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Overexpression of CD44 on alveolar eosinophils with high concentrations of soluble CD44 in bronchoalveolar lavage fluid in patients with eosinophilic pneumonia

Key words: bronchoalveolar lavage fluid; CD44; eosinophil; eosinophilic pneumonia; human; hyaluronic acid; interleukin-5.

Background: High levels of interleukin-5 (IL-5) are found in the alveolar space of patients with eosinophilic pneumonia (EP). IL-5 promotes the growth and differentiation of eosinophils, as well as activating these cells. IL-5 also induces the expression of CD44 on eosinophils *in vitro*. To evaluate the contribution of CD44 to the pathogenesis of EP, we examined the expression of CD44 on eosinophils in bronchoalveolar lavage fluid (BALF) and measured the concentration of soluble CD44 (sCD44) in BALF from patients with EP.

Methods: The concentrations of IL-5, sCD44, and hyaluronic acid (HA) were measured in BALF. The expression levels of CD44 on eosinophils in BALF and peripheral blood in patients with EP were compared.

Results: The expression of CD44 on alveolar eosinophils and the concentration of sCD44 were increased in BALF of patients with EP. There was a significant correlation between IL-5 and sCD44 in BALF. A high concentration of HA was observed in BALF of EP patients.

Conclusions: Our results suggest that the high expression of CD44 on eosinophils probably results from upregulation by IL-5 and could be important in the pathogenesis of EP.

Abbreviations: BALF: bronchoalveolar lavage fluid; EP: eosinophilic pneumonia; FCS: fetal calf serum; FITC: fluorescein isothiocyanate; HA: hyaluronic acid; HV: healthy volunteer; IL: interleukin; MFI: mean fluorescence intensity; PB: peripheral blood; PBS: phosphate-buffered saline; PE: phycoerythrin.

Eosinophilic pneumonia (EP) is characterized by infiltration of eosinophils into the alveolar spaces and interstitium of the lung that may or may not be associated with

eosinophilia in the peripheral blood (1). Several cytokines are known to enhance the production of eosinophils, including granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5 (2, 3). IL-5 is mainly effective on eosinophils in man (4), and its level is elevated in bronchoalveolar lavage fluid (BALF) in EP (5, 6). However, the immunopathogenic mechanisms of EP are still unclear.

Various adhesion molecules are known to be involved in the infiltration of eosinophils into the airway mucosa and alveolar space through the vascular endothelial cells (7, 8). One of these adhesion molecules, very late activation antigen-4 (VLA-4), may be important in eosinophil influx into the lung in EP (9). CD44 is a cell adhesion molecule that is widely distributed and is thought to be involved in several physiologic processes (10). For example, CD44 has been shown to be involved in homing to mucosal lymphatic tissue and lymphocyte infiltration into inflammatory tissues (11, 12). CD44 also functions as a receptor for hyaluronic acid (HA) (13, 14). These functions presumably result from the ability of CD44 to interact with extracellular matrix components (15). IL-5 can induce the expression and function of CD44 on lymphocytes (16, 17). The cytokine also induces the expression of CD44 on eosinophils *in vitro* (18). As a transmembrane protein, CD44 can potentially transmit signals (19). While little is known about the function of CD44 on eosinophils, the increased expression of CD44 on activated eosinophils by IL-5 suggests that CD44 signaling could be involved in their adhesion and activation. In the present study, to investigate the mechanisms of eosinophil accumulation in the airway and lung of EP, we measured the level of CD44 expression on eosinophils in the peripheral blood and BALF of EP patients. We also examined the relationship between soluble CD44 (sCD44) and IL-5 in BALF, and measured the levels of HA, representing one of the ligands of CD44, in BALF in EP.

Material and methods

Subjects

Eight patients with EP were enrolled in the present study. They included four men and four women, aged 38.1 ± 18.8 years (mean $\pm$ SD, two smokers and six nonsmokers). Diagnosis was based on clinical, laboratory, and chest radiographic data, and histopathologic findings of transbronchial lung biopsy. The latter was performed after bronchoalveolar lavage (BAL) in the same lung lobe or segment

showing radiologic abnormalities. EP patients were not on any treatment at the time of the study. They had no history of bronchial asthma or drug allergy. The pathogenesis of these cases of EP was unknown.

Eleven healthy volunteers (HV) were included as control subjects (10 men and one woman, aged 24.7 ± 5.8 years). They included one smoker and 10 nonsmokers. None of these subjects had a history of pulmonary disease. Human subject involvement in this study was approved by our institutional review board and with appropriate informed consent.

