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Endothelial cells modulate eosinophil surface markers and mediator release

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Endothelial cells modulate eosinophil surface markers and mediator release. M-J. Dallaire, C. Ferland, N. Pagé, S. Lavigne, F. Davoine, M. Laviolette. ©ERS Journals Ltd 2003.
ABSTRACT: Migration from blood to tissue modulates eosinophil function, possibly through interactions with endothelial cells.

The effects of contact with and migration through endothelial cells on eosinophil expression of surface markers and release of leukotriene C_4 were evaluated.

A small proportion (2.6%) of eosinophils spontaneously migrated through endothelial cell monolayers. Activation of endothelial cells by interleukin (IL)-4 or IL-1β slightly increased this migration (to 12.4%), which became much greater when a chemoattractant was placed in the lower chamber (84.3%). However, the chemotactic effect was downregulated by pretreating endothelial cells with interferon gamma (IFN-γ; 63.1%). At baseline, 5% of eosinophils expressed CD69; this increased to 30.7% in culture on untreated endothelial cells and to 50.9% on IL-1β-pretreated endothelial cells. This effect was mediated through intercellular adhesion molecule-1/CD11b interaction. Eosinophil migration through endothelial cells further increased CD69 expression to 63.9% and also increased CD35 expression from 83.3 to 91.3%. Upon stimulation, eosinophils that had migrated through endothelial cells produced more leukotriene C_4 than control cells (872.4 and 103.9 pg·mL^{-1}, respectively). Endothelial cell pretreatment with IL-4 or IL-1β further increased leukotriene C_4 release (1,789.1 and 2,895.1 pg·mL^{-1}, respectively), whereas pretreatment with IFN-γ decreased it (293.7 pg·mL^{-1}).

These data show that *in vitro* interactions with endothelial cells upregulate eosinophil membrane receptor expression and mediator release and that these effects are differently modulated by T-helper cell type 1 and 2 cytokines. These eosinophil modulations may play an important role in asthma pathogenesis.
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Eosinophils are recruited to tissue in various pathological conditions [1]. In asthma, eosinophils infiltrate the bronchial mucosa [2] and their counts in blood, sputum and bronchial mucosa correlate with indices of disease severity [3–4]. Blood eosinophils are thought to circulate in a quiescent state until they migrate into the tissue [5]. In healthy mucosa, eosinophils account for a small proportion of the cells, making it almost impossible to evaluate their activation status. In eosinophilic diseases in which their numbers increase, tissue eosinophils show increased expression of complement and immunoglobulin (Ig)G receptors compared to their blood counterparts [1, 5]. They also express intercellular adhesion molecule (ICAM)-1, human leukocyte antigen (HLA)-DR and CD69, which are barely or not detectable on blood eosinophils [6–9]. Tissue eosinophils show increased release of superoxide anion and leukotriene (LT)C_4 compared to blood eosinophils [10, 11]. Lung lavage eosinophils, obtained after segmental bronchoprovocation, show an increase in membrane receptors, adhesion, survival and generation of superoxide anions, which was not achieved by incubation of blood eosinophils with interleukin (IL)-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) [12–14]. These observations suggest that passage into tissue activates eosinophils, increasing their pro-inflammatory potential. The factors involved in this phenomenon have yet to be fully determined.

During their recruitment to tissue eosinophils pass through the endothelium. This is a complex process [1, 14, 15] and interactions with endothelial cells have been shown to modulate certain eosinophil functions [16]. Activation of endothelium is an important step in inflammatory processes and occurs in disease models, including those for allergic diseases [17]. Endothelial cells exposed to cytokines such as IL-1β, IL-4 and tumour necrosis factor-α increase their expression of ICAM-1 and vascular cell adhesion molecule (VCAM)-1 [18, 19], which serve as ligands for eosinophil integrins. Consequently, cytokines may modulate the transendothelial migration of eosinophils and amplify endothelial cell-induced changes in eosinophil functions [19, 20].

