CD69 surface expression on human lung eosinophils after segmental allergen provocation

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ABSTRACT: CD69 expression on eosinophils is observed in asthma and has been proposed as a marker of eosinophil activation. The role of allergens in the in vivo regulation of CD69 expression on eosinophils, however, remains incompletely understood. It was therefore investigated whether CD69 expression on eosinophils can be induced by allergen provocation in vivo.

Ten allergic asthmatics were studied by segmental allergen provocation. Two segments of the right and left lung were challenged with allergen or saline. CD69 expression was determined by flow cytometry and concentrations of interleukins were analysed by enzyme-linked immunosorbent assay in bronchoalveolar lavage (BAL) fluid.

Expression of CD69 on BAL eosinophils in the segments lavaged 10 min following saline instillation (28.3±8.8 specific mean fluorescence (SMF)) was not significantly different to segments lavaged 10 min after allergen (80.2±21.8 SMF) and segments lavaged 18 h after saline challenge (87.2±23.3 SMF). However, CD69 expression on eosinophils increased significantly 18 h after allergen challenge (128.6±21.9 SMF, p<0.03) which was accompanied by elevated granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations (114.9±42.9 pg·mL⁻¹, p<0.05). CD69 expression on eosinophils and GM-CSF concentrations correlated 18 h following allergen provocation (r=0.7, p=0.025).

These results suggest that in allergic asthma there is an allergen dependent, endobronchial upregulation of eosinophil activation as assessed by CD69 expression on eosinophils.


Infiltration of activated eosinophils into the bronchial mucosa is regarded as a specific feature of asthma. Expression of CD69 has been described as a marker of eosinophil activation [1, 2]. Cross-linking of CD69 with specific monoclonal antibodies can induce eosinophil apoptosis [3], suggesting a role for this surface antigen in the regulation of eosinophil inflammation. The natural ligand for CD69, however, remains unknown. Increased expression of CD69, a type II integral protein with a C-type lectin binding domain [4], on eosinophils has been reported in eosinophilic pneumonia and bronchial asthma. Bronchoalveolar lavage (BAL) eosinophils from patients with eosinophilic pneumonia had significantly increased expression of CD69, which was not observed on eosinophils from peripheral blood [5, 6]. Similarly, in allergic asthma elevated expression of CD69 on eosinophils has been reported in BAL, but not on peripheral blood eosinophils [1]. In contrast, a marked elevation of CD69 expression has been reported on eosinophils from patients with atopic dermatitis only following incubation of eosinophils in vitro [7], while in parasitic infections CD69 expression on peripheral blood eosinophils was significantly increased [8]. The factors regulating CD69 expression on eosinophils in vivo, however, remain unclear. In vitro granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, IL-5, IL-13 and phorbol ester have been shown to induce CD69 expression on eosinophils [1, 5, 9]. Incubation of freshly purified eosinophils with GM-CSF led to a very rapid induction of CD69 with expression being detectable after stimulation with GM-CSF for only 1 h [1]. In atopic dermatitis, CD69 expression on eosinophils in vitro has been associated with an autocrine production of cytokines, possibly GM-CSF or IL-5 [7].

Therefore, in order to elucidate whether CD69 expression on eosinophils can be induced by allergen provocation, which has been associated with increased concentrations of GM-CSF, IL-4 and IL-5 [10–13], CD69 expression on peripheral blood and BAL eosinophils was investigated following segmental allergen provocation in vivo.

Material and methods

Patients

Ten allergic asthmatics, eight males and two females, mean age 26.3±1.52 yrs with a duration of asthma of >2 yrs (mean duration 9.2±1.46 yrs) were studied. All patients suffered from allergic asthma as previously defined [14]. There was a history of intermittent wheeze, chest tightness, cough and sputum production either spontaneously or on allergen provocation, and a bronchial
hyperreactivity as determined by a modified broncho-
provocation test with carbachol [15]. Each patient had a
positive skin prick test to either birch pollen (n=3), rye
pollen (n=5), or house dust mite allergen (n=2) extracts
(Allergopharma, Reinbek, Germany) and almost all had
elevated total immunoglobulin (IgE) levels (626.8±249.32
kU·L⁻¹) as well as specific IgE levels (30.24±9.53 kU·L⁻¹)
(Kabi Pharmacia CAP System, Uppsala, Sweden) to their
respective allergen as well as a history of reversible
bronchoconstriction after inhalation of these particular
allergens. Only one patient had a low total IgE level, but a
clear history of allergen induced bronchoconstriction and
an elevated specific IgE concentration. There was no his-
tory or clinical evidence in any of the patients suggesting
a respiratory tract infection prior to or at the time of the
segmental allergen challenge. All patients were nonsmok-
ers. Baseline forced expiratory volume in one second
(FEV1) was 3.92±0.27 L (95.4±2.88% predicted) [16].

