Peripheral blood eosinophils priming and *in vitro* vascular endothelial growth factor stimulation in asthmatics

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Abstract

Introduction: Asthma is a complex airway disease with heterogeneity in molecular pathways. Hypersecretion of many cytokines (e.g. vascular endothelial growth factor – VEGF), inflammatory cells infiltration (e.g. eosinophils) and different genetic factors (e.g. gene polymorphism) might be responsible for physiological and pathological changes in the course of this chronic disease.

Aim: To reveal the possible expression of activation marker CD69 on eosinophils unstimulated and stimulated by VEGF in patients with asthma. Additionally, the influence of a genetic factor (del18 genotype at -2549 -2567 position in the promoter of the VEGF gene) was considered.

Material and methods: The study involved 122 participants (82 patients with asthma and 40 healthy controls). CD69 expression on peripheral blood eosinophils was detected by flow cytometry without exogenous stimulation and after *in vitro* stimulation with VEGF. Genotyping for VEGF-promoter region was performed using the polymerase chain reaction method.

Results: CD69 was strongly presented ($p < 0.05$) on unstimulated eosinophils of patients with asthma and del18 genotype in the promoter of the VEGF gene. Stimulation of peripheral eosinophils with VEGF did not induce CD69 expression in a dose-dependent manner.

Conclusions: Our results may suggest the potential contribution of the VEGF gene polymorphism to the spontaneous increase of eosinophils activity (priming) in patients with asthma. In addition, the results show that VEGF is unlikely to significantly activate eosinophils in asthmatics.

Key words: asthma, CD69, del/ins, eosinophils, vascular endothelial growth factor.

Introduction

Asthma is a prevalent chronic airway disease that is responsible for a large global disease burden. Additionally, asthma is increasingly considered as a complex respiratory disease in which both genetic and environmental factors determine the onset of symptoms. It is characterized by chronic airways inflammation in which heterogeneous pathophysiologic mechanisms involve hypersecretion of different cytokines, mucus overproduction simultaneously with neovascularization and inflammatory cells infiltration. This heterogeneity in molecular pathways is probably related to different endotypes of this disease [1–3].

Vascular endothelial growth factor (VEGF) is a homodimeric, heparin-binding glycoprotein of a molecular weight 46 kDa with the gene located on 6p21.3. VEGF is synthesized mainly by epithelial cells, platelets, neutrophils, macrophages and acts biologically by tyrosine-kinases receptors [4]. Many studies have reported that VEGF plays pleiotropic functions and is an essential regulator of vascular development and vessels function in health (i.e. post-natal and skeletal growth, reproductive functions, embryogenesis, endothelial cells growth, menstrual cycle, wound healing) and diseases (i.e. age-related macular degeneration, rheumatoid diseases, sepsis, coronary heart disease, chronic obstructive lung disease, carcinogenesis) [5, 6]. Furthermore, VEGF is considered as one of the agents important in asthma progress connected with lung remodelling and different cells activation [7].

Eosinophils account for approximately 0.5–1% of the leukocytes but this proportion is often raised in patients with allergy or exposed to parasites. Eosinophils play an important and varied role in the allergic inflammation. Along with mast cells and basophils, activated eosinophils control mechanisms associated with asthma pathogenesis, disease severity and exacerbation frequency [8]. Aided by the fact that they are present not only in the blood stream but can also be localized in tissues, eosinophil counts in peripheral blood and bronchoalveolar lavage (BAL) fluid in asthmatics are higher than in healthy controls. However, non-eosinophilic asthma phenotype is also recognized [9, 10]. Detection of activated eosinophils by indication of a different protein marker (e.g. CD69) on the membrane using the flow cytometry has been shown to be a useful diagnostic tool [11]. Following activation, eosinophils have the capacity to generate and release a number of growth factors, among others vascular endothelial growth factor which affects eosinophils function. Horiuchi and Weller [12] described presence of VEGF transcripts in purified peripheral eosinophils with the polymerase chain reaction, then VEGF protein was detectable in eosinophils through immunocytochemistry. Furthermore, there is strong evidence that VEGF-receptor is expressed on the cell surface and eosinophils activation with VEGF stimulates directed chemotaxis, migration and release of the eosinophil cationic protein [13].

