Summary

Introduction: In cell trafficking to the airways in asthma, among integrins the most important are those containing α4 and β2 subunits. We have previously shown that also blocking of collagen receptors, α1β1 and α2β1 integrins, inhibits transmigration of eosinophils of asthmatic subjects through a monolayer of skin microvascular endothelial cells seeded on collagen IV coated inserts. However, it was not clear whether this observation was limited to asthma or depended on the type of microvascular cell and collagen IV used as a base.

Materials & methods: In the current study we performed a transmigration assay using human lung microvascular endothelial cells seeded directly on a plastic surface as a base and blood cells isolated from 12 representatives of each of two groups, asthmatics and healthy donors, by gradient centrifugation, followed by immunomagnetic negative separation of eosinophils. Isolated eosinophils and peripheral blood mononuclear cells (PBMC) were inhibited by snake venom-derived integrin antagonists including viperistatin and VP12, as inhibitors of α1β1 and α2β1 integrin, respectively, and VLO5 and VLO4, as inhibitors of α4β1 and α5β1 integrin, respectively.

Results: All snake venom-derived anti-adhesive proteins were effective in inhibiting eosinophil transmigration, whilst only VLO5 and VLO4 reduced PBMC mobility in this assay. This observation was similar in both groups of subjects studied.

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Introduction

Bronchial asthma is one of the most common respiratory diseases. Its prevalence is constantly increasing, while the pathogenesis is still poorly understood [25]. The inflammatory foci of asthma are located in the airways, and eosinophil is considered as a major effector cell [10, 11, 34]. However, the mechanism of eosinophil recruitment and the pathway of its activation have not been fully elucidated yet. Although anti-IL-5 antibodies distinctly reduced blood eosinophilia, they caused only a slight clinical improvement of asthmatic symptoms [27]. On the other hand, helper T-lymphocytes appear to be pivotal in driving the disease development and progression. Adoptive transfer of Ag-primed T-cells into naïve animals induces eosinophilia, bronchial hyper-responsiveness and late airway response [26]. Moreover, helper T-cells are activated in the airways, even when the disease is asymptomatic [15].

Leukocyte migration through the blood vessel wall and into the inflammatory site is mediated by cell-surface receptors and their specific ligands located on the vascular endothelium and in extracellular matrix (ECM). Among these receptors, integrins play a very important role. Leukocyte-expressed integrins belong to a large family of heterodimeric glycoproteins, which in the active conformation are composed of two non-covalently associated α and β subunits. The 18 α and 8 β subunits identified to date are associated in a restricted manner to create 24 heterodimers for specific ligand binding [17]. By regulating cell-cell and cell-matrix interaction, they modify cell growth, migration, activation and survival [3, 17]. Firm adherence and rolling of leukocytes to the inflammatory site is supported mainly by interaction of β1 and α1 integrins. T cells abundantly express α1β1, but also α1β2 integrin. Both of them are known as the basic receptors for fibronectin, a major component of ECM [3, 28]. Eosinophil expresses constitutively a large amount of α1β1 integrin, but the presence of α2β1 integrin was also described on this cell [22]. Both of these integrins were previously characterized as important in the pathogenesis of asthmatic inflammation [3, 23].