Flow cytometric analysis

Peripheral blood and BAL samples were processed and
cells were counted by flow cytometry as described in detail
previously [17]. After lysis of erythrocytes, 20 µL of
either whole blood or cells from BAL were incubated in
the presence of saturating concentrations of phycoeryth-
rin (PE)-conjugated CD69 (Becton Dickinson, San Jose,
CA, USA) and fluorescein isothiocyanate (FITC)-con-
jugated CD16 (Immunotech, Marseille, France) or PE-
conjugated anti-IgG (DAKO, Hamburg, Germany) and
FITC-conjugated CD16, respectively, in the dark on ice
for 30 min. The cells were washed twice with phosphate-
buffered saline (PBS; Dulbecco, Berlin, Germany) con-
taining 2% foetal calf serum (FCS) and subjected to
cytocentrifugation which was performed on 1×
10⁶ cells from each sample by using laser excitation at
585 nm (PE) and 503 nm (FITC), respectively. Nonspec-
cific fluorescence was detected as previously described
[17] and subtracted from the mean fluorescence measured
with anti-CD69 antibodies. CD16 fluorescence was
used to separate CD16 negative and CD49d+ (Becton
Dickinson) positive eosinophils from CD16 positive neut-
rophils (fig. 1). In initial experiments it could be demon-
strated that within the cell population gated accordingly
only very few were CD14 positive.

Purification of eosinophils

Eosinophils were obtained from 100 mL ethylenedia-
mine tetraacetic acid (EDTA)-blood of healthy donors.
Cells were separated by negative immunomagnetic selec-
tion as previously described [9]. Comparing CD14+ and
CD16- cells according to granularity (side scatter) and size
(forward scatter) it could be shown that the cells
gated according to CD16- and CD49d+ contained only
very few CD14 positive cells.

Cell culture

Purified eosinophils (1×10⁶ cells·mL⁻¹) were cultured
as previously described [9] in culture medium alone or in
the presence of either GM-CSF or IL-4 (PBH). Before immu
nophotofluorescence labelling with anti-CD69 antibodies,
the cells were washed twice in PBS containing 2% FCS.

In vitro stimulation of eosinophils with GM-CSF, tu-
mour necrosis factor-α, and histamine

Purified eosinophils from peripheral blood of normal
donors were incubated in the presence of GM-CSF (Bio-
concept, Umkirch, Germany) (1 and 10 ng·mL⁻¹), tumour
necrosis factor (TNF)-α (Bioconcept) (10 ng·mL⁻¹), and
histamine (Sigma, Deisenhofen, Germany) (10⁻⁶-10⁻⁴ M)
for 10 min and then analysed for CD69 expression. In
addition, purified eosinophils from normal donors which
had been preincubated (“primed”) for 18 h with a mixture
of IL-3, IL-5 (both from Bioconcept) and GM-CSF (0.01
ng·mL⁻¹) each, which by itself did not induce CD69 ex-
pression, were incubated with GM-CSF (1 and 10 ng·mL⁻¹),
TNF-α (10 ng·mL⁻¹), and histamine (10⁻⁶-10⁻⁴ M) for 10
min and then analysed for CD69 expression.

Statistical analysis

Results are expressed as arithmetic mean±SEM. Differ-
ences between groups were analysed using the Wilcoxon
matched pairs test. Differences with p-values <0.05 were
considered statistically significant. Relationships are ex-
pressed using Pearson’s rank correlation.
Results

Eosinophils in peripheral blood prior to and after segmental allergen provocation

There was a significant increase in the total cell number in peripheral blood 18 h after segmental allergen provocation (8.5±0.6×10^3 cells·μL^-1) compared to baseline (5.9±0.4×10^3 cells·μL^-1; p<0.01). Among the different cell populations the total number of eosinophils showed a slight, but not significant, increase (0.2±0.1×10^3 cells·μL^-1 before allergen challenge and 0.3±0.1×10^3 cells·μL^-1 18 h after allergen challenge), while the relative number of eosinophils did not change (4.2±0.7% before and 3.9±0.7% 18 h after allergen challenge).

