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Original Article

CD44 on blood eosinophils as a novel marker of bronchial asthma management

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ABSTRACT

Bronchial asthma (BA) is characterized by infiltration of the respiratory tracts by eosinophils. A wide variety of adhesion molecules is involved in the binding of eosinophils to the vascular endothelium and their subsequent transmigration from the circulation to the airways, while little is known about CD44 expression on eosinophils. In the present study we introduce a novel staining combination with which surface markers on eosinophils can be analyzed without purification prior to staining and examined whether the expression of CD44 on peripheral blood eosinophils could be monitored as a marker of the control of BA. Staining of eosinophils with anti-CD16 and anti-VLA-4 monoclonal antibodies enabled us to delineate eosinophils as VLA-4^{high}CD16^{-} cells from other leukocyte populations in the whole blood. CD44 was constitutively expressed on resting eosinophils and expression increased following cytokine-mediated activation. In all BA patients examined, $CD44^{high}$ eosinophils were enriched in sputum relative to peripheral blood, indicating that eosinophils in sputum were more activated than those in the blood. By comparing the extent of CD44 expression on blood eosinophils from poorly controlled and well-controlled asthma patients, we unexpectedly found that the density of CD44 expression is lower on blood eosinophils from the poorly controlled group. Thus, the extent of CD44 expression on blood eosinophils defined as VLA-4^{high}CD16^{-} cells is a novel marker indicative of the management of BA.

Key words: bronchial asthma, CD44, eosinophils, flow cytometry.

INTRODUCTION

Bronchial asthma (BA) is an inflammatory disease of the respiratory tracts characterized by infiltration of the airways by a variety of inflammatory cells, including eosinophils and lymphocytes.[1–4] Adhesion molecules expressed on endothelial and inflammatory cells play a pivotal role in the initial stage of the translocation process.[5,6] CD44 was an adhesion molecule involved in lymphocyte transmigration from the circulation to inflammatory sites[7] or to the thymus,[8] although the CD44 expression on eosinophils has not been studied extensively.

Eosinophils comprise a minor leukocyte population in peripheral blood. Changes in the number and proportion of blood eosinophils have been used as indicators of the asthmatic condition.[9] Characteristic staining of eosinophil granules by various dyes enables discrimination of this subset from other leukocytes; however, the lack of specific surface markers for eosinophils has hampered the easy identification of the population by flow cytometry. No surface markers on eosinophils are monitored as a good marker of the course of deterioration and amelioration of BA.

In the present report we introduce a novel staining combination with which surface markers on eosinophils can be analyzed without purification prior to staining and examined whether the extent of CD44 expression on eosinophils correlated with the control of BA. CD44

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expression on eosinophils increased following cytokine-mediated activation. In BA patients, $CD44^{high}$ eosinophils were enriched in sputum relative to peripheral blood. Surprisingly, the level of CD44 expression on blood eosinophils was lower in poorly controlled patients compared with well-controlled patients. Thus, the level of CD44 expression on eosinophils could be a useful novel marker indicative of the management of BA.

METHODS

Subjects

Asthmatic patients who had been treated at the Tohoku University Hospital were chosen as subjects. The patients, with various BA severity, were separated into two groups based on peak expiratory flow (PEF), measured using the Assess Peak Flow Meter (HealthScan Products Inc., Cedar Grove, NJ, USA) at the time of blood sampling. Patients with PEF values of over 80% or less than 60% of the expected maximal value were categorized as well-controlled or poorly controlled, respectively. The expected maximal PEF was determined for each patient according to the manufacturer's data table. The mean (± SD) daily doses of corticosteroids taken by each patient in the well- and poorly controlled groups are as follows: oral prednisolone at 1.50 ± 2.41 and 0.58 ± 1.45 mg/day, respectively; inhaled beclomethasone at 850 ± 845 and 815 ± 600 μg/day, respectively. No significant difference in treatment was noted between the two groups.