Previously we hypothesized that also collagen integrin receptors, α1β1 and α2β1 integrins, could be involved in asthma pathology. Although α1β1 and α2β1 are structurally very homologous, their role in physiology and disease development displays many differences. The α1β1 integrin is a preferential receptor for basement membrane collagen type IV, whereas α2β1 is a preferential receptor for fibrillar collagens type I–III [35]. Collagen I (and III) is a major component of ECM in the lungs [10, 35], and for this reason it may be important in bronchial asthma-dependent airway remodelling. In the past we have reported that both α1β1 and α2β1 collagen integrin receptors can be found on peripheral blood eosinophils of asthmatic subjects [4] and α1β1 (but not α2β1) is up-regulated on blood T lymphocytes of patients with mild to moderate persistent asthma in comparison to healthy donors [6]. Moreover, integrin-dependent adhesive properties of eosinophil have been strongly inhibited by obustatin and viperistatin, snake venom KTS-disintegrins, potent and selective inhibitors of α1β1 integrin [4]. However, the role of both these collagen integrin receptors in asthma pathology has not been comprehensively studied yet. Beneficial effects of anti-α1β1 monoclonal antibodies have been described in certain animal models of immu-
nologically mediated diseases, including inflammatory bowel disease, arthritis, and allergen-induced leukocyte recruitment to the lungs associated with the late airway response in sheep [1, 3]. Recently, we also reported that inhibition of αβ1 and αβ6 integrins reduced transmigration of asthmatic eosinophils through a monolayer of human microvascular endothelial cells of skin origin seeded on collagen IV coated inserts [7]. However, in this study we did not test eosinophils obtained from healthy donors, and it was not clear whether our observation was limited to asthma or depended on the type of endothelial cells (skin origin) or collagen IV used as a base in the transmigration assay. For this reason we designed the current study with eosinophils purified from peripheral blood of subjects, asthmatics and healthy donors, in a transmigration assay through human microvascular endothelial cells of lung origin (L-HMVEC) seeded directly on a plastic surface. This basal cell, in our opinion, seems to be optimal for asthma research. For inhibition purposes we used functionally active mouse anti-human monoclonal antibodies (mAb) and snake venom-derived antagonists of integrins [24]. The presented study is a continuation of our previous research, but proposed experimental settings are original and have not been previously conducted.

**Materials and Methods**

**Subjects**

The transmigration assay was performed in 12 moderately persistent, well-controlled atopic asthmatics and 12 sex- and age-matched healthy donors. All asthma patients were in a stable clinical condition according to GINA guidelines [25]. Atopic status of all asthma patients was confirmed by a positive skin prick test for at least one standard inhaled allergen (Allergopharma, Germany). All of them were treated with a medium dose of inhaled glucocorticoid and long-acting β2-agonist and did not receive systemic steroids during the last 6 months. Additionally, two of the asthma patients received montelukast and four of them nasal steroids. Smokers and patients suffering from any chronic disease were excluded from the study. The control group consisted of non-atopic and non-smoking healthy volunteers. Our study was approved by the Jagiellonian University Ethical Committee and all subjects gave informed consent to participate in this study.

**Snake venom proteins**

Homodimeric RGD-disintegrin, VLO4 [13] blocking αβ1 integrin, and heterodimeric MLD-disintegrin, VLO5 [5] blocking αβ3 integrin, were purified from Vipera lebetina obtusa venom. Viperistatin, a monoclonic disintegrin [20], blocking αβ5 integrin, and VP12, a C-lectin type protein [33] blocking αβ6 integrin, were purified from Vipera palasitaevenom. Snake venom molecules were separated from crude venom using two steps of reverse phase HPLC. Specificity and selectivity of viperistatin for αβ1 were confirmed by Kisiel et al. [20] in ELISA and an adhesion system of transfected cell lines. In these assays, viperistatin inhibited only αβ1, and was not effective against αβ3, αβ5, αβ7, αβ6, or αβ8 [20]. Properties of VP12 for αβ3 were confirmed by Staniszewska et al. [33]. They proved that VP12 is a strong and specific αβ3 blocker, not effective against αβ1, αβ6, αβ7, αβ9, αβ1, or αβ5. VLO5 potently antagonizes αβ3, αβ6 and αβ3, but is also a weak inhibitor of αβ1 and αβ6 integrins [5,13]. VLO4 is a strong inhibitor of αβ3, but is less potent against αβ1 and αβ6 integrin [13].

**Monoclonal anti-human antibodies**

Blocking anti-human monoclonal antibodies against α1 (clone FB12) and α4 (clone P1E6) were purchased from Chemicon, Millipore Corporation (Temecula, CA, USA). The isotype control for the transmigration assay – mouse IgG1 antibody against keyhole limpet hemocyanin antigen (clone X40) – was purchased from Becton Dickinson Biosciences (San Jose, CA, USA).

