significant correlation was found between VEGF and endostatin levels in emphysema patients (fig. 3b). Moreover, the ratio of VEGF to endostatin levels was significantly

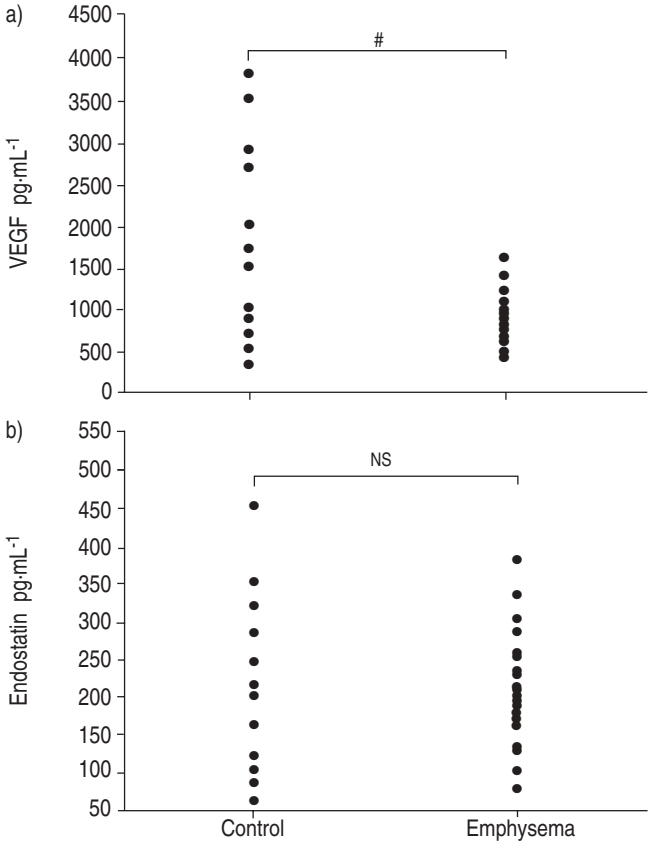

Fig. 1. – Comparison of the concentrations of a) vascular endothelial growth factor (VEGF) and b) endostatin in induced sputum in normal controls and emphysema patients. NS: nonsignificant. #: p=0.022.

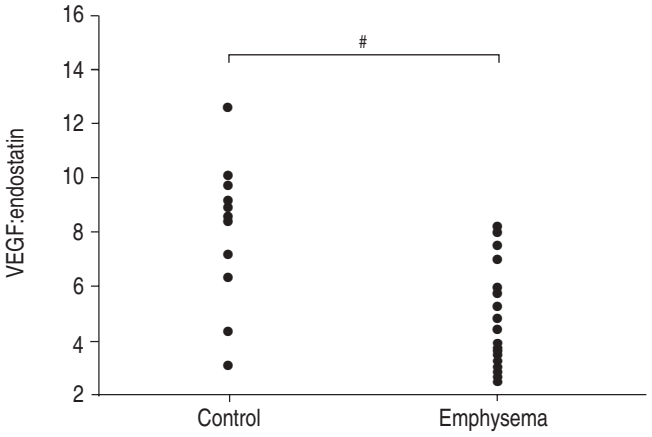

Fig. 2. – Comparison of the ratio of vascular endothelial growth factor (VEGF) to endostatin levels in induced sputum in normal controls and emphysema patients. #: p=0.0003.

