

Migration through basement membrane modulates eosinophil expression of CD44

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Summary

Background Tissue eosinophils express more membrane receptors and release more mediators than blood eosinophils, suggesting that migration from blood to tissue modulates eosinophil phenotype and functions.

Objective We postulated that eosinophil passage through endothelial basement membrane, an important step of eosinophil migration into tissue, may be responsible for some of these changes.

Method We previously showed that 5-oxo-6, 8, 11, 14-eicosatetraenoic acid (5-oxo-ETE) in combination with IL-5 promotes eosinophil migration through Matrigel®, a mouse tumour cell-derived basement membrane. Using this model, we evaluated the effect of trans-Matrigel migration on purified human blood eosinophil expressions of CD44, CD69 and HLA-DR that either increase or appear on activated eosinophils, and releases of peroxidase (EPO), leukotriene (LT) C₄ and granulocyte-monocyte colony stimulating factor (GM-CSF).

Results IL-5, but not 5-oxo-ETE, increased eosinophil expression of CD44 and CD69. Migration of eosinophils through Matrigel significantly increased CD44 expression level over the one induced by IL-5 ($P=0.0001$). Migration through Matrigel did not modify CD69 expression compared with the one obtained in the presence of IL-5 alone; however, incubation of eosinophils on Matrigel decreased IL-5-induced CD69 ($P=0.0001$). Trans-Matrigel migration did not modify HLA-DR expression, nor EPO, LTC₄ and GM-CSF releases.

Conclusion These data show that *in vitro* trans-Matrigel migration and Matrigel contact modulate eosinophil membrane receptor expression. Consequently, they suggest that migration through basement membrane mediates changes in cell-surface phenotype observed on activated eosinophils and probably prepares them for interactions with tissue components and cells.

Keywords CD44, CD69, eosinophil peroxidase, eosinophil, GM-CSF, HLA-DR, LTC₄, Matrigel, migration

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Introduction

Eosinophils are mostly tissue-dwelling leucocytes [1]. They have the capacity to express and/or release many mediators, cytokines, chemokines and ligands [reviewed in 2]. In tissue, they may act as very potent effector and immunomodulator cells. Blood eosinophils are thought to circulate in a quiescent state until they migrate into tissue [3]. In healthy mucosa, where they account for a small proportion of the cells, their activation status is hardly measurable due to their number. However, in diseases such as asthma, eosinophilic pneumonia and idiopathic hypereosinophilia syndrome, the number of tissue eosinophils is increased. These eosinophils show increased cell-surface molecule expression, such as complement and IgG receptors [1, 3] compared with their blood counterparts and expressed ICAM-1, HLA-DR and CD69, which are barely or not detectable on

blood eosinophils [4–7]. They also present an increased release of superoxide anion and leukotriene (LT)C₄ compared with blood eosinophils [8, 9]. Lung lavage eosinophils obtained after a segmental bronchoprovocation show an increase in membrane receptors, adhesion, survival and generation of superoxide anion, which was not achieved by incubation of blood eosinophils with IL-5 and granulocyte-monocyte colony stimulating factor (GM-CSF) [10]. Consequently, in diseases, tissue eosinophils present an increased cell-surface molecule expression, an increased capacity to release mediators, and probably an increased capacity to interact with other cells and extracellular matrix (ECM) components.

These observations suggest that the passage of eosinophils from blood to tissue is associated with modifications of eosinophil phenotype and upregulation of eosinophil functions via mechanisms not well understood. Blood eosinophils have to pass through an endothelium and a basement membrane and this process involves action of various chemotactic factors and cytokines, emergence of adhesion molecules, adherence to endothelial cells and to ECM components, modification of the cytoskeleton, activation of various proteinases, and movement

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of cells through the endothelium and the ECM components [1, 11]. Basement membrane is a thin structure constituted mostly of collagen IV, laminin, and proteoglycans [12]. This membrane, functioning as a barrier and a scaffolding, modulates cell proliferation, differentiation and orientation. During tissue migration, eosinophil interactions with various ECM components can influence their functions and phenotype. For example, collagen IV and laminin increase eosinophil adhesion and viability [13].

Given these observations, we postulated that eosinophil phenotype and functions are modulated by the passage of eosinophils through the endothelial basement membrane during their recruitment into the tissue. In this study, we evaluated the eosinophil expression of cell-surface receptors and the liberation of mediators and cytokines after their migration through a mouse tumour cell-derived basement membrane (Matrigel). We chose to evaluate expression of HLA-DR, an antigen presentation receptor [4], CD69, an early marker of activation [14], and CD44, a receptor that binds to hyaluronic acid and is likely to be involved in cell rolling [15, 16]. In addition, we studied the release of eosinophil peroxidase (EPO), a potent granule cationic protein, the release of LTC₄, a powerful bronchoconstrictor and pro-inflammatory mediator, and the production of GM-CSF, an important cytokine that promotes maturation and survival of eosinophils [2]. We found that CD44 and CD69 expressions, but not the other measured parameters, were modulated by trans-Matrigel migration or Matrigel contact.

