Increased vascular endothelial growth factor in acute eosinophilic pneumonia

Y. Nishigaki*, S. Fujiuchi*, Y. Yamazaki*, H. Matsumoto*, A. Takeda*, Y. Fujita*, K. Okamoto*, T. Fujikane*, T. Shimizu*, K. Kikuchi#

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ABSTRACT: Acute eosinophilic pneumonia (AEP) is associated with the presence of diffuse pulmonary infiltrates on the chest radiograph and an increased number of eosinophils and an elevation of interleukin (IL)-5 levels in bronchoalveolar lavage (BAL) fluid. Vascular endothelial growth factor (VEGF) is a constitutively expressed protein encoded by messenger ribonucleic acid in human eosinophils and is released following stimulation with IL-5. However, the roles of IL-5 and VEGF in the pathogenesis or activity of this disease have not been clarified.

The authors investigated the cells and the levels of these two factors in BAL fluid in five AEP patients and five normal controls before and after corticosteroid treatment.

The absolute number of eosinophils was higher than in controls (53.8 versus 0.3×10⁴·mL⁻¹, 490.1 versus 5.2 pg·mL⁻¹ and 643.0 versus 133.9 pg·mL⁻¹, respectively). IL-5 and VEGF rapidly decreased to the control level in parallel with clinical improvement. The relationship between eosinophilia and IL-5 and VEGF levels was strongly significant.

Elevated interleukin-5 in the lung may initiate the recruitment of eosinophils and enhance the release of mediators, such as vascular endothelial growth factor from eosinophils, which, in turn, increases the permeability of blood vessels.

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Materials and methods

Subjects

Five patients with acute eosinophilic pneumonia were seen in the Division between 1998–2001. Patients were selected for this study on the basis of clinical and pathological criteria. No patients suffered from organic disorders producing peripheral blood eosinophils, such as collagen diseases, allergic bronchopulmonary aspergillosis, sarcoidosis, Hodgkin’s disease, hypersensitive pneumonitis, helminthic infections, or bacterial and mycotic infections with the exception of bronchial asthma.

The patients ranged in age from 18–58 yrs. Four of the patients were males and one was female. Five normal control subjects (four males and one female, aged 55–70 yrs) were also examined. All of the male subjects smoked cigarettes.

The symptoms at admission to the hospital were the presence of dyspnoea and respiratory failure, i.e. mean arterial oxygen tension (PaO₂) level was 7.6 kPa (range 6.1–7.4 kPa; 57.1 mmHg, range 46.2–70.5 mmHg), mean arterial carbon dioxide tension level was 4.9 kPa (range 3.4–5.6; 36.8 mmHg, range 25.4–42.0 mmHg) and the alveolar/arterial PaO₂ difference was 6.1 kPa (range 3.6–7.8 kPa; 45.9 mmHg, range...
27.0-58.8 mmHg). The patients underwent corticosteroid treatment and oxygen therapy, but none needed mechanical ventilation therapy.

**Bronchoalveolar lavage**

The first BAL was performed on the day of admission or the next day, using a flexible fiberoptic bronchoscope (Olympus BF p-30; Olympus, Tokyo, Japan) under local anaesthesia of the upper airway with 2% lidocaine. The bronchoscope was wedged into the subsegmental bronchus of the right middle lobe and 150 mL of normal saline were instilled in 50 mL aliquots. BAL fluid was filtered through sterile nylon mesh and centrifuged at 160 x g for 2 min at 4 °C to obtain the cell preparation. The cells were later stained using the May-Giemsa method and a differential count was performed on 200 cells. The remaining fluid was centrifuged at 500 x g for 5 min and the supernatant was stored at -80 °C until used. Other specimens were used for flow cytometry to determine the CD4:CD8 ratio. Follow-up BAL was performed on days 10-29 after the first BAL when the clinical findings were improved.

**Determination of interleukin-5 and vascular endothelial growth factor**

IL-5 and VEGF were measured by a quantitative sandwich enzyme-linked immunosorbent assay, using antibodies recognising different epitopes of the cytokine molecules. The antibodies used for the assay of IL-5 (a monoclonal antihuman IL-5 capture antibody and a biotinylated monoclonal antihuman IL-5 detection antibody) were obtained from Endogen (Woburn, MA, USA). VEGF was measured using a VEGF-specific antibody (a polyclonal anti-VEGF capture antibody and a biotinylated monoclonal anti-VEGF detection antibody; R&D Systems, Minneapolis, MN, USA). This assay predominantly binds the monomeric VEGF165 but also detects the VEGF isoform containing 121 amino acids. The assay utilised a capture antibody, a biotinylated detection antibody conjugated to streptavidin horseradish peroxidase, and colour development with tetramethylbenzidine/hydrogen peroxide. All assays were performed according to the protocol of the manufacturer. The detection limit was 30 pg·mL⁻¹ for IL-5 and 31.25 pg·mL⁻¹ for VEGF.