Reagents used for flow cytometry

An anti-VLA-4 (CD49d) monoclonal antibody was kindly provided by Dr K Miyake (Saga Medical University).[10] The anti-VLA-4 monoclonal antibody (mAb) was partially purified from ascites by precipitation with 40% saturated ammonium sulfate and was conjugated to fluorescein isothiocyanate (FITC; Sigma Chemical Co., St Louis, MO, USA) in our laboratory. Phycoerythrin (PE)-conjugated anti-CD44, biotinylated anti-CD16, Quantum Red-conjugated streptavidin and propidium iodide were all purchased from Sigma Chemical Co.

Preparation of blood eosinophils and flow cytometry

Heparinized whole blood cells were centrifuged in dextran to remove red blood cells. The resulting leukocyte populations (2×10^6 cells) were stained with anti-VLA-4-FITC, anti-CD44-PE, anti-CD16-biotin and streptavidin-Quantum Red. Dead cells were excluded from analysis by propidium iodide staining. Stained cells were analyzed using FACScan (Becton Dickinson, Cockeysville, MD, USA). Monocyte and lymphocyte populations were discriminated by forward and side-scatter properties and were excluded from analysis. The remaining granulocyte population was composed primarily of neutrophils in addition to eosinophils. Eosinophils could be selectively acquired by gating on $VLA\text{-}4^+$, $CD16^-$ cells because neutrophils are $VLA\text{-}4^-$, $CD16^+$.[11–13] By this staining and gating combination, eosinophils comprising greater than 2% of whole leukocyte counts could be reproducibly demarcated from other leukocyte populations without eosinophil purification prior to staining. The stability and reproducibility of the flow cytometry analysis was verified by running fluorescinated calibration beads (Polysciences Inc., Warrington, PA, USA) before each analysis. Results are expressed as the mean fluorescence intensity (MFI).

Preparation of sputum eosinophils

Sputa expectorated spontaneously or induced by 10 min inhalation of 3% NaCl aerosol were collected from BA patients with varying PEF values. The obtained sputa were mixed with three volume of Sputasol (Unipath Ltd, Hampshire, England), shaken for 30 min at room temperature, filtered through nylon mesh to remove debris and used for staining. Pretreatment of eosinophils with Sputasol did not alter the level of CD44 expression (data not shown).

Activation of eosinophils *in vitro*

Blood taken from normal volunteers was depleted of erythrocytes, as described earlier, and was centrifuged through lymphocyte separation medium (Organon Teknita Corp., Durham, NC, USA) to remove mononuclear cells. The enriched granulocyte population, including neutrophils and eosinophils, was cultured at a concentration of 5×10^6 /mL in the presence of titrated concentrations of granulocyte–macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3 or IL–5. These cytokines were purchased from R & D Systems (Minneapolis, MN, USA). After 24 h, cells were harvested, stained and analyzed as described earlier.

RESULTS

Establishment of a novel method to demarcate blood eosinophils by flow cytometry

We started by establishing a method by which eosinophils could be differentiated from among heterogeneous blood leukocyte populations by flow cytometry. The three distinct populations of peripheral blood leukocytes, lymphocytes, monocytes and granulocytes, were differentiated by forward- and side-scatter properties and a gate was set to exclude lymphocytes and monocytes from analysis (Fig. 1a). The remaining granulocyte population could be clearly divided into two subpopulations based on the expression of CD16 and VLA-4 (CD49d; Fig. 1b). Neutrophils express high levels of CD16 and are negative for VLA-4 expression, while eosinophils are $CD16^-$ and $VLA\text{-}4^+$.[12,13] The validity of this method for eosinophil identification was confirmed with experiments using highly purified eosinophils. Forward- and side-scatter values for the eosinophils did not exceed the limits of the granulocyte gate (Fig. 1c). Staining of the purified eosinophils with anti-VLA-4 and anti-CD16 mAbs verified this method of eosinophil identification, as the majority of eosinophils were positive for VLA-4 expression and negative for CD16 expression (Fig. 1d). By means of this method, eosinophils comprising greater than 2% of peripheral leukocytes could be reproducibly isolated without prior purification steps (data not shown). In the following experiments, eosinophils were stained with anti-CD44 in addition to anti-CD16 and anti-VLA-4 and CD44 expression on gated eosinophils was examined.