Materials and methods

Reagents and monoclonal antibodies

Platelet activating factor (PAF) and activated complement fraction 5 (C5a) were purchased from Sigma Chemical Co. (St Louis, MO, USA), 5-oxo-6, 8, 11, 14-eicosatetraenoic acid (5-oxo-EETE) and kits for LTC₄ dosage from Cayman Chemical (Ann Arbor, MI, USA); kits for GM-CSF dosage from R & D Systems Inc (Minneapolis, MN, USA), monoclonal mouse antibody IgG₁ (peridinin chlorophyll protein, PerCP) (isotypic control) and human anti-HLA-DR (PerCP) from Becton Dickinson (San Jose, CA, USA), and monoclonal mouse antibody IgG₁ (phycoerythrin, PE) (isotypic control), human anti-CD69 (PE), monoclonal mouse antibody IgG_{2b} (FITC) (isotypic control) and human anti-CD44 (FITC) from Pharmingen (San Diego, CA, USA). Monoclonal mouse anti-human CD16 antibodies were obtained from Miltenyi Biotec (Auburn, CA, USA) and human recombinant IL-5 was purchased from Peprotech Inc (Rocky Hill, NJ, USA).

Subjects' evaluation

Healthy subjects without a history of allergy or asthma, and asthmatic subjects meeting the criteria of the American Thoracic Society for the diagnosis of asthma, were recruited for the study [17]. The asthmatic subjects had mild asthma defined by a morning pre-bronchodilator FEV₁ > 85% of the predicted value, and requirement for only short-acting β_2 -agonist on demand. The inclusion criteria included a stable treatment for more than 3 months, no use of inhaled steroids over the 3 months preceding the study, no use of other drugs, and no other disease than asthma. Approval for the study was obtained from the local ethics committee and all subjects agreed

to participate in the study. All subjects underwent blood sampling early in the morning. FEV₁ values were measured in the morning at least 8 h after any β_2 -agonist inhalation with a PFT II Vitalograph Spirometer (Vitalograph Ltd, Buckingham, UK) and were expressed as a percentage of predicted values.

Blood cell processing and eosinophil purification

Eosinophils were purified as we previously described [18]. Briefly, venous blood (150 mL) was collected in tubes with EDTA and centrifuged to remove platelet-rich plasma. Red cells were sedimented in presence of Dextran 6%. A centrifugation on Ficoll (1.077 g/mL) was performed to separate mononuclear cells from granulocytes. An osmotic shock lysed remaining red cells and granulocytes were recovered. Eosinophils were separated from neutrophils by negative selection using a magnetic cell sorter (MACS) from Miltenyi Biotec. An aliquot of cell suspension was used to determine cell counts (haemocytometer) and viability (trypan blue exclusion) and make smears for differential cell counts (Diff-Quik; Invitrogen Canada, Burlington, Ontario, Canada). Purity of eosinophil preparations used in this study was always greater than 98% and the contaminating cells were neutrophils and/or lymphocytes. Eosinophil viability was always greater than 99%.

Incubation conditions and transmigration assay

Eosinophils were resuspended at 1×10^6 cells/mL in culture medium (RPMI 1640 with 10% FBS and 1% penicillin-streptomycin; all from Dade Diagnostics, Aguada, Puerto Rico). To study membrane receptor expression, eosinophils were incubated or not with IL-5 (10 ng/mL) during 30 min and laid in inserts with bottom covered with Matrigel or not. These inserts were placed in 24-well Biocoat Matrigel Invasion Chambers (Becton Dickinson). 5-Oxo-EETE (1 μ M), a potent eosinophil chemoattractant, was added to the lower chamber of some wells to induce migration, as we have previously shown [10]. Plates were incubated at 37 °C under 5% CO₂ for 18 h. Wells with IL-5 alone served to evaluate the effects of IL-5, wells with IL-5 and 5-oxo-EETE to evaluate the potential additive effect of 5-oxo-EETE, wells with IL-5 and Matrigel served to evaluate the effect of the contact of eosinophils with the basement membrane, and wells with 5-oxo-EETE and Matrigel, with or without IL-5, served to evaluate the effect of migration through this membrane. To study mediator release, cells were stimulated with IL-5 for 30 min and laid in wells with or without Matrigel. Then, 5-oxo-EETE was added to the lower chamber and eosinophils were incubated at 37 °C under 5% CO₂ for 18 h. For this part of the study, all conditions were made in duplicate (two control wells without Matrigel and two wells with Matrigel). One pair of wells was used to determine cell counts and percentage of migration, and to stimulate eosinophils for measuring EPO release. The other pair served to measure GM-CSF release in the supernatants and to stimulate eosinophils for measuring LTC₄ release.