In order to further clarify the role of endothelial cells in the modulation of eosinophils during their migration to tissue, the effects of eosinophil migration through a human umbilical vein endothelial cell monolayer on expression of cell-surface markers and liberation of LTC_4, a powerful bronchoconstrictor and pro-inflammatory mediator, were evaluated. It was found that expression of CD69, an early marker of activation, and of CD35, a receptor for complement proteins, and LTC_4 release were modulated by endothelial cells and that treatment of endothelial cells with T-helper cell type 1 and 2 cytokines differently modified their effect on eosinophils.

Materials and methods

Reagents

Platelet-activating factor (PAF), activated complement fraction 5a (C5a) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Kits for LTC_4 determination and 5-oxo-6, 8, 11, 14-eicosatetraenoic acid (5-oxo-ETE) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Purified monoclonal mouse antibody IgG1κ and IgG2b (isotypic control), purified human anti-CD4, anti-CD16, anti-CD28, anti-CD86, anti-CD35, anti-CD69 and anti-HLA-DR and phycoerythrin-conjugated polyclonal antimouse antibodies were purchased from BD Biosciences (Mississauga, ON, Canada). In specific experiments, fluorescein isothiocyanate (FITC)-conjugated anti-CD69 antibodies were used. Monoclonal mouse antihuman CD16 antibodies were purchased from Miltenyi Biotec (Auburn, CA, USA) and human recombinant IL-1β, IL-4 and interferon gamma (IFN-γ) from Peprotech, Inc. (Rocky Hill, NJ, USA). Monoclonal mouse antihuman CD11b antibody (clone bear 1) was obtained from Beckman Coulter (Mississauga, ON, Canada) and fibronectin was from Roche Diagnostics (Laval, QC, Canada). Anti-ICAM-1 antibody (clone HA58) was purchased from BD Biosciences. Human umbilical vein endothelial cells cryopreserved from a single donor in primary culture were purchased from Clonetics (San Diego, CA, USA).

Subject selection

Seven normal subjects (two male/five female, median age 31 yrs (range 18–55 yrs)) without a history of allergy or asthma and 10 asthmatics (five male/five female, median age 23.5 yrs (range 19–35 yrs)) meeting the criteria of the American Thoracic Society for the diagnosis of asthma were recruited for the study [21]. The asthmatic subjects had a morning prebronchodilator forced expiratory volume in one second (FEV1) of >85% of the predicted value and required only a short-acting β_2-agonist on demand on less than four occasions per week. The inclusion criteria included stable treatment for >3 months, no inhaled steroids over the 3 months preceding the study, no use of other drugs and no disease other than asthma. Approval for the study was obtained from the local ethics committee and all subjects signed an informed consent form. FEV1 and provocative concentration of methacholine causing a 20% fall in FEV1 (PC20) were measured in the morning, $\geqslant$8 h after any β_2-agonist inhalation. Median FEV1 for normal and asthmatic subjects were 98.5 (91–110) and 96 (86–102)% pred, respectively (p=0.03), and geometric mean PC20 were 43.9±19.7 and 1.1±0.9 mg·mL^{-1}, respectively (p=0.006, Wilcoxon rank-sum test). Subjects underwent blood sampling early in the morning. Mean blood eosinophil counts were 0.2×10^9 and 0.3×10^9cells·L^{-1} for normal and asthmatic subjects, respectively.

Blood eosinophil purification

Eosinophils were purified as previously described [22]. They were separated from neutrophils by negative selection using a magnetic cell sorter. An aliquot of the cell suspension was used to determine cell number (haemocytometer) and viability (trypan blue exclusion; Sigma Chemical Co.) and differential cell counts (Diff-Quik; Dade Diagnostics, Aguada, PR, USA). The purity of the eosinophil preparations used in this study was >98% and the contaminating cells were neutrophils and/or lymphocytes. Eosinophil viability was always >99%.