**Statistical analysis**

Data are expressed as mean±SEM. For the comparison of data measured before and after treatment, a Wilcoxon-matched paired test was used to determine statistical significance. The Mann-Whitney U-test was used for comparisons between AEP patients and control subjects. Correlation coefficients were calculated using Spearman’s method. Values of p<0.05 were considered significant.

**Results**

**Characteristics of bronchoalveolar lavage fluid cells**

The total number and differential count of leukocytes in BAL fluid are summarised in table 1. The total number of cells·mL⁻¹ in the BAL fluid of AEP patients before treatment was not higher than that after corticosteroid treatment, but was higher than that in normal control subjects (p=0.0088). The percentage of eosinophils was >20% (46.4±8.9%) before treatment. The absolute number of eosinophils·mL⁻¹ in the BAL fluid of AEP patients before treatment was higher than that in normal control subjects (p=0.0071), and this value decreased significantly after corticosteroid treatment (p=0.0431). The absolute number of eosinophils·mL⁻¹ in BAL fluid after treatment, however, was still higher than that in normal control subjects (p=0.0343). The CD4:CD8 ratio in the BAL fluid of AEP patients before treatment was higher than that in the controls (p=0.0283), and decreased to the level of the controls after corticosteroid treatment (p=0.0431).

**Interleukin-5 and vascular endothelial growth factor levels in bronchoalveolar lavage fluid**

IL-5 levels in the BAL fluid of the patients were elevated before treatment and decreased significantly after corticosteroid treatment: 490.1±151.2 versus 6.9±2.7 pg·mL⁻¹ (p=0.0431; fig. 1a).

VEGF levels in the BAL fluid of the patients were also elevated before treatment and, again, decreased significantly

| Table 1. – Characteristics of patients, total and differential counts in bronchoalveolar lavage (BAL) fluid |
|------------------------------------------------------------------------------------------------|
| Patients with AEP | Normal controls |
|-------------------|-----------------|
| Age yrs | 29.8±7.2 | 60.4±2.9 |
| M/F | 4/1 | 4/1 |
| Smoking history | | |
| Smoked | 4 | 4 |
| Exsmoker | 0 | 0 |
| Never smoked | 1 | 1 |
| Total cells ×10⁶·mL⁻¹ | 108.0±41.6* | 41.6±5.7 | 28.0±2.5 |
| Macrophages % | 16.4±2.7 | 54.8±8.9* | 83.6±2.4* |
| Neutrophils % | 13.0±7.7 | 8.4±6.7 | 8.6±3.1 |
| Lymphocytes % | 24.2±6.0 | 21.5±5.4 | 9.4±5.1 |
| Eosinophils % | 46.4±8.9* | 15.8±4.5* | 0.4±0.4 |
| Eosinophils ×10⁴·mL⁻¹ | 53.8±26.4* | 6.3±2.2* | 0.3±0.3 |
| CD4:CD8 ratio | 3.5±0.9* | 1.2±0.2 | 1.6±0.2 |

Data are presented as mean±SEM. Total cell counts and percentage of cells in BAL fluid from acute eosinophilic pneumonia (AEP) and normal control subjects. M: male; F: female. *: p<0.05 comparison of data measured before and after treatment in patients with AEP, *: p<0.05 comparison between patients with AEP and control subjects.
The major findings of high-resolution computed tomography at the height of the disease process in AEP include peripheral ground-glass opacities distributed along pulmonary veins as well as along bronchovascular bundles, thickened interlobular septa, and pleural effusion were found in all cases. Therefore, hypoxaemia and an increase in the alveolar/arterial $P_aO_2$ difference was also present. The authors surmised that these conditions were caused from swelling of the alveolar interstitium with disturbances in the gas exchange.

Eosinophils are blood leukocytes that are recruited in helminthic infections and allergic diseases, especially bronchial asthma [19, 20]. Recent studies have shown that eosinophils synthesise a number of cytokines [21] that may contribute to the pathogenesis of asthma. VEGF is a potent, multifunctional cytokine that exerts several important actions on the vascular endothelium [13, 14]. VEGF was purified on the basis of its ability to induce transient vascular leakage and endothelial cell mitogenesis. It is a heparin-binding growth protein occurring in four molecular isoforms, generated by alternative splicing, which contain 121, 165, 189 or 206 amino acids [22]. VEGF is produced by many cell types, including peripheral blood mononuclear cells [23].