Increased expression of CD44 on activated eosinophils

To determine the effects of pro-inflammatory cytokines on the CD44 expression of eosinophils, we cultured peripheral blood granulocytes prepared from healthy donors

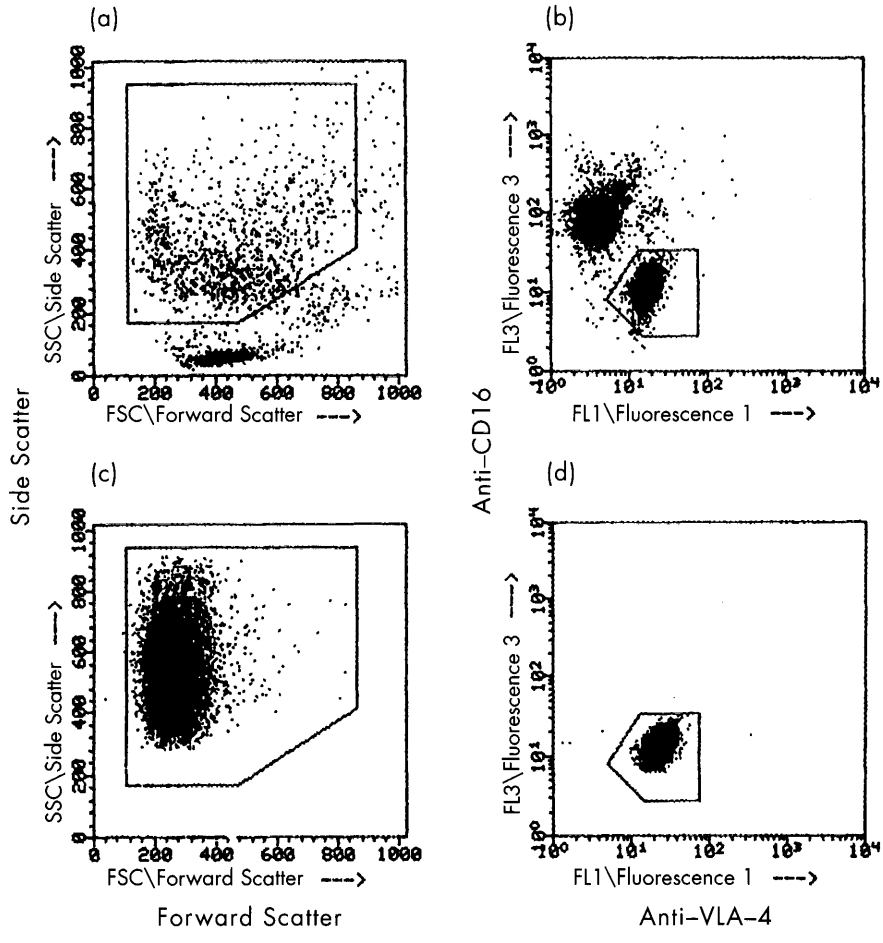

Fig. 1 Selective acquisition of eosinophils by flow cytometry. (a) Dot plot analysis of whole blood leukocytes is shown. Forward scatter/side scatter gating (granulocyte gate) was used to select a granulocyte fraction. (b) The correlation between VLA-4 and CD16 expression for the granulocyte fractions identified in (a). A $VLA\text{-}4^+$, $CD16^-$ fraction (eosinophil gate) was acquired for analysis of eosinophils using a FACScan. (c) Highly purified eosinophils from healthy subjects were analyzed by flow cytometry. The purified eosinophils fell precisely within the granulocyte gate. (d) The purified eosinophils were $VLA\text{-}4^+$, $CD16^-$ cells, which verified the propriety of the eosinophil gate in (b).