Endothelial cell culture

Endothelial cells were grown in endothelial growth medium supplemented with human recombinant epidermal growth factor, human fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, long-R^3 insulin-like growth factor-I, heparin, hydrocortisone, gentamicin and amphotericin B (Clonetics) and 10% foetal bovine serum (FBS) (Invitrogen Canada, Burlington, ON, Canada) in a 5% carbon dioxide (CO_2) atmosphere at 37°C. When ~80% confluent, cells were harvested, resuspended in fresh culture medium and seeded at a density of 2×10^5 cells·500 μL^{-1} on cell culture inserts (12-mm diameter polycarbonate membrane with 3.0-μm pores; BD Biosciences Labware, Mississauga, ON, Canada). The inserts were placed in 24-well culture plates (BD Biosciences Labware), culture medium without FBS (500 μL) was placed in the lower chamber to inhibit formation of an endothelial cell bilayer [23] and the cells cultured for 5 days. Endothelial cell monolayers were confirmed as being at confluence by toluidine blue staining on control inserts. All experiments were carried out on passage 4 cells. In specific experiments, endothelial cells were cultured on the bottom of 24-well culture plates.

Incubation of eosinophils with endothelial cells and transendothelial migration

Confluent endothelial cell monolayers were treated with IFN-γ (1,000 U·mL^{-1}) for 72 h, IL-4 (100 U·mL^{-1}) for 24 h or IL-1β (50 U·mL^{-1}) for 4 h in a 5% CO_2 atmosphere at 37°C. These cytokines were added to the upper compartment of the wells. Times of incubation and cytokine concentrations were chosen based on the results of previous studies [24–27]. Thereafter, the upper and lower compartments of the wells were washed three times with Hank′s balanced salt solution (HBSS) (37°C). Eosinophils (1×10^6 cells·mL^{-1} in Roswell Park Memorial Institute 1640 containing 10% FBS and 1% penicillin/streptomycin) were laid in inserts coated or not with endothelial cells. A potent eosinophil chemoattractant, 5-oxo-ETE (1 μM), was added to the lower chamber of some wells to induce migration [22]. After a 4-h incubation, eosinophils recovered from the upper chambers served to evaluate the effect of contact with endothelial cells and cells that had migrated through endothelial cell-coated inserts under the action of 5-oxo-ETE served to evaluate the effect of transendothelial migration. In these two sets of experiments, inserts without endothelial cells served as controls. In specific experiments, to further evaluate the effects of endothelial cell contact in the presence or absence of 5-oxo-ETE and to prevent migration through endothelial cells, eosinophils were incubated in 24-well culture plates coated or not with endothelial cells. Moreover, in order to evaluate the role of ICAM-1/CD11b ligation in eosinophil/endothelial cell interactions, an anti-ICAM-1 antibody (20 μg·mL^{-1}) was added to the incubation medium. The migration rate is presented as the percentage of cells in the upper chamber that migrate into the lower chamber. At the end of incubations, the viability of the eosinophils was always >98%.

Incubation on fibronectin- or anti-CD11b antibody-coated plates

In order to study the effect of CD11b and very late activation antigen-4 (VLA-4) ligation on eosinophil CD69 expression, 96-well culture plates (Nunc-Immuno Plate Maxisorp Surface; VWR International, Montreal, QC, Canada) were incubated with either 100 μL fibronectin (20 μg·mL^{-1}),

100 μL anti-CD11b monoclonal antibody (20 $\mu g \cdot mL^{-1}$), both fibronectin and anti-CD11b antibody, or 100 μL HBSS (control) overnight at 4°C. At the end of this incubation, the wells were washed twice with HBSS and nonspecific protein binding was blocked by addition of 100 μL HBSS containing 1% BSA for 1 h at 37°C. Before use, the coated wells were washed with HBSS and eosinophils (1×10^5 cells·100 μL^{-1}) were added to wells and incubated for 4 h at 37°C. Cells were then harvested and stained with FITC-conjugated antihuman CD69 as described below.

Cell-surface marker detection

Eosinophils were labelled (2×10^5 cells·100 μL^{-1} for 30 min at 4°C) with specific antibodies (1 $\mu g \cdot mL^{-1}$) directed against CD4, CD16, CD28, CD35, CD69, CD86 and HLA-DR or with their respective isotypic control, as previously described [28, 29]. After incubation, cells were washed with HBSS containing 1% BSA and fixed in 4% paraformaldehyde (10 min at 4°C). Phycoerythrin-conjugated antimouse secondary antibody (1 $\mu g \cdot mL^{-1}$) was then added (for 30 min at 4°C). In experiments with incubation on fibronectin- or anti-CD11b antibody-coated plates, FITC-conjugated anti-CD69 antibodies were used. Finally, eosinophils were washed, suspended in HBSS containing 1% BSA, kept at 4°C and immediately subjected to cytometric analysis using an EPICS® ELITE ESP flow cytometer (Beckman-Coulter, Miami, FL, USA). Mean fluorescence (MF) was measured on a logarithmic scale [28, 29] and the percentage of cells expressing a particular marker determined by counting the number of cells showing greater fluorescence activity than $\geqslant$96% of negative controls.