Reviewing the updated literature, it seems that data about the relationship between CD69 expression as a marker of activation on peripheral blood eosinophils unstimulated and after *in vitro* VEGF stimulation in patients with asthma are lacking.

Aim

Therefore, the study on that issue was undertaken and presented in this paper. Additionally, the influence of del18 genotype at -2549 -2567 position in the promoter of the VEGF gene was considered.

Material and methods

Study groups

The study population included 122 individuals (80 females; aged 20 to 70 years). In this group, 82 patients (54 females; aged 23 to 69 years) had the diagnosis of asthma established according to the Global Initiative for Asthma recommendations (GINA) [14]. The control group consisted of 40 healthy participants (26 females; aged 20 to 70 years) with neither allergy nor chronic pulmonary dysfunction in the medical history. After genotyping (del18 genotype at -2549 -2567 position in the promoter of the VEGF gene), all participants were divided into two cohorts: del (no mutation – genetic variant del/del) and ins (mutation – genetic variants ins/ins or ins/del). The

study protocol was approved by the Ethics Committee at the Wroclaw Medical University, Poland. The demographic characteristics are shown in Table 1.

Immunofluorescence and flow cytometry

Procedures performed for evaluation of eosinophils activation were coincident with those described by Ethier *et al.* [15] with own modification. Briefly, to investigate whether CD69 expression could be induced, eosinophils were cultured with: medium (non-stimulated negative control – patient background: Pb), N-formyl-methionyl-leucyl-phenylalanine (fMLP in concentration of 10^{-6} M – positive control: Pc) and VEGF (two samples in an experimentally selected concentration of 250 ng/ml and 500 ng/ml). All samples contained 100 µl blood taken to the 4.5 ml tubes with lithium heparin (Sarstedt AG & Co., Nümbrecht, Germany) and 100 µl RPMI-1640 Medium (Institute of Immunology and Experimental Therapy, Wroclaw, Poland), fMLP (Institute of Immunology and Experimental Therapy, Wroclaw, Poland) or VEGF (BD Biosciences Pharmingen, San Diego, USA). Samples were incubated in the atmosphere supplemented with 5% CO_2 at 37°C for 60 min (Incubator ASSAB, Stockholm, Sweden). Then, 20 µl of edentate disodium EDTA (BD Biosciences Pharmingen, San Diego, USA) was added and samples were centrifuged at 1600 rev/min for 10 min. Next, the supernatant was removed, 100 µl of PBS (Institute of Immunology and Experimental Therapy, Wroclaw, Poland) with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, USA) and 10 µl of anti-CD69 (Immunotech S.A.S., Marseille, France) were added and incubated in the darkness at 25°C for 30 min. After incubation, 2 µl of fluid for the cells lysis (BD Biosciences Pharmingen, San Diego, USA) was added and after 10 min at room temperature the samples were centrifuged at 1600 rev/min for 5 min. Afterwards, the supernatant was removed and 3 ml of PBS was added to each sample and centrifuged for 5 min at room temperature at 1600 rev/min. To preserve the cells, 200 µl of PBS with 1.5% paraformaldehyde (Sigma-Aldrich, St. Louis, USA) was added. From

Table 1. The study population data

Parameter	Asthmatics	Controls
N	82	40
Females, *n* (%)	54 (65.85)	26 (65)
Age, mean ± SD [years]	51.92 ±12.17	47.95 ±13.66
Age [years] Median (min; max)	53 (23; 69)	52.5 (20; 70)
Disease duration, mean [years]	17.27	–
Phenotype del vs. ins (*N*)	61 vs. 21	22 vs. 18

SD – standard deviation, min.; max. – minimum, maximum, del/ins – deletion/insertion.

each examined sample, cells were collected using a FAC-Scan flow cytometer (Becton Dickinson, San Diego, USA). The mean fluorescence of the eosinophils population was calculated and active cells were identified – expression of CD69 on the eosinophil surface was used for analysis of their activity.

Isolation of DNA

Peripheral blood lymphocytes and isolation kit (QiAamp DNA Blood Mini kit, Syngen Biotech, Wroclaw, Poland) were used for DNA isolation following the manufacturer's instructions.

