Increased Expression of CD69 Antigen on Human Peripheral Blood Natural Killer Cells in Patients with Allergic Rhinitis

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ABSTRACT

Allergic rhinitis (AR) is an inflammatory disorder of the nasal mucosa with high morbidity and prevalence. Natural killer (NK) cells might have a role in AR. We aimed to evaluate the changes of the markers and receptors on NK cells in AR patients compared to the non-atopic controls.

Flow cytometric analysis was used with double staining of the Peripheral Blood Mononuclear Cells (PBMCs) to examine the expression of CD25 and CD69 markers, and NKG2D and NKG2A receptors on NK cells of 20 patients with AR and 20 non-atopic controls. The serum total IgE level was measured by Enzyme-linked Immunosorbent Assay.

The expression of CD69 antigen on NK cells in AR patients was significantly higher than that of healthy group (p=0.03). No significant changes were observed between CD25, NKG2D and NKG2A expression on the surface of NK cells from healthy and AR subjects. Our study also showed that there was no significant correlation between the expression of CD69, CD25, NKG2D and NKG2A and level of serum total IgE in AR patients and normal subjects.

These results indicated that the expression of CD69 antigen on NK cells of AR patients was increased. The high expression of CD69 on NK cells in AR patients suggested that these cells were activated, probably due to the cytokines secreted from allergen-stimulated T' cells and activated monocytes.

Keywords: Allergic rhinitis; CD69 antigen; Natural killer cell

INTRODUCTION

Allergic rhinitis (AR) is an inflammatory disease of the nasal mucosa induced by an IgE-mediated reaction, following exposure to an allergen.1 AR is a
Increased CD69 on NK Cells in Allergic Rhinitis

global health problem, with a prevalence of 9-42% among general population.3

The characteristic morphology in AR includes presence of basophils and eosinophils in nasal secretions, infiltration of mast cells and eosinophils into the epithelium, increased mucosal antigen-presenting cells, activated T lymphocytes, and upregulation of endothelial adhesion molecules.3

NK cells can be distinguished from other lymphocytes with the lack of the T cell receptor and surface immunoglobulins, and many NK cell-specific surface molecules with different functions. Human NK cells express structurally and functionally two distinct families of major histocompatibility complex (MHC) class I receptors: killer cell immunoglobulin-like receptors (KIR) and lectin-like receptors. These receptors are also divided into two families that include activating and inhibitory receptors.4,5 Inhibitory receptors (e.g. CD94/NKG2A) recognize and engage MHC class I molecules on the surface of the target cell, thereby initiating an inhibitory signal. Activating receptors (e.g. CD94/NKG2C and NKG2D) bind to their ligands on the target cell surface and trigger NK cell activation and target cell lysis.6

Kruszewski et al. have shown that the activity of NK cells is increased in patients with atopic respiratory diseases.7 Another study showed that patients with AR have a higher percentage and enhanced cytotoxicity of NK cells compared to nonatopic subjects.8

CD69 is a member of a supergene family of type-II integral membrane proteins with C-type lectindomains.9 It is one of the earliest markers induced upon activation in T and B lymphocytes, NK cells, macrophages, neutrophils, and eosinophils. In addition, it is constitutively expressed on monocytes, platelets, Langerhans cells, and a small percentage of resident lymphocytes in thymus and secondary lymphoid tissues.10,11 CD69 is a useful marker for evaluation of cytotoxic activity of NK cells, whereas proliferative potential is indicated by CD25 expression.12 CD25 (the α chain of the IL-2 receptor) is a phosphorylated 55-60 KD cell surface glycoprotein.13

However, to our knowledge, no study has investigated the changes of these markers and receptors on NK cells in AR. We have previously shown that the percentage and cytotoxicity of NK cells are increased in patients with AR.8 Expression of CD69 on NK cells in antigen-stimulated culture has been shown.13 In the present study we evaluated the expressions of activation markers CD69 and CD25, and activation receptor NKG2D and inhibitory receptor NKG2A on NK cells and the relationship of their changes with serum total IgE in patients with allergic rhinitis compared to non-atopic controls.

MATERIALS AND METHODS

Study Population

In a case-control study, 20 patients (11 men and 9 women; 16-40 years old, mean age 31.5 ±5.1 years) with AR, referred to Allergy clinic of Vali-Asr Hospital, School of Medicine, Tehran University of Medical Sciences, were studied. Table 1 shows the subjects’ demographic features and medical histories. The diagnosis of AR was made according to ARIA guidelines, based on the patients’ history of nasal discharge, blockage, sneezing and itching (2 or more of these symptoms for at least 1 hour on most days) plus at least 1 criterion of atopy (family history of allergy, high plasma total IgE, or positive skin prick test).1

Past medical history of other allergies was recorded. All cases entered the study right after the diagnosis, before starting any therapy, and they had been symptomatic for at least 1 year before diagnosis.

Twenty non-atopic healthy controls (11 men and 9 women; 16-40 years old, mean age 28.6 ± 6.6 years) were also evaluated. They had no history of allergy or any other chronic diseases. The case and control groups were age-and sex-matched. All participants signed an informed consent before entering the study.