Leukotriene C_4 assay

Eosinophils (1×10^6 cells·mL^{-1} in HBSS/$CaCl_2$ (1.6 mM)) were incubated with PAF (1 μM) for 10 min at 37°C under 5% CO_2 and thereafter with C5a (10 nM) under the same conditions. Tubes were then put on ice for 5 min to stop the reaction. Cells were centrifuged and LTC_4 measured in the supernatants by quantitative enzyme immunoassay according to the manufacturer's recommendations.

Statistical analyses

Mediator and cell-surface marker expression data were analysed using a crossed-nested design (analysis of variance). Data are presented as mean±SEM and results were considered significant when p was <0.05. There was no difference between the eosinophils of normal and asthmatic subjects for all measured parameters under all tested conditions. Their data were, therefore, pooled together for analysis and presentation.

Results

Migration through endothelial cells

Spontaneous eosinophil migration through uncoated inserts, untreated endothelial cells and IFN-γ-pretreated endothelial cells was 1.4±0.2, 2.6±0.3 and 2.1±0.4%, respectively (fig. 1). Pretreatment of endothelial cells with IL-4 or IL-1β increased eosinophil migration to 11.5±0.9 and 12.4±0.8%, respectively (p=0.0001). Addition of 5-oxo-ETE to the lower chambers dramatically stimulated eosinophil migration through endothelial cells (84.3±1.0%; p=0.0001). Pretreatment of endothelial cells with IFN-γ decreased the effect of 5-oxo-ETE on eosinophil migration (63.1±1.3%; p=0.0001). In contrast, pretreatment of endothelial cells with IL-4 or IL-1β did not modify 5-oxo-ETE-induced migration (82.8±1.3 and 81.8±2.0%, respectively).

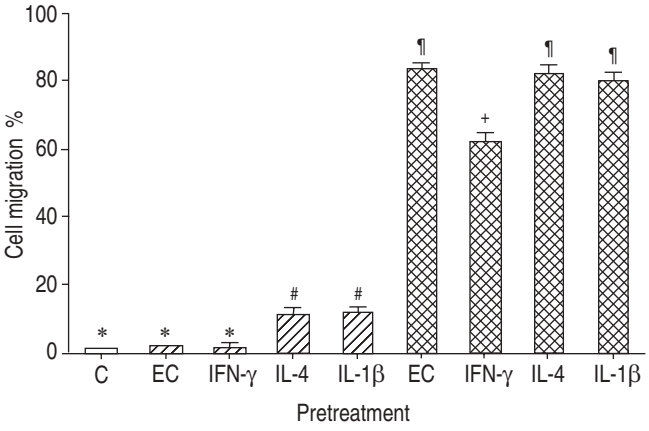

Fig. 1. – Migration of eosinophils through endothelial cell monolayers. Eosinophils were laid on cell culture inserts coated (▨; ■) or not (□) with endothelial cells. Data are presented as mean±SEM. The eosinophils barely migrated through uncoated inserts (C) and inserts coated with untreated endothelial cells (EC). Pretreatment of endothelial cells with interleukin (IL)-4 or IL-1β but not interferon gamma (IFN-γ) increased eosinophil migration. When 5-oxo-6, 8, 11, 14-eicosatetraenoic acid was added to the lower chambers (■), most eosinophils migrated through inserts coated with endothelial cells, but this effect decreased with IFN-γ-pretreatment of endothelial cells. *, #, ¶, +: p=0.001 *versus* each other (n=17 for each).