Cell recuperation and percentage of migration through Matrigel

To evaluate the number of cells that had migrated in the lower chamber, eosinophils from upper and lower chambers of wells were recuperated with cold RPMI 1640 without FBS and the chambers were washed with cold RPMI-EDTA 5 mM to detach

adherent cells. Recuperated cells were centrifuged, re-suspended in cold HBSS-BSA 1% and counted. For each condition, percentage of migration was calculated as the number of cells in the lower chamber of the Matrigel invasion chamber divided by the number of cells in the lower chamber of the control invasion chamber without a Matrigel membrane. In the absence of Matrigel, the number of cells found in the lower chamber of the wells was greater than 95% of the total number of eosinophils added to the upper chamber; in the presence of Matrigel with both IL-5 and 5-oxo-ETE it was greater than 74%. The viability of eosinophils was greater than 98% at the end of the incubations.

Cell surface receptor detection

Eosinophils from all different incubation conditions were suspended in HBSS-BSA 1% at a concentration of 250 000 cells/100 μ L. Eosinophils were then labelled with antibodies for each membrane receptor, namely CD44, CD69 and HLA-DR, or with their isotypic controls. After 30-min incubation at 4°C, eosinophils were washed, centrifuged and re-suspended in 100 μ L of HBSS-BSA 1%. They were fixed with paraformaldehyde 4% and incubated at 4°C for 10 min. Eosinophils were then washed with HBSS-BSA 1%, centrifuged and re-suspended in HBSS-BSA 1% and kept at 4°C up to the analysis by flow cytometry (EPICS ELITE ESP, Coulter Electronics, Hialeah, FL, USA). Receptor expression was expressed as the percentage of positive cells. A cursor was placed where the negative control has no more than 4% of the measured events observed above that cursor. With the tested antibodies, the percentage of positive cells was then determined using the same cursor.

Eosinophil adhesion assay to hyaluronic acid

Adhesion to hyaluronic acid was evaluated using a MTT assay kit (Sigma). The MTT system measures enzymatic activity of living cells via mitochondrial dehydrogenase. Briefly, 96-well plates (Nunc-Immuno Plate Maxisorp Surface) were coated overnight at 4°C with 100 μ L of hyaluronic acid (100 ng/mL). After incubation, the wells were washed twice with HBSS buffer. Non-specific protein binding was blocked by addition of 100 μ L of HBSS + 1% BSA for 1 h at 37°C. Eosinophils (1×10^6 cells/mL) were pre-incubated for 30 min with or without IL-5 (10 ng/mL) and placed in the insert upper chamber coated or not with Matrigel; 5-oxo-ETE was added or not to the lower chamber. The cells were incubated for 18 h at 37°C. Migrated cells (1×10^5 /100 μ L) were recovered and immediately added into hyaluronic acid-coated plates in duplicate for 1 h at 37°C. Thereafter, non-adherent cells of test wells were removed by gentle aspiration and washing with pre-warmed HBSS buffer. Control wells were not washed. MTT solution (10 μ L) was added to the wells. After a 4-h incubation period, the resulting MTT formazan was dissolved by adding MTT solvent. The resulting purple solution was spectrophotometrically measured and the optical density obtained. The number of adherent cells was expressed as percentage of optical density of total cell number added to the control wells; optical density of tested condition/optical density of control well $\times$ 100.

Eosinophil peroxidase assay

A kinetic, colourimetric assay was used to measure EPO concentration as a function of maximum velocity of reaction ($V_{\max}$) [19]. Eosinophils that migrated through inserts with and

without Matrigel were re-suspended in HBSS-CaCl₂ 1.6 mM + 0.1% gelatin. Cells (200 000–500 000-cells/100 μ L) were distributed in wells of a 96-well plate. PAF (1 μ L, 1 μ M) was added to selected wells to initiate degranulation of eosinophils. The plate was shaken for 30 min, o-phenylenediamine dihydrochloride substrate was added (100 μ L/well) and the supernatants were analysed by spectrophotometry at 490 nm every 6 s for 4 min.