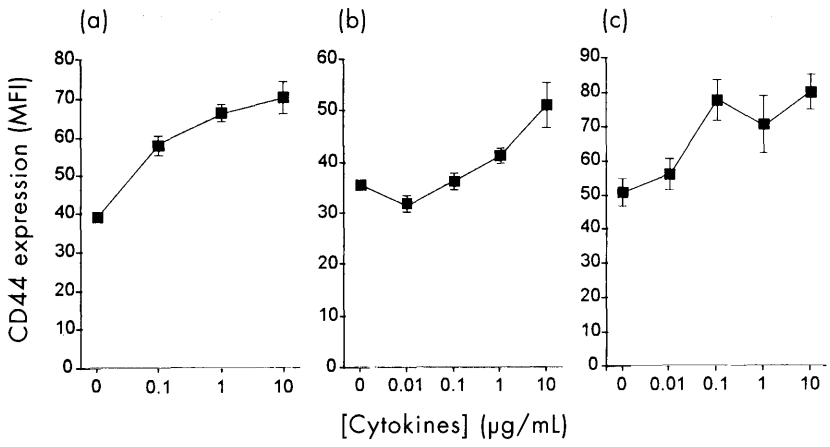

Fig. 2 Increased expression of CD44 on eosinophils activated by cytokines. Human blood eosinophils prepared from healthy subjects were cultured for 24 h in the presence of titrated concentrations of interleukin (IL)-5 (a), IL-3 (b) and granulocyte–macrophage colony stimulating factor (c). The CD44 expression on the activated eosinophils was determined by flow cytometry. Individual points represent the mean ± SD of triplicate cultures. MFI, mean fluorescence intensity.

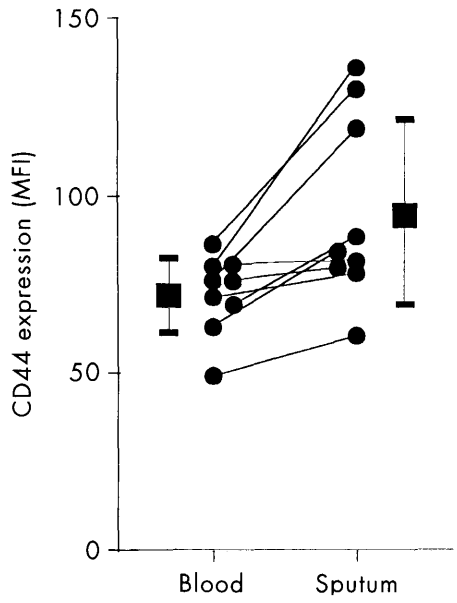

Fig. 3 Enrichment of CD44^{high} activated eosinophils in sputum from asthmatic patients. Leukocytes from the sputum and peripheral blood of asthmatic patients with various peak expiratory flow values were stained with a combination of anti-VLA-4, anti-CD44 and anti-CD16 monoclonal antibodies and the expression of CD44 on gated eosinophils was compared in each patient (●—●). The mean (■) ± SD (—) of each group is also shown. The CD44 expression on sputum eosinophils was significantly higher than that on blood eosinophils ($P < 0.01$; paired *t*-test). MFI, mean fluorescence intensity.

in the presence of titrated concentrations of IL-5, IL-3 or GM-CSF for 24 h and examined the expression of CD44 on eosinophils by flow cytometry. Eosinophils constitutively expressed CD44 and expression was increased in a dose-dependent manner by IL-5 (Fig. 2a), IL-3 (Fig. 2b) or GM-CSF (Fig. 2c). Thus, eosinophils, in an activated state, were found to express increased levels of CD44.

Enrichment for CD44^{high} eosinophils in sputum relative to peripheral blood

We compared the level of the CD44 expression on eosinophils prepared from sputum to that of peripheral blood eosinophils. In all BA patients with varying PEF values, CD44 expression on eosinophils from sputum was higher than that of peripheral blood. The average MFI of CD44 on sputum eosinophils (95.1 ± 26.1) was significantly higher than that of blood eosinophils (72.5 ± 11.0; $P < 0.01$; Fig. 3). This result indicates that eosinophils are more activated in sputum than in the peripheral blood of asthmatic patients.