Table 1. Demographic and clinical characteristics of the patients with AR and non-atopic controls

<table>
<thead>
<tr>
<th></th>
<th>Non-atopic Controls (n=20)</th>
<th>AR patients (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Male/Female)</td>
<td>11/9</td>
<td>11/9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28.6±6.6</td>
<td>31.5±5.1</td>
</tr>
<tr>
<td>Type of allergy, perennial/seasonal</td>
<td>None</td>
<td>12/8</td>
</tr>
<tr>
<td>Serum total IgE (IU/ml)</td>
<td>36.3±11.8</td>
<td>78.1±92.7</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation.
Peripheral Blood Mononuclear Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood by density gradient centrifugation over Ficoll (Histopaque, gradient 1.077, Innotrain, Taunus, Germany) and washed twice and resuspended in Phosphate buffered saline (PBS).

Cell Surface Staining

The following monoclonal antibodies (mAbs) were used in this study: PE-conjugated anti-human NKG2D, PE-conjugated anti-human NKG2A and PE-conjugated anti-human CD25, purchased from R&D Systems (MN, USA). PE-conjugated anti-human CD69 and PEcy5-conjugated anti-human CD56 were obtained from BD Pharmingen (CA, USA). Isotype matched control antibodies were purchased from R&D and BD.

For surface staining, PBMCs were incubated for 30 min at 4 °C with appropriate amounts of the fluorochrome-conjugated mAbs. The cells were washed, resuspended in PBS and collected on a FACS Calibur (Becton Dickinson Immunocytometry Systems, CA, USA). Only CD56+ cells were gated and analyzed for presence of each of the following markers: CD69, CD25, NKG2A, and NKG2D. The data were analyzed by using WinMDI software (Tree Star Inc., OR, USA).

IgE Measurement

Total serum IgE was measured using ELISA method (Genesis Diagnostics, UK) according to the instructions of the manufacturer. After collection of venous blood, the blood was centrifuged and serum was stored at -20°C until carrying out the measurement.

Statistical Analysis

Data are presented as mean ± S.D. The results were analyzed using Statistical Package for the Social Sciences (SPSS; version 15). Comparisons were performed using Student’s t- test, and Pearson’s correlation coefficient was used for bivariate correlations. P<0.05 was considered as statistically significant.

RESULTS

The Percentage of CD56+ NK Cells in PBMCs

Patients with AR had 21.74%±6.24% CD56+ cells in their PBMCs which was significantly (p=0.02) higher than the control group (15.88%±9%). The percentage of CD56+ cells did not correlate with serum IgE levels in patients nor in controls.

Surface Expression of Receptors and Activation Markers on Peripheral Blood NK Cells

In patients with AR, CD69 expression was elevated in CD56+NK cells compared to non-atopic control subjects, 6.18% ± 2.36% versus 4.47% ± 2.54% (p=0.03). The expression of CD25, NKG2D and NKG2A in CD56+NK cells did not differ between patients with AR and non-atopic control subjects (Table 2). Actual FACS dot plots from one representative experiment are represented in Figure1.

To investigate further the association of atopic status with the receptors and markers of NK cells, the correlation between serum total IgE level and the expression the receptors and markers of NK cells were evaluated in patients and non-atopic control subjects.

We observed no significant correlation between the percentages of cells expressing CD69, CD25, NKG2D and NKG2A in CD56+NK cells to the level of serum total IgE in the AR patients and non-atopic control subjects (Table 3).

Table 2. Expression of CD25 and CD69 markers, and NKG2D and NKG2A receptors on peripheral blood NK cells in patients with allergic rhinitis (AR) and comparison with non-atopic subjects.

<table>
<thead>
<tr>
<th>Markers</th>
<th>AR patients (n = 20) (Percentage)</th>
<th>Non-atopic controls (n = 20) (Percentage)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25</td>
<td>24.43 ± 6.29</td>
<td>24.49 ± 8.63</td>
<td>0.98</td>
</tr>
<tr>
<td>CD69</td>
<td>6.18 ± 2.36</td>
<td>4.47 ± 2.54</td>
<td>0.034</td>
</tr>
<tr>
<td>NKG2A</td>
<td>12.07 ± 4.09</td>
<td>8.97 ± 5.89</td>
<td>0.06</td>
</tr>
<tr>
<td>NKG2D</td>
<td>57.11 ± 10.70</td>
<td>49.14 ±16.49</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation of percentage values.
Figure 1. Expression of CD25, CD69, NKG2D and NKG2A on CD56+ NK cells. The expression of CD25, CD69, NKG2D and NKG2A on CD56+ NK cells in PBMCs obtained from allergic rhinitis patients and non-atopic controls were evaluated by flow cytometry, using double staining. Flow cytometry dot plots showing expression of CD69 (b) and NKG2D (c), CD25 (e) and NKG2A (f) on CD56+ NK cells from a representative individual with allergic rhinitis. Left dot plots (a, d) show results with isotype control antibodies, (a) isotype control for CD69 and NKG2D and (d) isotype control for CD25 and NKG2A.