**Isolation of eosinophils and peripheral blood mononuclear cells**

Eosinophils and peripheral blood mononuclear cells (PBMC) were purified from the buffy coats as described previously [16,30]. Briefly, 50 ml of blood was collected on EDTA from the antecubital peripheral vein. Blood was diluted and layered onto 1.119 and 1.077 g/ml Histopaque gradient (Sigma Chemicals Co., St Louis MO, USA), then centrifuged at 300 g for 30 min at 20°C. Both cell layers – the upper one containing mononuclear cells and the lower one containing granulocytes – were collected and washed twice in PBS with 0.5% albumin. The upper layer (containing PBMC) was incubated in RPMI 1640 (Sigma Chemicals Co., St Louis MO, USA) with 10% FCS (Sigma Chemicals Co., St Louis MO, USA) at 37°C in a humidified atmosphere of 5% CO2 for 1 hour in a 25 cm2 tissue culture flask (Corning Costar Co, Cambridge, Mass), in order to adhere and discard monocytes. Remaining red blood cells were removed by lysis with 155 mM NH4Cl and 10 mM KHCO3 buffer. Afterwards, granulocytes were incubated with a cocktail of biotin-conjugated mouse mAb against keyhole limpet hemocyanin antigens (clone X40) – was purchased from Becton Dickinson Biosciences (San Jose, CA, USA). Purification of eosinophils from monocytes was performed with magnetic microbeads (Eosinophil isolation kit, Miltenyi Biotec, Auburn CA, USA) in a manner and a concentration recommended by the product supplier. Finally, negative magnetic separation of pure eosinophils was performed with a magnetic column CS and magnet-activated cell sorter system purchased from Miltenyi Biotec (Auburn CA, USA). Purity of eosinophils was > 95% (May-Grunwald-Giemsa staining) and viability > 97% (trypan blue). Contaminating cells included neutrophils (1-4%) and lymphocytes (3-4%).

**ECP and IgE level measurement**

Serum concentration of ECP and IgE was measured using UniCAP System, Pharmacia, Sweden.
Table 1. Clinical and laboratory characteristics of the subjects studied in transmigration assay

|                                | Asthmatics (n=12) | Healthy controls (n=12) | Difference |
|--------------------------------|-------------------|-------------------------|------------|
| Male/female ratio              | 3/9               | 4/8                     | NS         |
| Age (years), mean ± SE         | 44.4 ± 4.1        | 41.2 ± 1.8              | NS         |
| Duration of asthma (years), mean ± SE | 11.9 ± 4.4        | not done                |            |
| FEV1 (% of predicted), mean ± SE | 77.3 ± 3.9        | not done                |            |
| Blood eosinophilia (mm3), mean ± SE | 170 ± 36          | 126 ± 18                | NS         |
| Total IgE (IU/l), mean ± SE    | 192 ± 75          | 27 ± 10.1               | p<0.05     |
| Serum ECP (μg/l), mean ± SE    | 8.1 ± 1.3         | 7.9 ± 2.0               | NS         |

Data are presented as a mean and a standard error of a mean. The difference was calculated using U Mann-Whitney test; NS- non significant, SE - standard error, FEV1- forced expiratory volume in 1 second, ECP - eosinophil cationic protein

Transmigration assay

The lung-derived human microvascular endothelial cell (L-HMVEC) line was purchased from Lonza (Walkersville Inc, USA). Cells were grown in EGM-2-MV media also purchased from Lonza. Passages 5 to 9 were used for experiments. L-HMVEC were seeded onto 3 μm pore size polyethylene terephthalate transwell plate inserts (BD Biosciences, Franklin Lakes, NJ, USA) with the density of 1 x 10^5 cells and after reaching confluence stimulated with 3 ng/ml TNFα (Sigma-Aldrich, Saint Louis, Missouri, USA). Isolated PBMC or eosinophils (1 x 10^6) were suspended in serum-free RPMI 1640 and incubated at room temperature for 30 minutes with specific integrin inhibitors. Monoclonal antibodies and snake venom proteins were applied at a concentration of 10 μg/1 ml. Each transmigration assay was started by washing upper and lower chambers with RPMI 1640. Afterwards, 250 μl of cell suspension was placed into the upper chamber (above the L-HMVEC monolayer). The lower chamber contained 900 μl of RPMI 1640 supplemented with 10% FCS as a chemoattractant. The transmigration assay was performed in duplicate, at 37°C, in a humidified atmosphere of 5% CO2 for 3 hours. After that, cells in the lower chamber were counted in a haemocytometer. The percentage of transmigrated eosinophils was calculated using the formula: (number of migrated eosinophils)/(total eosinophil added to the upper chamber) x 100 (%).

After the transmigration assay, presence of eosinophils in the lower chamber was confirmed by cytoospin followed by May-Grunwald-Giemsa staining, with 85% cell viability (trypan blue) in upper and lower chambers – similar in samples with isotype control and all anti-adhesive proteins used.