Cell preparation

BAL was performed by the method described previously (20). Briefly, after we obtained informed consent from each subject, BAL was done with 150 ml of sterile saline in 50-ml aliquots with immediate gentle aspiration after each instillation in either the middle lobe (control subjects) or in the radiologically affected lobe (EP patients). Immediately after collection, BALF was filtered through several layers of surgical gauze and centrifuged at 160 g for 2 min to obtain the cell fraction. The total cell count was determined, and the cells were stained with May-Giemsa, followed by differential cell count with 200 cells.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD44 monoclonal antibody (mAb) was obtained from Seikagaku Co. (Tokyo, Japan). FITC-conjugated anti-VLA-4 (CD49d) mAb, mouse IgG1, and phycoerythrin (PE)-conjugated anti-CD16 mAb, mouse IgG1 were purchased from Coulter Co. (Tokyo). FITC-conjugated anti-CD9 mAb was obtained from Cosmo Bio Co. (Tokyo).

Flow cytometry

After lysis of red blood cells, peripheral blood leukocytes and BALF cells were washed with phosphate-buffered saline (PBS) and stained with anti-CD44-FITC, or anti-VLA-4-FITC. Then they were analyzed by FACScan[®] (Becton Dickinson). Eosinophils were gated by flow cytometry based on forward and side light scatter. The purity of eosinophils in the sample was assessed by staining with anti-CD9-FITC and anti-CD16-PE, as described by Azuma et al. (21). More than 95% of leukocytes in the eosinophils gate were CD9-positive and CD16-negative.

Stimulation of eosinophils with IL-5

Ethylenediamine tetraacetic acid (EDTA)-anticoagulated blood samples were obtained from patients with EP before BAL. Granulocytes were separated by the dextran sedimentation method, and centrifuged on Ficoll-Paque-gradient (Pharmacia Biotech, Tokyo). Contaminating red blood cells were eliminated by hypotonic lysis. Eosinophils were purified (>95%) by discontinuous density gradient centrifugation with Percoll (Pharmacia Biotech), as previously described by Matsumoto et al. (18). Cells (1×10^6 /ml) were cultured with or without IL-5 (1 ng/ml) for 3 days in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% fetal calf serum (FCS).

Measurement of IL-5, sCD44, and HA

The concentration of IL-5 was measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 50 μ l (4 μ g/ml) of mAb to human IL-5 (TRFK5, Pharmingen, San Diego, CA, USA) was bound to microtiter plates by incubation at 4°C overnight. The wells were washed twice with PBS containing 0.05% Tween 20. After blocking with 400 μ l of PBS/10% FCS for 2 h, 200 μ l BAL supernatant and recombinant human IL-5 (Pharmingen) were added to each well and incubated at room temperature overnight with gentle agitation. After washing twice with 0.05% Tween/PBS, 100 μ l (4 μ g/ml) of biotinylated anti-human IL-5 mAb (JES1-5A10, Pharmingen) was added to each well and incubated at room temperature for 2 h, followed by further 1-h incubation with 100 μ l of peroxidase-conjugated

streptavidin. Wells were subsequently washed five times with 0.05% Tween/PBS and incubated with 0.11 M sodium acetate buffer (pH 5.5) containing tetramethylbenzidine for 15 min. The reaction was stopped by adding 100 μ l of 1.8 M H₂SO₄ to each well. Absorbance of the plates was read at 450 nm in an ELISA reader. The assay was linear over the range of approximately 10–320 pg/ml and was specific for IL-5 without cross-reactivity with recombinant IL-3 or GM-CSF. The average level from duplicate assays was reported. The concentration of sCD44 in BALF was measured with an ELISA kit (Bender Meal Systems, Vienna, Austria). HA was measured by the sandwich binding protein assay kit (Chugai Pharmaceutical Co., Tokyo). The absolute values of soluble factors were reported in this study, because the level of the cytokines was not influenced by the concentration of albumin in our previous study (20).

Statistical analysis

All data were expressed as mean $\pm$ SE. Differences between groups were examined for statistical significance by the paired Student's *t*-test or Mann-Whitney test. Pearson's correlation coefficient was used to examine the correlation between various parameters. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

Results

High concentrations of IL-5 in BALF

We first compared the total and differential cell counts in BALF of EP patients to those from HV (Fig. 1). In general, hypercellularity was found in BALF samples obtained from EP patients. The absolute number of BAL cells was significantly elevated in EP compared with HV ($P < 0.001$). Furthermore, approximately half of BAL cells from patients with EP were eosinophils, compared to a very small number in controls (Fig. 1). The numbers of lymphocytes and neutrophils in BALF samples from EP patients were also significantly higher than in HV ($P < 0.001$ and $P < 0.05$, respectively).