GM-CSF and LTC₄ assays

Lower chamber suspension of eosinophils that migrated through inserts with or without Matrigel were centrifuged. As adhesion to ECM components may trigger autocrine generation of GM-CSF by eosinophils [20], the supernatants were aliquoted for dosage of GM-CSF release by eosinophils using a quantitative immunoenzymatic assay according to manufacturer recommendations (R & D Systems Inc.). For LTC₄ assay, eosinophils were re-suspended in HBSS-CaCl₂ 1.6 mM at a concentration of 1×10^6 cells/mL and incubated with PAF (1 μ M) for 10 min at 37°C, 5% CO₂ and thereafter with C5a (10 nM) in the same conditions. Tubes were then put on ice for 5 min to stop the reaction. Cells were centrifuged and supernatants used for the dosage of LTC₄ by a quantitative immunoenzymatic assay according to manufacturer recommendations (Cayman Chemical).

Statistical analysis

Results of respiratory functions are presented as medians with range and were analysed using the Wilcoxon's rank sum test, as normality and variance assumptions were not fulfilled. Geometric means of PC₂₀ were analysed by a Student's unpaired *t*-test. Mediator, cytokine and cell-surface receptor expression data were analysed using a crossed-nested design. These data are presented as means and standard errors. *A posteriori* multiple comparisons were performed using the Tukey's technique. Results were considered to be significant when $P < 0.05$. These statistical analyses were performed with a statistical package (SAS Institute Inc., Cary, NC, USA).

Results

Subjects' characteristics

Thirteen normal subjects (5 M/8F, median age 31 years, range 17 to 54) and 17 asthmatics (9 M/8F, median age 23.5 years, range 18 to 48) were recruited for this study. Median (range) FEV₁ for normal and asthmatic subjects were, respectively, 98.5% (91 to 134) and 96% (85 to 111) of predicted values ($P = 0.23$). Geometric means of PC₂₀ for normal and asthmatic subjects were, respectively, 43.9 ± 19.7 mg/mL and 1.4 ± 0.2 mg/mL ($P = 0.0001$).

Cell surface receptor expression

Figure 1 shows typical cytometry log scaled plots of cell surface CD44 expression modulation by IL-5 and migration through Matrigel. Eosinophil expression of CD44 (expressed as percentage of positive cells) was significantly increased by IL-5 ($62.6 \pm 5.6\%$; mean fluorescence: 1.17 ± 0.14) compared with unstimulated cells and cells incubated on Matrigel ($46.2 \pm 5.5\%$ and $48.7 \pm 5.3\%$, $P = 0.0001$, Fig. 2a; mean fluorescence: 0.84 ± 0.11 and 0.88 ± 0.24 , respectively). 5-Oxo-ETE and contact with Matrigel did not amplify CD44 expression.