Statistical analysis

Values in Table 1 are presented as the mean and standard error of the mean. The distribution of results of the transmigration assay cannot be assessed, due to the low number of subjects studied (n=12). Therefore, for statistical purposes we used the Wilcoxon signed-rank test (anti-adhesive proteins vs. isotype control) and Mann-Whitney U test (asthma subjects vs. healthy donors) – non-parametric tests usually applied for non-normally distributed values. Moreover, we applied an agglomerative hierarchical clustering approach (Ward’s minimum variance method) in order to study similarity in mode of action of all anti-adhesive proteins used. In all instances the two-sided 5% level of significance was used. All statistical testing was performed with Statistica StatSoft (Tulsa, OK, USA) software.

Results

Clinical and laboratory characteristics of the subjects studied in the transmigration assay are shown in Table 1.

The inhibitory effect of anti-adhesive proteins on PBMC and eosinophil transmigration is presented in Fig. 1 and Fig. 2.

In Fig. 1 data are shown as percentage of transmigrated cells, calculated using the formula: (number of migrated cells)/(total cells added to the upper chamber) x 100 (%). In Fig. 2 results of the transmigration assay are presented as the percentage of inhibition with respect to the isotype control (number of transmigrated cells in isotype control was assumed as 0% inhibition).

Snake venom disintegrins, VLO5 and VLO4, were active to block transmigration of PBMC in both groups of subjects,
whereas inhibitors of collagen receptors were not effective. All integrin antagonists blocked migration of eosinophil of asthmatic subjects to a statistically significant level. Interestingly, a similar pattern of inhibition was also observed for control eosinophil, except for anti-α2 mAb, which showed no effect.

Using the Mann-Whitney U test we did not find any difference in mode of action of anti-adhesive proteins between asthma and control subjects.

The agglomerative clustering procedure (Ward’s method) showed similarity in mode of action for: 1) anti-α1 mAb, anti-α2 mAb and viperistatin, 2) VP12, VLO4 and VLO5 for eosinophils of asthmatic subjects; and 1) anti-α1 mAb and viperistatin, 2) anti-α2 mAb and VP12; 3) VLO4 and VLO5 for eosinophil of healthy donors. For PBMC in both groups of subjects, anti-adhesive proteins were similarly divided into two separate groups: 1) anti-α1 mAb, anti-α2 mAb, anti-α2 mAb, viperistatin and VP12; 2) VLO4 and VLO5.

**DISCUSSION**

The collagen receptors in asthma pathology still require intensive studies to elucidate their role in progression of this inflammatory disease. Collagens are the major structural matrix proteins, which are abundantly found in the majority of human tissues. Therefore, their receptors could be involved in pathology of many chronic inflammatory diseases, especially those leading to architectural changes, e.g. asthma remodelling. Recently, we obtained experimental evidence of possible α1β1 and α2β1 integrin involvement in transmigration of eosinophils of asthmatic subjects through a monolayer of human microvascular endothelial cells of skin origin, seeded on collagen IV coated inserts [7]. However, this study was performed only with eosinophil obtained from asthmatic subjects, and it was not clear whether its results depended on eosinophil physiology or pathology limited to asthma, or type of microvascular endothelial cell and collagen IV used as a base in the transmigration assay. For this reason in the current study we examined cells purified from both asthmatics and healthy donors in a transmigration assay through a monolayer of lung-derived HMVEC seeded directly on a plastic surface. We found that anti-α1 mAb, viperistatin and VP12 significantly inhibited eosinophil movement in a similar manner in both asthmatics and healthy donors. In contrast, anti-α2 mAb was effective only for eosinophils of asthmatic subjects. For cells obtained from healthy donors, migratory reduction
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Fig. 2. Effect of monoclonal antibodies and snake venom inhibitors of integrins on transmigration of peripheral blood mononuclear cell (PBMC) and eosinophil through monolayer of lung-derived human microvascular endothelial cell. Data are presented as a percentage of inhibition in relation to the isotype control samples. Results are expressed as a mean with standard error of a mean. Statistical significance was calculated using Wilcoxon signed-rank test (in comparison to isotype control).

approached but did not reach statistical significance. In our previously published paper, anti-α1 mAb was also not able to significantly inhibit asthmatic eosinophil movement (although the difference was close to reaching significance). This discrepancy in effectiveness of anti-α1 mAb in eosinophil mobility inhibition could be explained by selection of patients and the higher number of subjects studied in the current project, as well as the different type of microvascular endothelial cells used as a base. Also important is the fact that VP12 recognizes α1β1 integrin regardless of activation stage, whereas mAb may possibly bind only activated integrin, and this alteration of integrin activation in asthmatic eosinophils could be crucial for our results.