To evaluate the role of IL-5 in lung eosinophilia, we compared the concentrations of IL-5 in BALF and serum from each patient. As shown in Fig. 2, higher levels of IL-5 were observed in unconcentrated BALF than in serum taken from the same patients ($P < 0.01$). These data suggest that locally produced IL-5 could play an important role in EP.

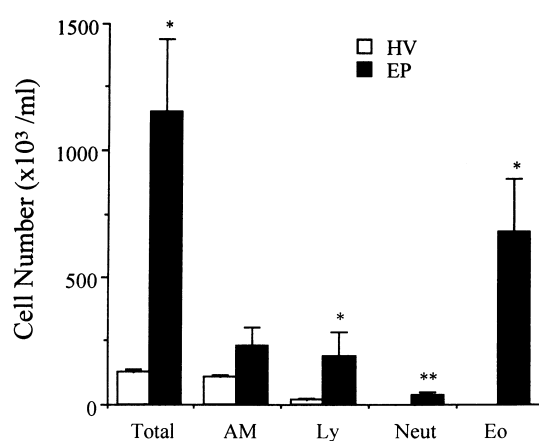


Figure 1. Cellular composition of BALF in healthy volunteers (HV) and patients with eosinophilic pneumonia (EP). Total: total cell count, AM: alveolar macrophages, Ly: lymphocytes, Neut: neutrophils, Eo: eosinophils. Data represent mean $\pm$ SE of eight patients and 11 control subjects. * $P < 0.001$, ** $P < 0.05$, compared with respective values in HV.

High CD44 concentration and its overexpression on eosinophils in BALF

In the next step, we compared the expression of CD44 on alveolar eosinophils to that on peripheral blood eosinophils in EP patients with eosinophilia. In three samples, we also examined the expression of VLA-4, which is primarily expressed on the surface of eosinophils, but not on neutrophils. As shown in Fig. 3, alveolar eosinophils, as compared to eosinophils from peripheral blood, overexpressed CD44 ($P < 0.01$, $n = 5$). In contrast, the levels of VLA-4 on alveolar eosinophils were lower than those found on peripheral blood eosinophils ($P < 0.05$, $n = 3$).

The soluble form of CD44 is thought to originate from CD44 on the cell surface by a proteolytic cleavage mechanism (22). Therefore, we examined concentrations of sCD44 in BALF of patients with EP. Significantly elevated concentrations of sCD44 were detected in BALF in EP as compared with normal controls ($P < 0.001$, Fig. 4). There was no statistically significant difference between sCD44 in serum of patients with EP and that of controls (data not shown). We also examined the relationship between the percentages of eosinophils and the concentrations of sCD44 in BALF (Fig. 5A). There was a significant correlation between the percentage of eosinophils and the concentration of sCD44 in BALF ($r = 0.88$, $P < 0.0001$). Similarly, the correlation between the absolute number of eosinophils and concentration of sCD44 was also significant ($r = 0.62$, $P = 0.0041$) (data not shown). Furthermore, we found a significant relationship between concentrations of IL-5 and amounts of sCD44 in BALF ($r = 0.69$, $P = 0.0007$) (Fig. 5B).

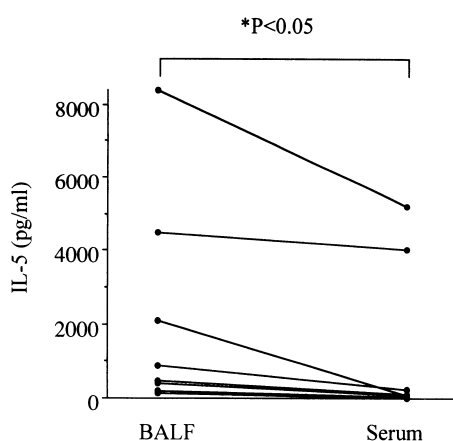


Figure 2. Comparison of IL-5 concentrations between BALF and serum in patients with EP. IL-5 concentrations were measured by ELISA in BALF and serum from eight patients with EP. Data represent values of paired samples obtained from same patient. Note that IL-5 levels in BALF are significantly higher than those in serum (* $P < 0.05$).