Eosinophils in BAL following segmental allergen provocation

Following allergen provocation a marked increase in eosinophils was observed in the allergen challenged segment after 18 h which was significantly elevated compared to the other segments lavaged 10 min after allergen challenge or 10 min and 18 h after saline challenge (table 1). This increase in eosinophils, which was consistently observed in all patients, was associated with a statistically significant increase in the relative number of eosinophils 18 h after segmental allergen challenge compared to the other segments (1.5±0.7% and 3.8±1.5% 10 min and 18 h after NaCl instillation, and 1.7±0.5% and 28.7±6.1% 10 min and 18 h after allergen challenge; p<0.03).

CD69 expression on eosinophils and neutrophils following segmental allergen provocation

CD69 expression on peripheral blood eosinophils was 16.2±3.2 specific mean fluorescence (SMF) before allergen challenge and increased only slightly 18 h after allergen provocation (22.5±6.3 SMF). However, this difference failed to reach statistical significance. In contrast to peripheral blood eosinophils there was a marked upregulation in the expression of CD69 on eosinophils obtained from BAL fluid following segmental allergen provocation (fig. 2).

Fig. 1. – CD16, CD49d and CD69 expression on bronchoalveolar lavage (BAL) eosinophils 18 h after allergen provocation. Cells were obtained from BAL 18 h after allergen provocation and incubated with fluorescent anti-CD16 (fluorescein isothiocyanate (FITC)), anti-CD49d (phycoerythrin (PE)) and anti-CD69 antibodies. The dot plot (a) shows ungated BAL cells. Eosinophils (R8) were gated and their CD16 (b), CD49d (c) and CD69 (d) expressions (-----) are shown in comparison to an unspecific control antibody (Immunoglobulin (Ig)G; ::::). SSC: side scatter.
Cytokines in BAL fluid

Among the different cytokines measured in BAL fluid a weak, although statistically significant, correlation was observed between CD69 expression on bronchoalveolar eosinophils and the GM-CSF concentrations measured in BAL fluid 18 h after segmental allergen provocation ($r=0.697$, $p<0.025$) (fig. 3a). Furthermore, there was a trend towards a correlation of CD69 expression on eosinophils and IL-4 concentrations in BAL fluid. However, this relationship failed to reach statistical significance ($r=0.58$, $p<0.077$) (fig. 3b).

Effect of GM-CSF and IL-4 on CD69 expression on isolated eosinophils

In order to investigate the significance of the observed correlation between CD69 expression and GM-CSF or IL-4 concentrations in BAL fluid, freshly purified eosinophils from healthy volunteers were incubated with two different concentrations of GM-CSF and IL-4 (1 and 10 ng mL$^{-1}$) in vitro for 6 h. CD69 expression was then measured by flow cytometry. Only a weak expression of CD69 was observed when freshly isolated eosinophils from normal subjects were incubated in medium alone for 6 h. However, incubation with either 1 or 10 ng mL$^{-1}$ of GM-CSF resulted in a marked increase in CD69 expression as shown in figure 4a, which was also observed following incubation with IL-4, although to a lesser extent. In addition, when the effects of GM-CSF and IL-4 (both 10 ng mL$^{-1}$) on the induction of CD69 expression on eosinophils were measured over time, a similar increase was observed between 2 and 6 h. This increased expression of CD69 on eosinophils persisted for 24 h after incubation with GM-CSF, but showed a small decline with IL-4 (fig. 4b).

In vitro stimulation of eosinophils with GM-CSF, TNF-α, and histamine

In order to analyse which factors might be involved in the rapid upregulation of CD69 on BAL eosinophils recovered 10 min after allergen challenge, purified cells from peripheral blood of normal donors were incubated for 10 min in the presence of GM-CSF, TNF-α, and histamine which can be released from mast cells immediately following allergen challenge. As shown in table 3, none of these mediators influenced CD69 expression on peripheral blood eosinophils from normal donors.

Discussion

Infiltration of activated eosinophils into the bronchial lumen is a prominent feature of asthma [18] and has been associated with specific, eosinophil-mediated damage to the respiratory epithelium. CD69 which is expressed on a number of cells has been described as a marker for eosinophil activation [1, 2]. Several studies have reported elevated expression of this surface antigen on BAL eosinophils compared to peripheral blood eosinophils in diseases such as eosinophil pneumonia and asthma [1, 5, 6]. While CD69 expression on eosinophils in vitro can be induced by incubation with cytokines such as IL-3, IL-5, and GM-CSF [1], the factors leading to CD69 expression on eosinophils in vivo remain unclear. Therefore, in this study, it was investigated whether allergen exposure can modify CD69 expression and thus indicate eosinophil activation in vivo.