We have also noted in the past that α1β1 and α2β1 integrins can be found on peripheral blood eosinophils of asthmatic subjects [4]. However, presence of both collagen receptors on resting eosinophils appears to be not completely confirmed [2]. It is also possible that α1β1, detected by us on blood eosinophils comes from blood platelets adhering to eosinophils. Platelets are known to bind to eosinophils to a varying degree [19]. However, platelets abundantly express the α2 subunit, and not α1 [12]. Therefore α1 expression on isolated eosinophils could not be related to platelet “satellitism”, but to eosinophils themselves. Although we focused on collagen receptors expressed on leukocytes, it is also likely that inhibitors may affect integrins expressed on endothelial cells. The α1β1 and α2β1 integrins are abundantly expressed on the HMVEC, but their role in the immune response is not completely confirmed [31, 32]. Therefore, we consider that leukocytes after pre-incubation with integrin inhibitors contain specifically blocked collagen receptors, which are unable to interact with ECM produced by endothelial cells during a seeding procedure [21]. In this study, L-HMVEC were seeded on a plastic surface not coated with any ECM compounds, but the results were similar to those with HMVEC of skin origin seeded on collagen IV coated inserts [7]. Both these studies support previously published research showing blocking of the recruitment of eosinophils in a sheep model of bronchial asthma by specific anti-α1 mAb [1].

Potent inhibition of PBMC migration by VLO5 is secondary to the antagonistic effect on α4β1 integrin and its interaction with VCAM-1 on activated endothelium. VLO4 potently antagonizes α5β1 integrin, and its activity to block leukocyte migration suggests that the fibronectin receptor may also play a role in the inflammatory response.
Involvement of $\alpha_1$, $\beta_1$ and $\alpha_2$, $\beta_1$ integrins in eosinophil but not PBMC transmigration may also have a therapeutic implication. To date, anti-integrin asthma treatment has focused mainly on $\alpha_1$, $\beta_1$ antagonists [18], but it was interrupted due to the observed side effects [9]. We hope that collagen receptor therapy could be supportive for future asthma treatment. Viperinatin or VP12 seems to be an excellent template for the design of a new $\alpha_1$, $\beta_1$ and $\alpha_2$, $\beta_1$ integrin low molecular weight inhibitor with potential therapeutic utility. It is important to note that up to 5 mg/kg KTS-disintegrins (such as viperinatin) are not toxic at least in a mouse experimental model [33]. Currently, disintegrin-based anti-integrin treatment is used widely only in cardiovascular medicine for the prevention of platelet thromboembolism. Two RGD/KGD integrin-derived drugs, tirofiban and eptifibatide, are successfully applied as inhibitors of $\text{IIb/IIIa}$ glycoprotein on the platelet surface [8]. A similar strategy may be used to design a disintegrin-based therapy for chronic inflammation. Ligands for $\alpha_1$, $\beta_1$ and $\alpha_2$, $\beta_1$ integrins, such as collagen I and IV and laminin, are important components of the lung ECM, and participation of this integrin in tissue remodelling during asthma development is considered [14]. This suggestion is supported by previous reports demonstrating $\alpha_1$, $\beta_1$ integrin as a stimulator of collagen and fibronectin accumulation in the airways [14, 29]. Moreover, inhibitors of collagen receptors may also influence airway remodelling. Current chronic asthma therapy has no direct impact on the remodelling process. Recent studies revealed that in a murine asthma model both effects could be achieved by blocking of a tyrosine kinase using imatinib [29]. This compound inhibits collagen deposition also [29]. A similar situation may occur for integrin inhibitors. It is well established that integrins are associated with cellular signalling pathways, which activate various tyrosine kinases. It is tempting to speculate that such anti-adhesive snake-venom derived disintegrins could be used as an anti-inflammatory therapy in the future, especially in eosinophil-dependent diseases. However, before such a therapeutic option is possible, a comprehensive understanding of integrin/ligand interactions is required to specifically limit the inflammatory response without adversely affecting other physiological functions of integrins.

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