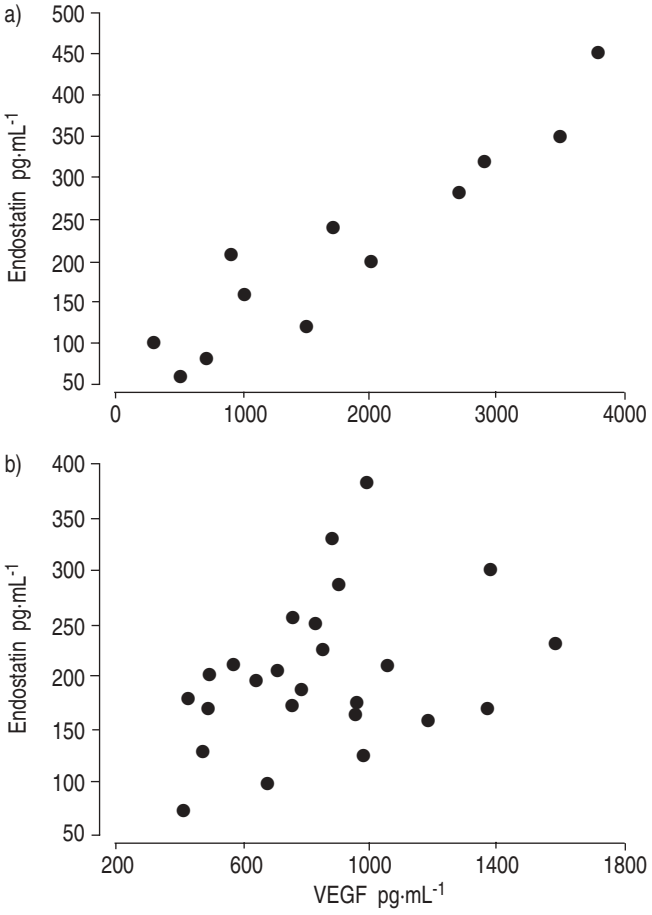

Fig. 3. – Relationship between the concentrations of vascular endothelial growth factor (VEGF) and endostatin in induced sputum in a) normal controls (r=0.916, p=0.0024) and b) emphysema patients (NS).

correlated with FEV1 % pred (r=0.669, p=0.0011), FEV1/forced vital capacity (r=0.482, p=0.018) and $D_{L,CO}$ (r=0.654, p=0.0014) in emphysema patients (fig. 4).

Discussion

The novel aspect of this investigation is the finding of a decreased ratio of VEGF to endostatin levels in emphysema

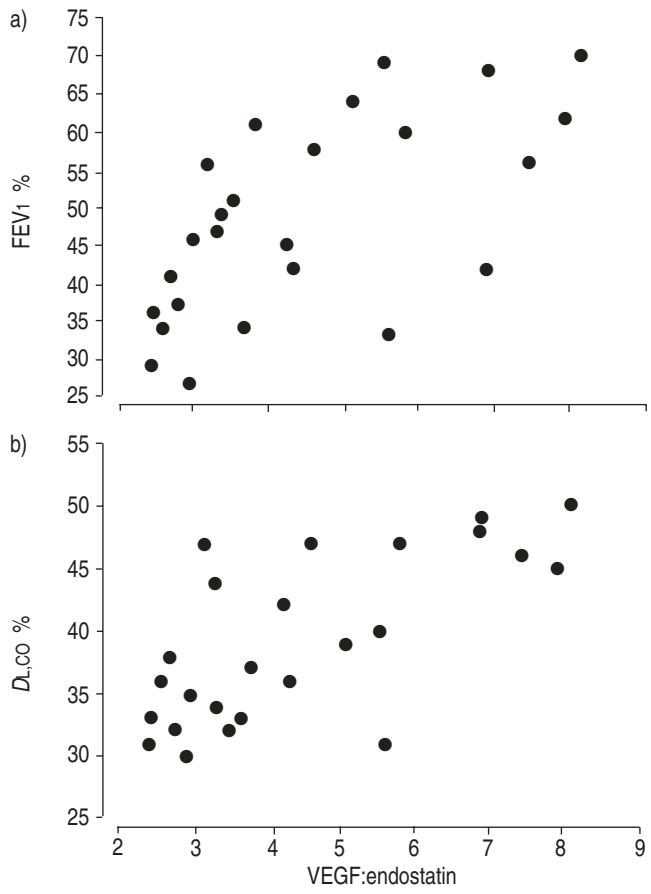

Fig. 4. – Relationship between the ratio of vascular endothelial growth factor (VEGF) to endostatin levels in induced sputum and a) forced expiratory volume in one second (FEV_1) (r=0.669, p=0.0011) and b) carbon monoxide diffusing capacity of the lungs ($D_{L,CO}$) (r=0.654, p=0.0014) in emphysema patients.