Table 1. Cellular composition in bronchoalveolar lavage fluid after segmental allergen provocation (cells x 10$^3$ mL$^{-1}$; mean±SEM)

<table>
<thead>
<tr>
<th>Time</th>
<th>Macrophages</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 10 min</td>
<td>75.4±28.2</td>
<td>0.5±0.3</td>
<td>3.1±1.9</td>
<td>27.2±9.5</td>
</tr>
<tr>
<td>C 18 h</td>
<td>190.2±59.2</td>
<td>13.4±4.5</td>
<td>6.8±2.3</td>
<td>31.3±5.5</td>
</tr>
<tr>
<td>P 10 min</td>
<td>61.4±13.1</td>
<td>0.7±0.3</td>
<td>1.6±0.6</td>
<td>17.2±4.8</td>
</tr>
<tr>
<td>P 18 h</td>
<td>129.2±29.2</td>
<td>41.7±11.9</td>
<td>125.0±44.7</td>
<td>77.1±15.0</td>
</tr>
</tbody>
</table>

C 10 min: saline challenged control segment lavaged 10 min after instillation of 2.5 mL normal saline; C 18 h: saline challenged control segment lavaged 18 h after instillation of 2.5 mL normal saline; P 10 min: allergen challenged segment lavaged 10 min after instillation of allergen 10×PD$_{20}$; P 18 h: allergen challenged segment lavaged 18 h after instillation of allergen 10×PD$_{20}$. Cell numbers are given in absolute numbers.
Using the model of segmental allergen provocation this is the first study to show that CD69 expression is indeed markedly upregulated on BAL eosinophils following allergen provocation. The study thus extends the observations of HARTNELL et al. [1] by showing that the low basal expression of CD69 on BAL eosinophils increases following allergen exposure and therefore provides further evidence for the hypothesis that eosinophil activation in allergic asthma in vivo is a dynamic process which is allergen dependent. In contrast to eosinophils, BAL neutrophils showed a baseline expression of CD69 which did not change significantly following allergen provocation. Therefore, from the present study it was concluded that CD69 expression in this model of allergic asthma appears to be specific for eosinophils.

Interestingly, a small increase in CD69 expression on eosinophils was also observed in the segment lavaged 10 min after allergen provocation, at a timepoint where infiltration of newly activated eosinophils is unlikely. Although this difference in CD69 expression between the two segments lavaged after 10 min following either saline or allergen challenge was not statistically significant these findings would support the concept that CD69 expression on eosinophils can be upregulated rapidly, possibly due to exposure of intracellularly stored receptors [9]. In order to elucidate factors which might regulate this rapid increase in CD69 expression, peripheral blood eosinophils from normal donors were incubated with GM-CSF, TNF-α, and histamine. These factors which are rapidly released following allergen-dependent mast cell activation, however, did not change CD69 expression on eosinophils in

Table 2. Cytokine concentrations in bronchoalveolar lavage fluid after segmental allergen provocation (pg·mL⁻¹; mean±SEM)

<table>
<thead>
<tr>
<th></th>
<th>IL-4</th>
<th>IL-5</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 10 min</td>
<td>8.2±6.2</td>
<td>9.7±5.7</td>
<td>7.9±4.4</td>
</tr>
<tr>
<td>C 18 h</td>
<td>11.5±9.2</td>
<td>4.8±4.3</td>
<td>8.5±3.6</td>
</tr>
<tr>
<td>P 10 min</td>
<td>5.6±3.5</td>
<td>8.3±5.1</td>
<td>8.2±3.3</td>
</tr>
<tr>
<td>P 18 h</td>
<td>131.6±56.1*</td>
<td>198.0±67.2*</td>
<td>114.9±42.9*</td>
</tr>
</tbody>
</table>

Cytokine concentrations of interleukin (IL)-4, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in BAL fluid in the segments lavaged 10 min and 18 h after saline instillation (C 10 min and C 18 h) as well as the segments lavaged 10 min and 18 h after allergen provocation (P 10 min and P 18 h). *: p<0.05 for P 18 h compared to P 10 min, and C 18 h for IL-4 and for P 18 h compared to all other segments for IL-5 and for GM-CSF.
Therefore, it could be speculated that mucosal eosinophils with elevated CD69 expression are preferentially detected in BAL fluid following allergen provocation. Alternatively, unlike eosinophils from normal donors, eosinophils from asthmatic patients might have been exposed to a variety of "priming" factors in vivo that could facilitate a rapid upregulation of CD69 in vivo. However, even the incubation of peripheral blood eosinophils from normal donors with a mixture of IL-3, IL-5, and GM-CSF (which did not cause CD69 upregulation) failed to reproduce the rapid upregulation of CD69 similar to that observed 10 min following allergen provocation. Thus, the mechanisms responsible for CD69 upregulation in vivo remain to be elucidated. The large, statistically significant increase in CD69 expression 18 h after segmental allergen challenge which coincided with a large increase in the numbers of eosinophils present in the BAL fluid suggests a selective, allergen-dependent infiltration of activated eosinophils into the site of allergen challenge. Alternatively, it might be possible that eosinophils which are attracted to the site of allergic inflammation are activated by local factors present in the inflamed microenvironment 18 h following allergen provocation.