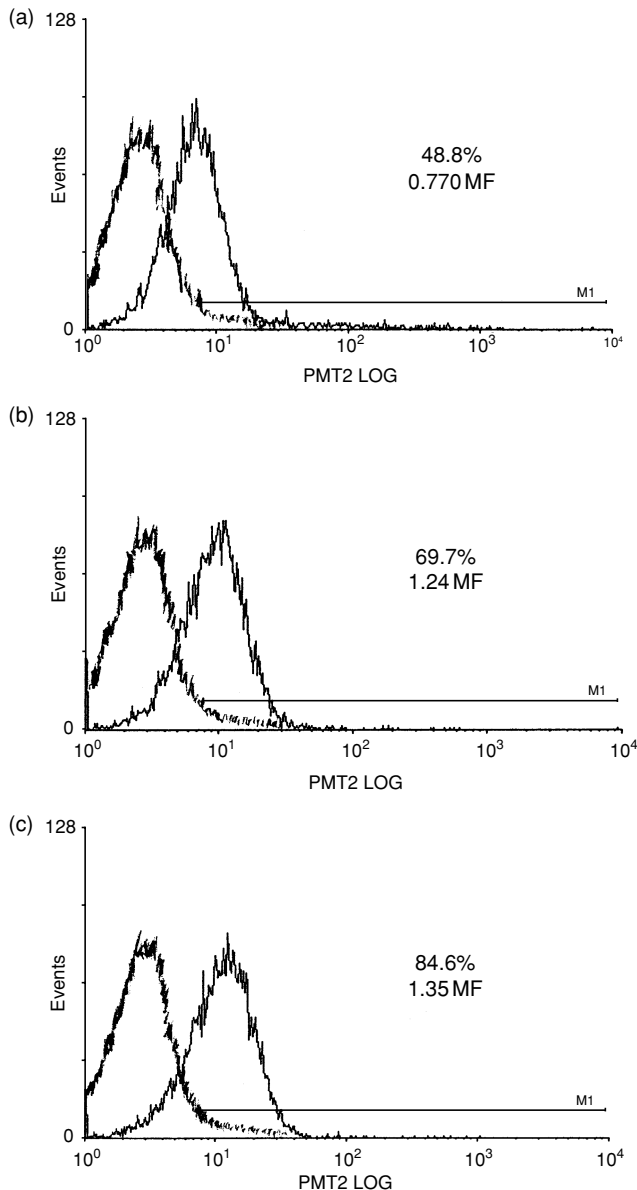


Fig. 1. Typical flow cytometry log scaled plots of CD44 expression on eosinophils of an asthmatic subject. (a) Unstimulated eosinophils; (b) eosinophils incubated with IL-5; (c) eosinophils incubated with IL-5 after 5-oxo-ETE-induced migration through Matrigel. CD44 expression is augmented in the presence of IL-5 and further increased after migration through Matrigel. All these conditions are compared with an isotypic control (percentage of positive cells: 3.6% and mean fluorescence (MF): 0.274).

5-Oxo-EET (data not shown) did not modify IL-5-mediated increase of CD44 expression. However, migration through Matrigel further increased IL-5-stimulated CD44 expression ($75.8 \pm 5.3\%$, $P = 0.0001$; mean fluorescence: 1.34 ± 0.16). In order to evaluate the functional significance of the CD44 expression, we measured eosinophil adherence to hyaluronic acid. Cells treated with IL-5 and 5-oxo-EET presented a higher adherence to this ECM component than untreated cells: $53.2 \pm 22.1\%$ and 30.0 ± 11.3 , respectively ($n = 3$, $P = 0.023$). In these experiments, migration through Matrigel on the effects of IL-5 and 5-oxo-EET did not further increase eosinophil adherence to hyaluronic acid: $47.1 \pm 15.6\%$.

IL-5 also increased cell surface CD69 expression ($67.3 \pm 5.9\%$; mean fluorescence: 1.4 ± 0.28) compared with unstimulated cells and cells incubated on Matrigel ($4.1 \pm 0.8\%$ and $7.1 \pm 1.5\%$, $P = 0.0001$, Fig. 2b; mean fluorescence: 0.13 ± 0.04 and 0.19 ± 0.11 , respectively). 5-Oxo-EET did not modify this effect but the contact of eosinophils with Matrigel significantly decreased IL-5-induced expression of CD69 ($43.1 \pm 5.9\%$, $P = 0.0001$; mean fluorescence: 0.69 ± 0.17). However, after trans-Matrigel migration in the presence of IL-5, CD69 expression level ($70.3 \pm 5.6\%$; mean fluorescence: 1.49 ± 0.51) was similar to the one observed after incubation with IL-5. Migration through Matrigel in the absence of IL-5 did not modify CD69 expression ($10.7 \pm 1.4\%$; mean fluorescence: 0.25 ± 0.11) compared with control unstimulated cells. Expression of HLA-DR was not modified by IL-5 incubation, contact with Matrigel or trans-Matrigel migration (data not shown). The observed effects on each parameter were the same for normal and asthmatic subjects (Fig. 3).

Mediator and cytokine releases

Baseline and PAF-stimulated eosinophil EPO releases were similar after migration through uncoated and Matrigel-coated inserts for normal subjects (Table 1, $n = 9$, $P = 0.14$). Similar results were obtained with asthmatic subjects' eosinophils that migrated through uncoated and Matrigel-coated inserts (Table 1, $n = 9$, $P = 0.14$). In all conditions, there was no difference between normal and asthmatic subjects ($P = 0.66$). PAF and C5a-induced liberation of LTC₄ was similar in conditions with and without Matrigel for normal subjects (Table 2, $n = 12$, $P = 0.38$), and for asthmatic subjects (Table 2, $n = 14$, $P = 0.38$). No differences were observed between normal and asthmatic subjects for LTC₄ release ($P = 0.40$). GM-CSF assay on supernatants of eosinophils from normal and asthmatic subjects demonstrated that these cells did not release detectable amounts of GM-CSF in the experimental model used for migration. All values were under the detection limit of 3 pg/mL.