Activated eosinophils in peripheral blood were reduced in poorly controlled asthmatic patients

Increased numbers of eosinophils migrate into the respiratory tracts in exacerbated disease.[13] This fact led us to speculate that blood eosinophils may be more activated in exacerbated compared with well-controlled cBA and that this increased activation would be reflected by increased CD44 expression. To examine this hypothesis, CD44 expression was compared on peripheral blood eosinophils from BA patients with well-controlled (PEF > 80% expected value) and exacerbated (PEF < 60% expected value) disease. The level of CD44 expression on peripheral blood eosinophils from well-controlled patients ranged from 45.0 to 124.6 MFI, with a mean (± SD) of 73.4 ± 23.2 MFI (Fig. 4a). This result was almost

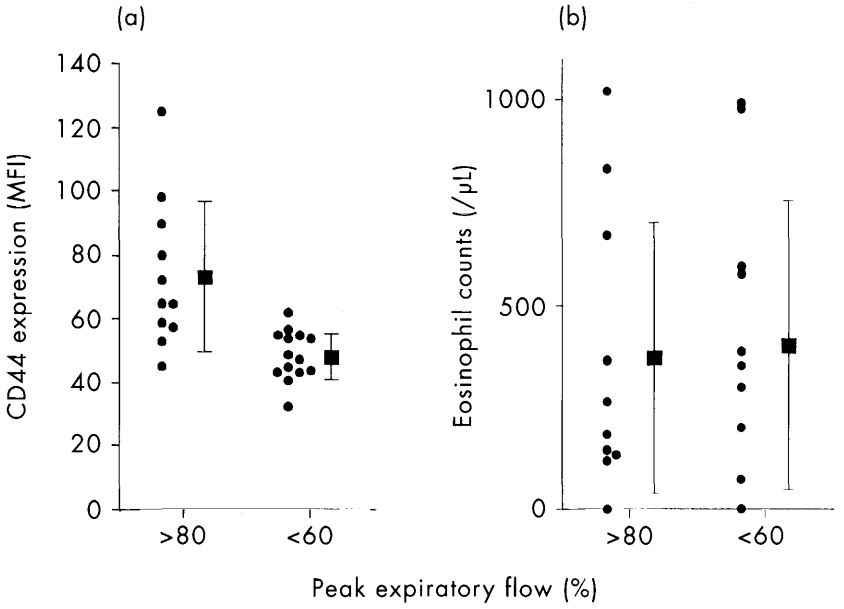

Fig. 4 A decrease in activated peripheral eosinophils in exacerbated asthmatic patients. (a) Peripheral blood eosinophils from either well-controlled asthmatic subjects (> 80% expected peak expiratory flow; PEF) or poorly controlled subjects (< 60% expected PEF) were analyzed for CD44 expression by flow cytometry. The expression of CD44 on gated eosinophils in each patient (●) and the mean (■) ± SD (—) of each group is shown. It should be noted that CD44 expression on peripheral eosinophils was lower in exacerbated asthmatic patients than in well-controlled patients ($P < 0.002$). (b) The number of peripheral blood eosinophils between the two groups was compared. No difference was noted. MFI, mean fluorescence intensity.

comparable to that of normal subjects (67.7 ± 18.1 MFI). In sharp contrast, the level of CD44 expression on peripheral blood eosinophils from poorly controlled patients was significantly lower ($P < 0.002$), with an average MFI of 48.3 ± 7.9 and a range of 32.1–61.8. There was not a significant difference in the mean number of peripheral blood eosinophils between well-controlled (410 ± 328) and poorly controlled (403 ± 408) groups (Fig. 4b). Thus, we found that eosinophils in peripheral blood from poorly controlled patients were in a less active state than those from well-controlled patients, with respect to CD44 expression. This result indicates that the extent of CD44 expression on blood eosinophils could be a novel marker indicative of the management of BA.

Discussion

One of the characteristic features of BA is pathogenic invasion of the respiratory tract by eosinophils that are lured from the circulation.[14] Activation of eosinophils in response to cytokines such as IL-3, IL-5 or GM-CSF[15–17] leads to the increased expression of adhesion molecules, including LFA-1 and Mac-1, which is an essential step in the transmigration from circulation to respiratory tract. CD44, an adhesion molecule specific for hyaluronic acid, is known to be expressed on various leukocyte populations. Its critical role in lymphocyte homing to inflammatory sites[7] and the thymus[8] has been well documented, while little is known about CD44 expression on eosinophils.