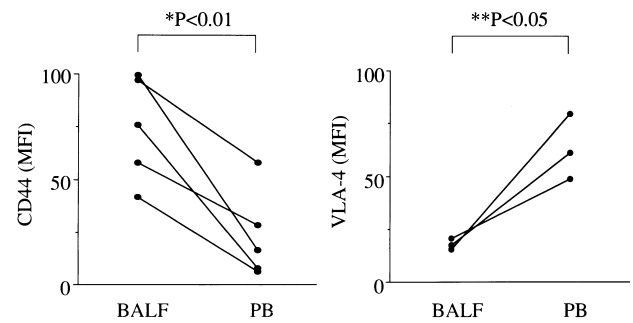


Figure 3. Comparison of expression levels of CD44 and VLA-4 on eosinophils from BALF and peripheral blood (PB). Data of CD44 (left panel) and VLA-4 (right panel) represent values of paired samples obtained from same patient and determined by flow cytometry. Statistical significance of differences between BALF and PB was evaluated by paired Student's *t*-test. Cell surface CD44 and VLA-4 values are expressed as mean fluorescence intensity (MFI).

These findings suggest that sCD44 in BALF in EP probably originated from CD44 on the surface of eosinophils, and that high expression levels result from abundant IL-5 present in the lung. To test this possibility, we stimulated peripheral blood eosinophils from EP patients with IL-5 *in vitro* and then examined the expression of CD44 and VLA-4 by flow cytometry. As shown in Fig. 6, IL-5 treatment increased the expression of CD44 but reduced VLA-4 on these cells.

High levels of HA in BALF

Previous studies have demonstrated potential binding sites for HA in all CD44 isoforms and leukocytes stimulated by HA (23, 24). We measured and compared the concentrations of HA in BALF of EP patients and HV. As shown in Fig. 7, elevated levels of HA were found in BALF in EP ($P < 0.001$).

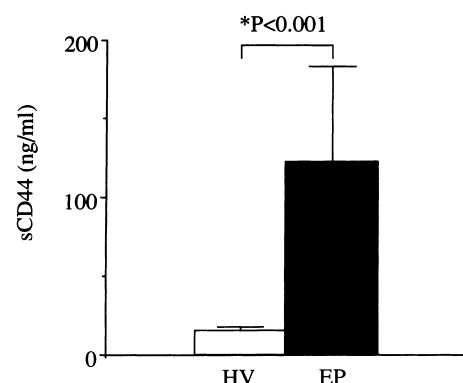


Figure 4. Soluble CD44 (sCD44) levels in BALF from healthy volunteers (HV) and patients with EP. Concentration of sCD44 in BALF from HV (open bar) and EP (closed bar) was determined by ELISA. Values represent mean $\pm$ SE of eight patients and 11 control subjects.

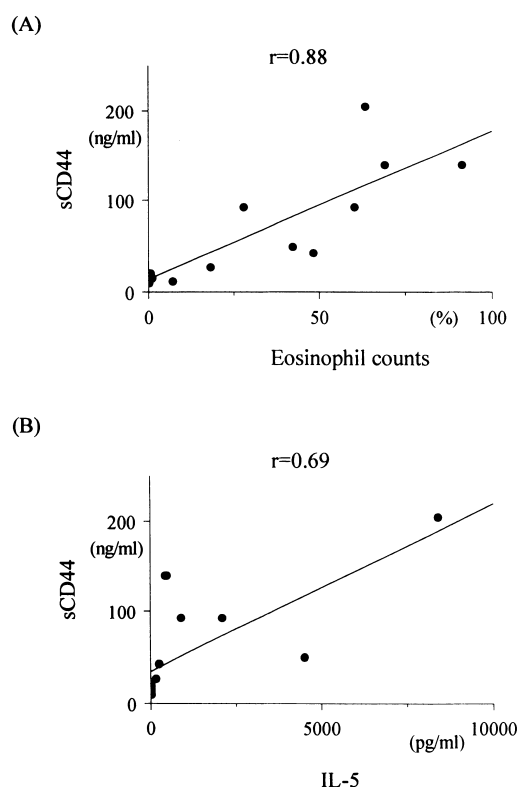


Figure 5. Relationship between concentration of sCD44 and percentages of eosinophils in BALF (A), and IL-5 concentrations in BALF (B). Note significant correlations between sCD44 concentrations and percentages of eosinophils as well as IL-5 concentrations in BALF.