Table 1. EPO release (mOD/min) by eosinophils of normal and asthmatic subjects ($n = 9$ in both groups of subjects)

	Uncoated inserts	Matrigel-coated inserts
Baseline		
Normals	1.8 ± 0.5	1.5 ± 0.5
Asthmatics	1.7 ± 0.4	2.1 ± 1.1
PAF-induced release		
Normals	40.1 ± 6.6	38.1 ± 7.2
Asthmatics	31.7 ± 4.1	28.3 ± 5.5

Table 2. PAF and C5a-induced release of LTC₄ (pg/ 1×10^6 cells) by eosinophils of normal and asthmatic subjects ($n = 12$ and 14, respectively)

	Uncoated inserts	Matrigel-coated inserts
Normals	318.3 ± 79.7	193.2 ± 42.1
Asthmatics	306.3 ± 60.8	344.5 ± 74.4

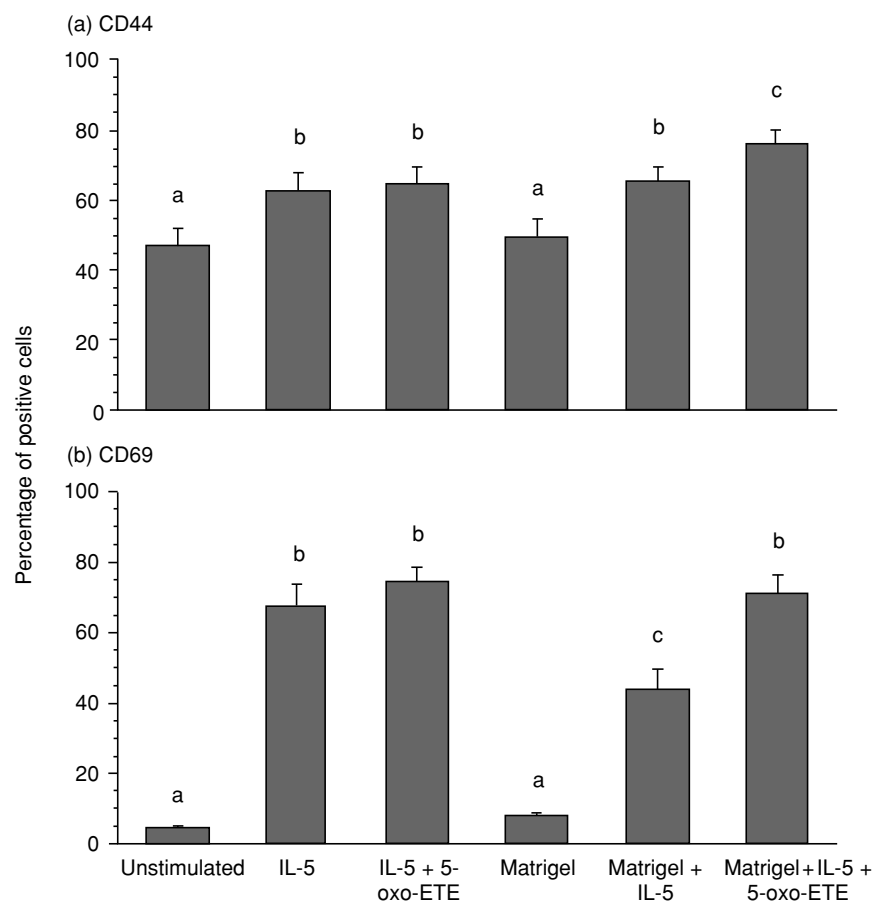


Fig. 2. Eosinophil expression of membrane receptors. (a) CD44 ($n = 12$); (b) CD69 ($n = 10$). Eosinophils were incubated in different conditions: unstimulated, with IL-5, with IL-5 and 5-oxo-ETE, with IL-5 or not on Matrigel and after 5-oxo-ETE-induced migration through Matrigel. CD44 expression is augmented in the presence of IL-5 and further increased after migration through Matrigel. CD69 expression is markedly increased by IL-5, it decreased in contact with Matrigel but goes back to previous levels after trans-Matrigel migration. In each graph, bars marked with different letters are significantly different, $P < 0.001$ (multiple comparison: Tukey's technique).

Discussion

This study shows that, *in vitro*, migration through Matrigel increases eosinophil expression of functional CD44. Moreover, it shows that contact with Matrigel decreases eosinophil CD69 expression. These data suggest that, *in vivo*, passage through a basement membrane modulates specific eosinophil cell-surface marker expression, modifying eosinophil phenotype. In an extensive evaluation of eosinophil surface phenotype, Matsumoto et al. showed that CD44 and CD69 represent activation markers for human eosinophils [21]. They showed that expression of CD44, constitutively present on fresh eosinophils, increased after *in vivo* and *in vitro* activation, whereas CD69, absent on fresh eosinophils, was present after *in vivo* activation.