patients. Moreover, VEGF levels were significantly correlated with endostatin levels in normal controls but not in emphysema patients. These findings suggest that there is an imbalance between VEGF and endostatin levels in induced sputum from emphysema patients. Since the survival of pulmonary endothelial cells is thought to depend upon the local balance of VEGF and endostatin, endostatin produced in the lungs may participate in negative feedback regulation of the activity of VEGF. Therefore, a decrease in the ratio of VEGF to endostatin levels may result in the loss of pulmonary endothelial cells in emphysema patients.

GERBER *et al.* [11] previously reported that VEGF induces expression of antiapoptotic proteins and that VEGF acts as a survival factor for endothelial cells. Moreover, cigarette smoke may alter maintenance of pulmonary endothelial cells by altering VEGF and VEGF receptor expression and signalling, and result in emphysema due to pulmonary endothelial death, followed by progressive disappearance of alveolar septa due to apoptosis [12]. If the amount of VEGF is sufficiently reduced by cigarette smoking, then alveolar endothelial cells could indeed die as a consequence of a failing VEGF-dependent maintenance programme of endothelial cells. However, if VEGF is critical for maintenance of the alveolar compartment and there is less alveolar compartment tissue in emphysema patients, VEGF levels may be expected to be reduced, since there are fewer distal alveolar septa that require VEGF signalling. Therefore, it cannot be determined whether the decrease in VEGF levels is the cause or consequence of the pathogenesis of emphysema. However, previous

observations have suggested that rats treated with the VEGF-receptor blocker SU5416 develop emphysema, which was preceded by alveolar septal death [13]. Accordingly, a decreased ratio of VEGF to endostatin may be clearly responsible for the pathophysiological manifestations of emphysema. In agreement with this hypothesis, a positive correlation was found between the ratio of VEGF to endostatin levels and FEV_1 and $D_{L,CO}$ values in emphysema patients.

The induced sputum method is not thought to be the best way of evaluating the biochemical markers associated with pulmonary emphysema, as induced sputum generally represents secretions in the airways rather than the lung parenchyma. However, evaluation of the lower respiratory tract and parenchymal lungs by means of induced sputum has been extensively studied in emphysema patients, and the sputum induction method has proved to be a useful and reproducible tool in emphysema patients. A recent study also confirmed that the introduction of sputum induction into the regular work-up of emphysema patients could be useful [14].

Although the dose of saline used in sputum induction did not affect the difference in VEGF levels in individual subjects, as the dose of saline was equal in all, there is a possibility that VEGF was diluted by the larger amount of airway secretions in emphysema patients. Therefore, there is a limitation in the measurement of VEGF levels in this study since these data were not normalised. MEYER *et al.* [15] have demonstrated that the VEGF levels in bronchoalveolar fluid decline with age in normal subjects. However, in this study, emphysema patients and normal controls were well matched with respect to age, although it is possible that ageing is an important factor that influences the VEGF levels in induced sputum. In addition, the emphysema patients in the current study had stopped smoking >1 yr previously. Thus, smoking and ageing effects cannot explain the reduced levels in VEGF in induced sputum from emphysema patients.

In conclusion, in this study, an imbalance was found between vascular endothelial growth factor and endostatin levels in emphysema patients. However, serial measurements of vascular endothelial growth factor and endostatin levels in induced sputum, the imbalance between other angiogenic and antiangiogenic factors, and the measurement of vascular endothelial growth factor and endostatin levels in bronchoalveolar fluid and tissues of lung biopsy in emphysema patients should be examined in future studies.

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