In this study, anti-inflammatory therapy was withheld for 7 days prior to segmental allergen challenge. Although an even longer period might have been desirable to exclude any effects of inhaled corticosteroids or cromoglycate on CD69 expression it was considered to be unethical to withhold therapy for longer periods of time, although this was well tolerated by all subjects. However, with respect to this study no differences were observed between patients treated with corticosteroids, cromoglycate or β₂-agonists suggesting that the influence of corticosteroids or cromoglycate on eosinophil CD69 expression did not influence the findings when they were withheld for at least 7 days.

In this study, the increase in CD69 expression on eosinophils was accompanied by an increase in GM-CSF concentration in the allergen challenged segment 18 h following allergen challenge. A similar increase, which correlated with the number of infiltrating eosinophils in BAL 18 h after allergen challenge [11], has been reported following allergen provocation [11–13]. Furthermore, several studies have reported an increase in CD69 expression on eosinophils following incubation with GM-CSF in vitro [1, 19]. In the present study this data was confirmed [1, 19] by demonstrating that CD69 expression on eosinophils can be induced by incubation of isolated cells with GM-CSF. This does suggest that the increase in CD69 expression and GM-CSF levels observed 18 h following allergen provocation in this study might be causally related, but supports the assumption that this cytokine plays an important role in the regulation of eosinophil activation in vivo.

Several cells such as mast cells, macrophages and T-lymphocytes have been implicated in the production of GM-CSF [20, 21]. However, in view of previous studies which have shown that eosinophils themselves can produce GM-CSF [22–24] and that the ability of cultured eosinophils to induce CD69 expression by autocrine mechanisms can be inhibited by anti-GM-CSF antibodies

| Table 3. CD69 expression following incubation with different concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α, histamine and a mixture of interleukin (IL)-3, IL-5 and GM-CSF (mean±SEM) |
|---------------------------------|----------------|----------------|
| Priming with GM-CSF/IL-3/IL-5   | No priming    |
| Control                        | 3.6±1.39      | 1.04±0.55      |
| GM-CSF ng·mL⁻¹                 | 5.0±1.71      | 1.86±0.85      |
| Histamine M                    | 2.04±0.65     | -0.21±0.46     |
| 10⁻⁵                           | 1.23±0.52     | 4.25±2.22      |
| 10⁻⁴                           | 0.1±0.14      |                |

CD69 expression following incubation with different concentrations of GM-CSF, TNF-α and histamine for 10 min after "priming" with a mixture of IL-3, IL-5 and GM-CSF.
[7], eosinophils cannot be excluded as a major source of GM-CSF in vivo which might cause autocrine modulation of CD69 expression.

Since there was also a weak, statistically nonsignificant correlation between IL-4 concentrations and CD69 expression on BAL eosinophils 18 h following allergen provocation it was investigated whether IL-4 can induce CD69 expression on cultured eosinophils in vitro. Interestingly, a small, concentration dependent effect was demonstrated for IL-4 on CD69 expression in this setting, suggesting that in addition to GM-CSF other cytokines with a Th2 phenotype can enhance eosinophil activation. Thus, in view of these in vitro results and the observed relationship between IL-4 and CD69 expression in vivo the hypothesis that IL-4 might also contribute to eosinophil activation cannot be refuted as assessed by CD69 expression following allergen provocation.

In this study, the expression of CD69 on eosinophils in BAL fluid 10 min following saline instillation was not significantly different to the expression observed on peripheral blood eosinophils. This suggests that, in the absence of allergen eosinophil activation (assessed by CD69 expression) CD69 expression does not differ between BAL and peripheral blood in mild, asymptomatic asthma.

In conclusion, this is the first study to provide evidence that in patients with allergic asthma segmental allergen provocation causes an allergen dependent upregulation of CD69 expression on eosinophils which might, in part, be regulated by locally released cytokines such as granulocyte-macrophage colony-stimulating factor and interleukin-4.

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References