CD44 is a proteoglycan expressed on all leucocytes, and exists in multiple isoforms [22]. CD44 plays an important role in cell trafficking by mediating rolling and firm adhesion to ECM constituents, notably hyaluronic acid [15, 21]. Binding of CD44 to hyaluronic acid mediates various cell-cell and cell-ECM interactions, such as lymphopoiesis, cell homing and tumour metastasis [23, 24]. In the present report, the increased expression of CD44 was associated with higher adherence to hyaluronic acid, showing that the newly expressed CD44 were functional. In addition to hyaluronic acid, CD44 binds to other ligands, including collagen, fibronectin, serglycin and osteopontin [21]. Eosinophil expression of CD44 augments upon activation by the cytokines IL-3, GM-CSF or IL-5, as observed herein [16, 21, 22]. In asthmatic patients, eosinophils

of sputum expressed higher levels of CD44 than cells of peripheral blood [22]. As for other leucocytes, CD44 may be a primary adhesion molecule required for eosinophil migration within tissue [15, 25]. Recently CD44 has been shown to bind gelatinase-A and -B and serve as cell membrane receptor for active proteinases [26]. This CD44-dependent cell surface localization of gelatinase occurs in normal and malignant cells and promotes tumour invasion, angiogenesis and growth [26]. These observations suggest that gelatinase-B binding to CD44 could serve as a mechanism for localization of gelatinase-B proteolytic activity at the front of migrating cells during eosinophil migration into tissue. Moreover, CD44 may mediate adherence between T lymphocytes and airway smooth muscle cells, suggesting that interactions between leucocytes and smooth muscle cells occurring via CD44 may play a significant role in the pathophysiology of airway diseases [27]. The mechanisms by which trans-Matrigel migration increases CD44 expression remains undefined but eosinophil interaction with Matrigel components during migration could increase its expression.

In contrast to CD44, CD69 was decreased by contact with Matrigel. CD69 mRNA is constitutively expressed in isolated peripheral blood eosinophils of normal subjects [28]. CD69 is not detectable on freshly isolated eosinophils; however, CD69 is rapidly expressed after stimulation with low concentrations of the cytokines IL-3, GM-CSF and IL-5 and is increased on bronchoalveolar lavage eosinophils of asthmatic individuals [14, 21]. Our data show that CD69 expression is increased by IL-5 but that trans-Matrigel migration did not further increase

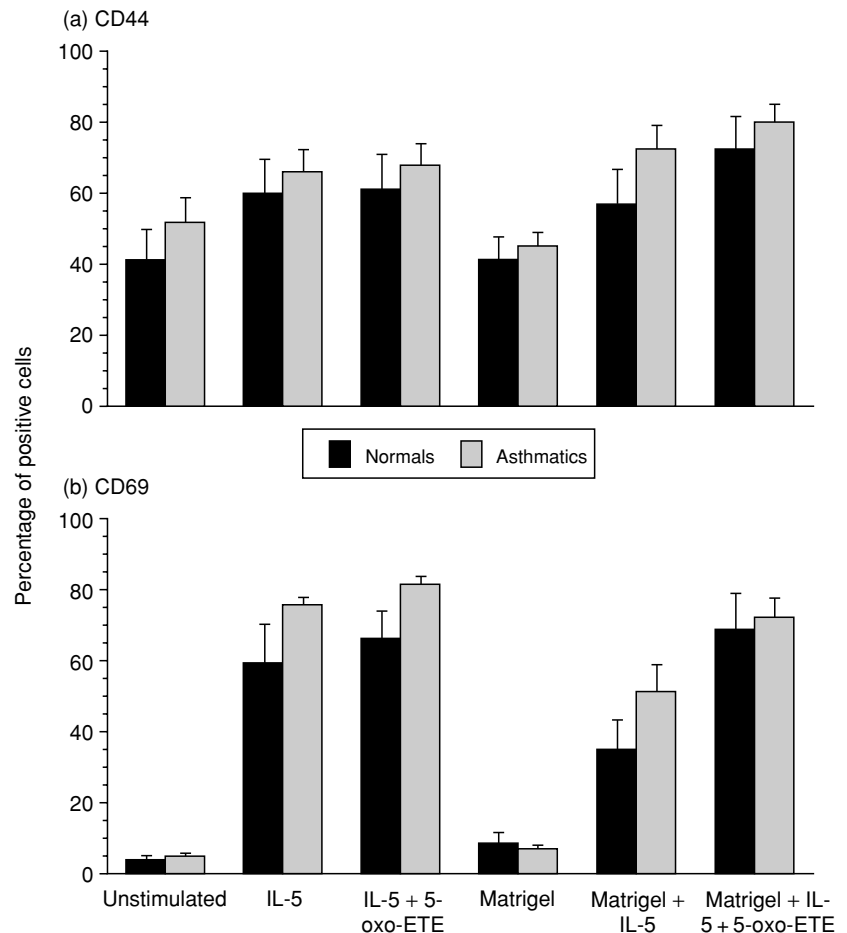


Fig. 3. Expressions of CD44 (a) (six normals, six asthmatics), and CD69 (b) (five normals, five asthmatics) on eosinophils of normal (black bars) and asthmatic (shaded bars) subjects. Cells were incubated in different conditions: unstimulated, incubated with IL-5, with IL-5 and 5-oxo-ETE, with IL-5 or not on Matrigel and after 5-oxo-ETE-induced migration through Matrigel. Eosinophils of asthmatic subjects responded similarly to cells of normal subjects for CD44 and CD69 expressions, $P > 0.412$.