Genotyping of the VEGF polymorphism

All participants were genotyped using the polymerase chain reaction (PCR) following a protocol by Lachheb *et al.* [16] for the verification of the VEGF polymorphism (addition or loss of 18-bp in the promoter region at position -2549 -2567). In the first step, the concentration of the isolated DNA and its purity were identified using a spectrophotometer (NanoDrop, Thermo Fisher Scientific). Next, the PCR mixture was prepared (total volume 25 µl) – this contained 100 ng of genomic DNA, 1 × Taq Buffer, 0.5 mmol/l of nucleotide, 3 pmol of suitable starter, and 0.5 units of Taq-DNA polymerase (Taq DNA Polymerase E2500-02 – 5000u, EURx); the final $MgCl_2$ concentration was 4 mmol/l. The PCR comprised an initial denaturation step (95ºC for 15 min), then 35 cycles (95ºC for 30 s), primer annealing (54ºC for 30 s and 72ºC for 30 s), and a final extension step (72ºC for 10 min.). The primers were as follows: F, 5'-CCTGGAGCGTTTTGGTTAAA-3' and R, 5'-ATATAG-GAAGCAGCTGGAA-3' (DNA primers, Polgen). Then, the PCR products underwent electrophoresis in agarose gel stained with ethidium bromide. DNA in the form of strips was visible by fluorescence under a UV light transilluminator. The fragment sizes were 234 bp when the 18-bp insertion was present and 216 bp when the 18-bp deletion was present.

Statistical analysis

The data were analysed using Statistica Software Package, version 10 (Polish version; StatSoft, Poland). Laboratory data were reported as means. Comparisons between groups were performed using Kruskal-Wallis ANOVA test. Statistical significance refers to $p < 0.05$.

Results

To determine the expression of CD69 in examined asthmatics and controls, eosinophils from peripheral blood were incubated with a medium (as a negative control), fMLP (as a positive control) or a different concentration of VEGF (Table 2).

Effect of the medium and fMLP on CD69 expression

Freshly isolated eosinophils expressed CD69 but culture in the medium alone or in fMLP had a little effect on the expression of this receptor. The mean specific fluorescence of active CD69+ eosinophils was at a similar level in all tested persons and this difference was not statistically significant ($p > 0.05$).

Effect of VEGF on CD69 expression

To investigate whether CD69 expression could be induced by VEGF, eosinophils were incubated with this cytokine in an experimentally established concentration of 250 ng/ml and 500 ng/ml for 30 min. There was no dose-dependent induction of CD69 expression by VEGF. The mean activated CD69+ eosinophils in asthmatics and controls were similar and this difference was not statistically significant ($p > 0.05$).

Effect of del18 genotype at -2549 -2567 position in the promoter of the VEGF gene on CD69 expression

In the non-stimulated (incubated with medium – patient background: Pb) sample in asthmatics with del phenotype mean of activated CD69+ eosinophils was higher than in asthmatics with ins phenotype and this result was statistically significant ($p = 0.0383$). By contrast, other tested groups (i.e. asthmatics and controls, cultured with fMLP or with VEGF in a concentration of

Table 2. CD69 expression on eosinophils from examined groups – values shown as a specific fluorescence presented as the mean ± SD

Incubation placement	Asthmatics	Controls	Asthmatics del phenotype	Asthmatics ins phenotype
Patient background (Pb – medium)	128.52 ±41.46	138.66 ±88.65	142.50 ±42.25*	114.53 ±40.81*
Positive control (Pc – fMLP)	161.66 ±71.95	177.58 ±126.32	165.90 ±88.76	157.42 ±50.76
VEGF (250 ng/ml)	127.37 ±45.76	131.23 ±88.72	135.61 ±55.49	132.90 ±41.74
VEGF (500 ng/ml)	134.25 ±48.80	148.90 ±107.41	128.33 ±50.08	126.41 ±41.67

p < 0.05, SD – standard deviation, fMLP – N-formylmethionyl-leucyl-phenylalanine, del/ins – deletion/insertion, VEGF – vascular endothelial growth factor.

250 ng/ml and 500 ng/ml for 30 min.) did not show a difference in a level of active CD69+ eosinophils ($p > 0.05$), independently from del or ins phenotype.