Table 3. Pearson correlation analysis between the expression of surface markers and receptors on NK cells in AR patients and non-atopic control subjects with serum total IgE.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Subjects</th>
<th>r</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25</td>
<td>Patients</td>
<td>0.288</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0.378</td>
<td>0.10</td>
</tr>
<tr>
<td>CD69</td>
<td>Patients</td>
<td>0.151</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>-0.220</td>
<td>0.35</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Patients</td>
<td>-0.148</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>-0.231</td>
<td>0.32</td>
</tr>
<tr>
<td>NKG2A</td>
<td>Patients</td>
<td>0.310</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>-0.360</td>
<td>0.11</td>
</tr>
</tbody>
</table>

DISCUSSION

Allergic rhinitis is an IgE-mediated reaction in nasal mucosa, happening following exposure to the allergen the patient had been previously sensitized.\textsuperscript{14} Rhinitis is a global health problem that affects 9%-42% of general population in developed countries and the incidence is still rising.\textsuperscript{5} It can be induced by different mechanisms because of several etiological agents.\textsuperscript{15-17} Allergens induce Th2 lymphocyte proliferation leading to release of their characteristic profile of cytokines. These cytokines promote IgE synthesis and mast cell activation. Inflammatory mediators and cytokines upregulate endothelial cell adhesion markers and...
Chemotactants lead to the characteristic infiltration by immune cells in chronic allergic rhinitis.\textsuperscript{18,19}

Patients with AR have been shown to have a higher percentage and enhanced cytotoxicity of NK cells compared to non-atopic subjects.\textsuperscript{8} In this study, it was also shown that patients with AR had a higher percentage of CD56+ cells. It could therefore be speculated that some mediators of allergic reactions would stimulate the maturation and/or activity of NK cells. While histamine slightly inhibits NK activity, the stimulatory activity of leukotriene B4 (LTB4) on NK cells has been demonstrated.\textsuperscript{20} LTB4 is shown to activate monocytes and these cells produce LTB4, IL-18, IL-15, and IL-12, which can contribute to the functional activation of T cells and NK cells.\textsuperscript{21} The lipid mediator LTB4 can enhance the sensitivity of NK cells to IL-2, and it stimulates the cell surface expression of IL-2Rβ and the IL-2Rβ gene message in CD56 and CD8+ human lymphocytes.\textsuperscript{22} Studies of the responses of these cells to IL-2 suggest that IL-2Rβ mediates the initial phase of induction of lymphokine activated killer (LAK) cells and proliferative activities.\textsuperscript{23}

In this study, we found that CD69 expression on NK cells was higher in AR patients compared to non-atopic controls, which indicated that NK cells from patients with AR were activated and this could be a reason of their enhanced cytotoxicity.\textsuperscript{8} Other researchers have shown that CD69 is persistently expressed on leukocyte infiltrates of different chronic inflammatory diseases.\textsuperscript{24-26} Certain results indicate that CD69 may be involved in the pathogenesis of some diseases such as rheumatoid arthritis, chronic inflammatory liver diseases, mild asthma, and acquired immunodeficiency syndrome.\textsuperscript{27} Expression of CD69 has been analyzed in several diseases and has been shown to be increased in some such as cow's milk allergy,\textsuperscript{28} Systemic lupus erythematosus,\textsuperscript{29} and diabetes in the NOD mouse model.\textsuperscript{30}

CD69 plays a critical role in the induction of both Ag-induced eosinophilic airway inflammations and airway hyperresponsiveness. Furthermore, administration of anti-CD69 antibody resulted in a dramatic reduction in the extent of airway inflammation and hyperresponsiveness, suggesting that the CD69 mAb could be used for the treatment of asthmatic patients.\textsuperscript{31}

CD69 is one of the earliest specific activation markers expressed during large granulated lymphocyte activation, including NK cell.\textsuperscript{10,27,32} The high expression of this marker on NK cells in antigen-stimulated cultures suggests that NK cells are easily activated by cytokines from antigen-stimulated T cells.\textsuperscript{33} NK cells express CD69 after activation by different stimuli such as phorbol 12-myristate 13-acetate (PMA), IL-2, IL-12, IFN-α or anti-CD16 mAbs.\textsuperscript{34}

Although CD69 expression was higher in patients with AR comparing with non-atopic subjects, it did not correlate with serum total IgE level in patients and healthy controls. These two findings were not conflicting, since serum total IgE is an indicator of allergy, it does not have direct relationship with allergies.

We also evaluated the expression of CD25, NKG2D and NKG2A receptors on NK cells in AR patients and control groups. The total expressions of CD25, NKG2D and NKG2A on the surface of NK cells from non-atopic and AR subjects were not significantly different. This could show that these markers and receptors were not seemed to be effective or affected during the process of AR.

Our results indicated that the expression of CD69 antigen on NK cells of AR patients was increased. The high expression of CD69 on NK cells in AR patients suggests that these cells might be activated in patients with AR, which could be due to the cytokines secreted from allergen-stimulated T cells and activated monocytes.

ACKNOWLEDGEMENTS

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