Discussion

Transmigration of eosinophils from the circulation into the alveolar spaces is a characteristic feature in EP (1), and IL-5 is one of the important factors contributing to pulmonary eosinophilia (5). Locally high levels of IL-5 were reported in two patients with acute EP (6). We now describe higher concentrations of IL-5 in BALF than serum, in eight patients with EP (Fig. 2). A previous study (6) and our data suggest that IL-5 is locally produced in the lung in EP. Since IL-5 in BALF exhibits a chemotactic activity toward eosinophils (25), locally produced IL-5 may play a key role in the pathogenesis of EP.

Recent studies demonstrated that stimulation with IL-5 increases the level of CD44 expression on eosinophils from peripheral blood of normal subjects, and that there are high levels of CD44 on eosinophils present in the sputum, relative to those in peripheral blood, of patients with bronchial asthma (18, 26). We now demonstrate that IL-5 treatment *in vitro* upregulates CD44 on eosinophils obtained from peripheral blood in EP. Our study also showed increased expression of CD44 on alveolar eosinophils and high concentrations of sCD44 in BALF in patients

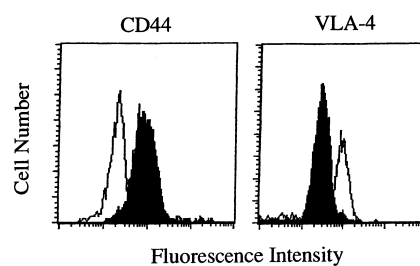


Figure 6. Effect of IL-5 on expression of CD44 and VLA-4 on eosinophils from peripheral blood in patients with EP. Peripheral blood eosinophils were cultured with (shaded area of graph) or without (unshaded area) 1 ng/ml of IL-5. Expression levels of CD44 and VLA-4 were analyzed by flow cytometry. Representative results of three independent experiments are shown.

with EP. Furthermore, we demonstrate a positive correlation between the percentage/number of eosinophils and sCD44 in BALF, and between IL-5 and sCD44 in BALF. These findings suggest that IL-5 upregulates the expression of CD44 on eosinophils in patients with EP. Increased expression of CD44 on B cells activated by IL-5 has been reported previously (16, 17). Furthermore, CD44 is involved in the infiltration of lymphocytes into the inflammatory tissues (12). Thus, it is possible that the high density of CD44 on eosinophils in EP is responsible for their recruitment to the lung.

CD44 is a broadly expressed cell adhesion molecule on leukocytes and can certainly function as a signal transmitting receptor for hyaluronic acid (13, 14). For example, ligation of CD44 on monocytes triggers production of insulin growth factor-1 (IGF-1) and several other cytokines (27, 28). CD44- and HA-dependent extravasation of activated T cells has recently been reported (12). Furthermore, interactions between HA and neutrophil CD44 might

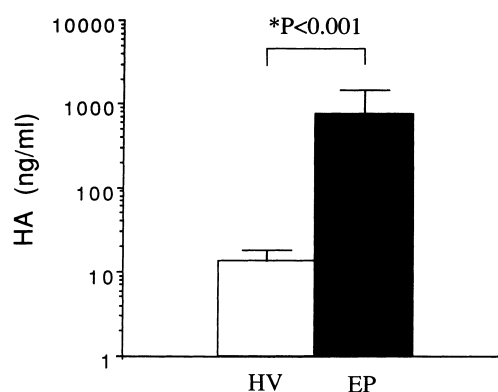


Figure 7. Comparison of concentrations of hyaluronic acid (HA) in BALF from healthy volunteers (HV) and patients with EP. HA levels were determined as described in Material and methods. Values represent mean $\pm$ SE of eight patients and 11 control subjects. Note significant differences in levels of HA between HV and EP ($P < 0.001$).

initiate cytotoxic or inflammatory responses (24). However, little is known about the function of CD44 on eosinophils. Enhanced proliferation of eosinophil precursors was found when they were cultured with HA (29). We have now found overexpression of CD44 on eosinophils and correspondingly increased levels of HA in BALF in EP. Interactions between HA and CD44 on eosinophils might contribute to the pathogenesis of EP. Further studies are necessary to clarify the role of HA in the function of CD44 expressed on eosinophils in patients with EP.

In conclusion, we demonstrated locally produced IL-5 and overexpression of CD44 on alveolar eosinophils compared

with peripheral blood eosinophils in patients with EP. We also demonstrated high levels of sCD44 and HA in BALF in EP. These findings suggest that IL-5 and CD44 on eosinophils play an important role in eosinophilic inflammation of the lung.

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