Discussion

CD69 (Cluster of Differentiation 69) is a human, disulfide-linked homodimer protein combined of two glycosylated subunits (28–32 kDa). Each subunit consists of an extracellular C-Type lectin domain that binds ligands, a single-spanning transmembrane region with a cytoplasmic tail that relays signals to the cell interior. The CD69 gene is located in the natural killer gene cluster on chromosome 12p13 in humans [17]. CD69 is an early activation marker in most leukocytes (mainly hematopoietic stem cells, T cells, B cells, and NK cells). Transcriptional expression of this molecule is detected 30–60 min after cells activation, protein presence might be detected as early as 2–3 h after stimulation and it declines after 4–6 h. CD69 is expressed on leukocytes in human chronic inflammatory conditions, e.g. asthma, atopic dermatitis, rheumatoid arthritis, lupus erythematosus or systemic sclerosis. *In vivo* strategies on mice models showed that CD69 expression modulates the severity of inflammation disorders, including asthma, contact hypersensitivity, inflammatory bowel disease, arthritis or myocarditis [18, 19].

Different studies have shown that CD69 can be presented on eosinophils. This protein is either absent or expressed at a low level on unstimulated peripheral blood eosinophils but is induced *in vitro* by cytokines i.e. PAF, IL-5, GM-CSF, IFN-γ [20–22]. Further, CD69 expression on peripheral blood eosinophils was significantly increased in asthmatic patients after a whole-lung specific inhalation challenge with high or low molecular weight agents (pollens, house dust mites, Penicillia, isocyanates), supporting the suggestion of CD69 acting as an eosinophil activation marker [23]. Moreover, Miki-Hosokawa *et al.* [24] described that CD69 plays a crucial role in the pathogenesis of allergen-induced eosinophilic airway inflammation and hyperresponsiveness, therefore this molecule could be a possible therapeutic target for asthmatics.

In the present study we revealed that peripheral blood eosinophils from the patient with asthma and with genetic variant del18 of the VEGF-promoter region showed primarily a higher level of activation expressed as presence of CD69 marker on the membrane. Furthermore, incubation of donor eosinophils with increasing VEGF concentrations did not lead to higher CD69 expression in examined groups of patients. These findings may suggest the potential contribution of VEGF gene polymorphism -2549 -2567 del18 to the spontaneous increase of eosinophils activity (priming). In addition, the results show that VEGF is unlikely to significantly activate eosinophils in a dose-dependent manner. In the available literature there are not many data about eosinophils stimulation with VEGF and influence of genetic factors on eosinophils priming in asthmatics [25, 26]. However, taking into account the results of our research, it can be assumed that presence of del18 variant leads to up-regulation of CD69 expression (cells priming and standby) and because of that eosinophils respond to various stimuli.

Eosinophils priming linked with a higher expression of receptors on the cell surface in patients with asthma might be associated with greater predisposition to release mediators from these cells by various – outer (allergens) and inner (cytokines) factors. Furthermore, if eosinophils present higher susceptibility to production and degranulation of pro-inflammatory factors, priming may be associated with severity of symptoms and therapy effectiveness. Airway eosinophilia has been suggested to play a role in airway inflammation and exacerbation in asthma. Peripheral blood eosinophils potentially being in different activation states including pre-activated or primed, may circulate to the airway endothelium leading to the process of airway remodelling and narrowing [11, 27, 28]. In another paper [29], we revealed a connection between presence of the del18 genotype in the promoter region of the VEGF gene and the risk of irreversible bronchoconstriction in asthmatics. Because the same genetic variant is demonstrated in the current study as a possible factor for eosinophils priming, it may suggest some new, complex pathway of progress in airway remodelling and bronchoconstriction in asthmatics. Interestingly, in our previous article [30] we also showed that basophils VEGF-activation is characteristic for patients with asthma and might be associated with presence of the VEGF-polymorphism mentioned above. In turn, it might indicate to influence this particular genetic region on different cells activation state in asthmatics.

Our present study does have potential limitations – expression of only one possible membrane marker chosen to the flow cytometry protocol, experimental selection of two VEGF concentrations and a comparatively small size of the investigated group. Therefore, it would be interesting to extend our work and assess a phenotyping panel with other eosinophils receptors (e.g. CD11b, CD54, CD162) and other cytokines on a greater sample of asthmatics. In conclusion, we present our findings to draw attention to the potential role of VEGF and its genetic variants in the eosinophil priming and activation which is currently undervalued. CD69-mediated signalling during the development of the inflammatory responses might be the goal of future pharmacological endeavours in order to achieve more efficient treatment of asthma.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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