CD69 as it did with CD44. The greater expression of CD69 in trans-Matrigel migrated eosinophils compared with eosinophils in contact with Matrigel is likely to be due to the effect of IL-5 that diffused through Matrigel on eosinophils that were then not anymore in contact with Matrigel. The mechanisms of decreased CD69 expression on cells in contact with Matrigel are unknown. As for T cells, CD69 may be a coreceptor for eosinophil activation, which involves protein synthesis, or, as for platelets, it may facilitate mediator release or degranulation [14]. The function of CD69 on eosinophils and other cell types is not entirely clear, although engagement of this marker leads to eosinophil apoptosis *in vitro* and cytokine release in other cells [29]. This receptor could be involved in triggering eosinophil activation and/or mediator release in tissue and therefore may be a pathway by which eosinophils exert their pro-inflammatory effects.

HLA-DR expression was not modified by IL-5, Matrigel contact and trans-Matrigel migration. Bone marrow eosinophil precursors, but not circulating eosinophils, express class II proteins of the major histocompatibility complex. However, blood eosinophils can be stimulated to express HLA-DR protein by combination of interferon- γ with IL-3, IL-5 or GM-CSF [29]. In accordance with Hansel et al. [30], we showed that IL-5 alone had no effect on HLA-DR expression. HLA-DR expression is not an early marker of activation, but HLA-DR expression might develop after prolonged homing in tissue [31]. Our data

and previous observations suggest that a tissue factor other than basement membrane migration is responsible for the induction of HLA-DR expression observed in pulmonary eosinophils [6].

The passage through Matrigel did not modify the eosinophil EPO, LTC₄ and GM-CSF releases. Major constituents of Matrigel are collagen IV, laminin, entactin and heparan sulphate proteoglycan [12]. However, in asthma, ECM composition is modified with subepithelial and submucosal deposition of fibronectin, collagen, tenascin and modifications of proteoglycans [32]. Adhesion to fibronectin augments eosinophil release of oxygen radicals and LTC₄ and increases eosinophil survival and release of GM-CSF [20, 33, 34]. These observations suggest that contact with asthma-modified subepithelial and submucosa ECM after their migration through the capillary endothelial wall may modulate eosinophil functions differently compared with ECM of normal tissue and that not only trans-endothelial migration but also interactions with tissue ECM may be important in the modulation of migrating eosinophil functions.

As blood eosinophils from asthmatic subjects are activated compared with normal eosinophils [2, 35], we postulated that asthmatic eosinophils could respond differently to migration through Matrigel. No difference was observed between normal and asthmatic blood eosinophils for the effects of trans-Matrigel migration on mediator release and receptor expression. However, subjects recruited in our study had mild asthma

and blood eosinophils from subjects with more severe asthma could respond differently to trans-Matrigel migration.

Migration of eosinophils through endothelial cells also modulates eosinophil receptor expression and this phenomenon is upregulated by activation of endothelial cells [36]. These results and ours suggest that, beside ECM components, both trans-endothelial and trans-basement membrane migration may modulate eosinophil phenotype. The combination of trans-endothelial cell and trans-basement membrane occurring *in vivo* might have an additive or synergistic effect on eosinophils. This issue needs to be further investigated. On the other hand, the demonstration of increased CD44 expression after trans-Matrigel migration *in vitro* suggests that *in vivo* tissue eosinophils of normal subjects are likely to present some hallmarks of activation compared with their blood counterparts. This issue awaits study of the few eosinophils found in normal tissue.

In conclusion, this study shows that migration through a reconstituted basement membrane (Matrigel) increases CD44 expression. This observation and the one showing increased eosinophil adhesion molecule expression following adhesion to pulmonary microvascular endothelial cells [36] suggest that *in vivo* the combination of trans-endothelial and trans-basement membrane migration have more extended effects on eosinophils. Therefore, the study of the activation of eosinophils following their passage from blood to tissue and the augmentation of their capacity to interact with cells and ECM components is a very important step for our comprehension of the role of these cells in tissue homeostasis and in asthma pathophysiology.

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