In the present report we examined CD44 expression on eosinophils from BA patients. CD44 is constitutively expressed on eosinophils and its expression level increases following activation by cytokines. Because a higher degree of respiratory tract infiltration by eosinophils is observed in exacerbated BA compared with well-controlled disease, we originally speculated that CD44 expression on blood eosinophils would be higher in poorly controlled asthmatic patients than in well-controlled patients. Surprisingly, we found that this is not the case. CD44 levels on blood eosinophils were lower in cases of poorly controlled disease than in well-controlled subjects, indicating a reduction of activated blood eosinophils in exacerbated BA. This result could be at least in part ascribed to the enhanced transmigration of the activated, $CD44^{high}$ eosinophils to the respiratory tract, because eosinophils in sputum expressed higher levels of CD44 than those in peripheral blood for all BA patients examined. The enhanced accumulation of activated eosinophils in airways with a concomitant decrease in peripheral blood implies that increased expression of adhesion molecules on endothelium in the case of exacerbated disease would facilitate transmigration of activated eosinophils to local inflammatory sites while leaving behind unactivated eosinophils in the blood. A similar shift of activated cells to the airway was reported by Barnes *et al.*, who described a fall in peripheral blood counts of activated CD4 and CD8 T lymphocytes with corresponding increases in the airway.[18] The difference in

CD44 expression between poorly controlled and well-controlled groups was not due to differences in medication. Patients were separated into two groups on the basis of the PEF values at the time of blood sampling, which reflected the control but not the severity of the disease, and, therefore, there was no significant difference in treatment between two groups.

In exacerbated BA, the activated eosinophils were reported to increase in the peripheral blood, which was reflected in an increase in the serum level of eosinophilic cationic protein (ECP).[19,20] This observation appears contradictory to our finding that eosinophils in the peripheral blood were less activated in poorly controlled patients with respect to CD44 expression. However, these discrepant findings could be reconciled if serum ECP derived from airway inflammatory foci where ECP was released from activated eosinophils.

The number of blood eosinophils has been used as an indicator of the asthmatic condition.[9] In the present study, we suggest that CD44 expression on blood eosinophils is also a diagnostic indicator of BA. In this regard, it is useful to monitor and compare the extent of blood eosinophil CD44 expression in each individual throughout the course of disease progression and amelioration. Levels of blood eosinophil expression clearly increased following treatment of an asthma attack in two cases examined (data not shown). In both cases, the restoration of $CD44^{high}$ eosinophils in blood lagged behind the improvement in PEF (data not shown). Recurrence of inflammatory responses in the respiratory tract could also be heralded by decreased levels of blood eosinophil CD44 expression. Further studies are required to analyze this issue.

In the present study, we were able to reproducibly and accurately examine the surface phenotype on eosinophils without purification prior to staining. The method of direct staining of whole leukocyte populations for analysis of eosinophil could have advantages over gradient density based purification procedures that may lead to removal of an activated, low-density eosinophil fraction that could be critical to disease. In addition, this method is simpler and more time effective. The method described here enabled us to obtain reproducible data for populations in which eosinophils comprised at least 2% of total leukocytes.

A major ligand of CD44 is hyaluronic acid, which is a constituent of the extracellular matrix. The importance of CD44 function in various processes, including tumor metastasis,[21] T lymphocyte migration to inflammatory sites[7] and T lymphocyte precursor homing to the thymus,[8] has been demonstrated. Signals through CD44 were reported to stimulate functions of lymphocytes and NK cells.[22,23] Of particular interest is the reported involvement of CD44 in eosinophil development from bone marrow precursors,[24] suggesting the existence of possible stimulatory signals transmitted by CD44. In this regard, the increased expression of CD44 on activated eosinophils may suggest that CD44 signaling is involved in adhesion and transmigration. The verification in the present report of an inverse relationship between CD44 expression on blood eosinophils and the extent of BA management implies that CD44 may be a primary adhesion molecule required for eosinophil transmigration.

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