The mRNA encodes for these splice variants in human peripheral blood mononuclear cells and eosinophils, as was demonstrated using reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern hybridisation. RT-PCR of a mononuclear cell population demonstrated three alternatively spliced forms (VEGF121, VEGF165, VEGF189), as previously reported [23], while the analysis of eosinophil-derived VEGF mRNA by RT-PCR yielded two amplification products, which were shown by Southern hybridisation to correspond to VEGF121 and VEGF165 [11]. The VEGF-specific antibody that was used in this assay predominantly binds VEGF165 but will also detect the VEGF isoform containing 121 amino acids.

The percentage of eosinophils and the absolute number of eosinophils--mL$^{-1}$, IL-5 and VEGF levels in the BAL fluid of AEP patients, were elevated before treatment and decreased significantly after treatment in parallel with clinical improvement. The relationship of the absolute number of eosinophils and IL-5 levels in the BAL fluid was strongly significant, as was the relationship of the absolute number of eosinophils and VEGF levels. These data suggest that eosinophils are strongly correlated with VEGF production in BAL fluid in AEP patients.
In humans, eosinophils have the capacity to generate VEGF, a vascular endothelial cell-specific cytokine that mediates angiogenesis and vascular permeability. Human eosinophils constitutively transcribe mRNA encoding VEGF, and store the product, probably in cytoplasmic granules, releasing it following stimulation with GM-CSF or IL-5. VEGF production by activated eosinophils at sites of allergic inflammation may contribute to vascular permeability and subsequent tissue oedema in patients with bronchial asthma [15].

Pulmonary oedema is defined as an excessive amount of extravascular water in the lungs [24]. The major consequence of pulmonary oedema is in the gas exchange units, where swelling of the alveolar interstitium and flooding of the alveoli cause disturbances in lung mechanical functions and gas exchange [25, 26]. Although there are many causes of pulmonary oedema, the pathophysiology of pulmonary oedema can be generally classified as due to increased pressure or increased permeability [27, 28].

Permeability of pulmonary oedema is most commonly linked to local or systemic inflammatory processes, such as those associated with acute infection of the lung or systemic conditions including sepsis, the postoperative state, pancreatitis, burns, and trauma [27]. Overexpression of VEGF in the lung may be one mechanism of increased pulmonary vascular permeability in the early stages of acute lung injury [29]. Production by eosinophils of VEGF, which enhances vascular permeability, might contribute to the pulmonary oedema and disturbances in gas exchange in AEP.

It has been reported that AEP patients respond satisfactorily to steroids and become symptom-free with essentially normal respiratory function without relapse [16]. In the present study, the clinical symptoms of all patients promptly decreased and the photographic findings were improved, along with a decrease in the absolute number of eosinophils, IL-5 and VEGF levels in BAL fluid with glucocorticoid therapy.

The authors hypothesised that the decreases in eosinophils, IL-5 and VEGF levels were caused by the following: IL-5 and VEGF levels in BAL fluid with glucocorticoid treatment, IL-5 and VEGF levels rapidly diminished the steady-state levels of eosinophil VEGF expression by activated eosinophils and subsequent tissue oedema in patients with bronchial asthma [15].

Although the percentage of eosinophils and the absolute number of eosinophils·mL⁻¹ in the BAL fluid of AEP patients did not decrease to the level of normal control subjects after corticosteroid treatment, IL-5 and VEGF levels rapidly returned to the control level in parallel with clinical improvement. The persistence of eosinophil accumulation in the alveolar space was considered to be caused by the increased survival of eosinophils due to IL-5, because the IL-5-induced prolongation of eosinophil survival persists for several weeks [7, 8].

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Fig. 2. – a) Interleukin (IL)-5 level in bronchoalveolar lavage (BAL) fluid correlated with the absolute number of CD4⁺ lymphocytes·mL⁻¹ in BAL fluid from acute eosinophilic pneumonia (AEP) patients (R²=0.502, p=0.0194). The relationships between b) IL-5 level and absolute number of eosinophils in BAL fluid from AEP patients (R²=0.762, p=0.0004), c) absolute number of eosinophils and vascular endothelial growth factor (VEGF) level in BAL fluid from AEP patients (R²=0.971, p=0.0011) were all significant.
The major findings of the present study were: 1) interleukin-5 could be recruited into the lung by eosinophils in acute eosinophilic pneumonia patients; and 2) a correlation was found between increased interleukin-5 and vascular endothelial growth factor levels and eosinophilia in bronchoalveolar lavage fluid. In conclusion, it has been demonstrated that vascular endothelial growth factor may play an important role in the pathogenesis of pulmonary oedema in patients with eosinophilic pneumonia.

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