Eosinophil surface marker expression

Among eosinophils incubated on uncoated inserts and recovered from the upper chambers, the percentage of CD69-positive cells was 5.0±2.2% (0.06±0.02MF) and increased to 30.7±8.4% (0.34±0.11MF) on inserts coated with untreated endothelial cells (p<0.0001) (fig. 2a). Contact of eosinophils with endothelial cells in culture plates in the presence of 5-oxo-ETE induced similar CD69 expression (27.2±6.2%; 0.2±0.04MF) to incubation on endothelial cells alone. Pretreatment of endothelial cells with IFN-γ or IL-4 did not modify eosinophil expression of CD69 (36.3±7.6% (0.35±0.06MF) and 45.4±7.3% (0.43±0.05MF), respectively). However, pretreatment of endothelial cells with IL-1β increased CD69-positive eosinophil percentages (50.9±10.8%; 0.58±0.14MF) (p<0.0001). Compared to cells incubated on endothelial cells, eosinophils of the lower chambers that had migrated through endothelial cells under the action of 5-oxo-ETE showed a further increase in CD69 expression (63.9±7.0%; 0.88±0.18MF; p=0.004) (fig. 2b). Migration of eosinophils through cytokine-pretreated endothelial cells did not further modify CD69 expression compared to migration across untreated endothelial cells.

The effect of CD11b and VLA-4 ligation on eosinophil CD69 expression is presented in figure 3. Eosinophils incubated on fibronectin showed low expression of CD69 (6.2±1.5%), similar to control wells (4.2±1.4%). In contrast, cells incubated on anti-CD11b antibody-coated wells exhibited increased expression of CD69 (27±6%; p=0.007; n=6). Addition of fibronectin did not modify this expression (26.8±11.5%). These values were similar to those observed with eosinophils incubated on untreated endothelial cells (fig. 2a). Furthermore, in two experiments eosinophils were incubated on endothelial cells in the presence of an anti-ICAM-1 antibody. This antibody decreased endothelial cell-induced CD69 expression from 36.6 to 2.4%.

The expression of CD35 in eosinophils incubated in uncoated

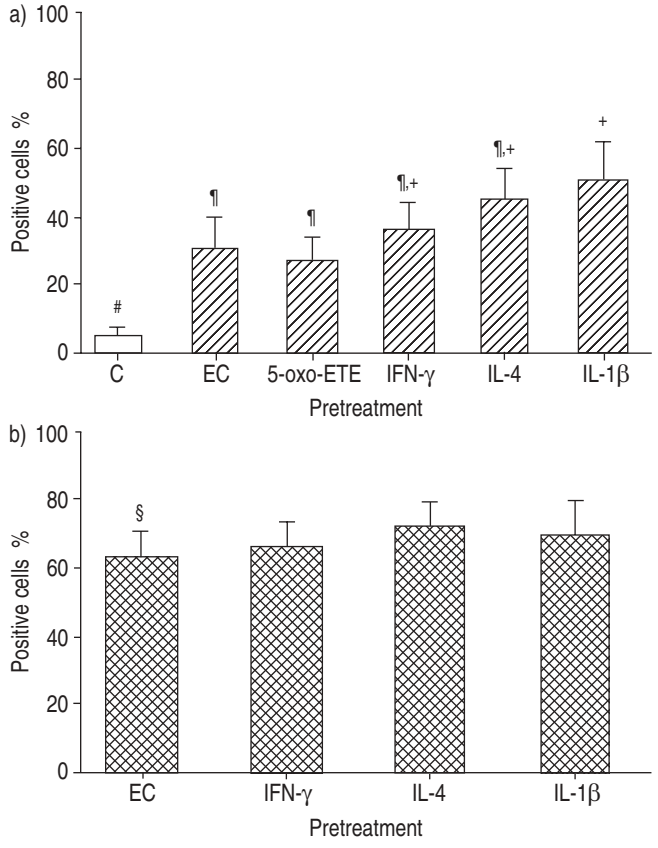

Fig. 2. – Expression of CD69 receptors on a) eosinophils incubated on inserts or culture plates coated (▨) or not (□) with endothelial cells and b) cells that migrated through inserts coated with endothelial cells under the effect of 5-oxo-6, 8, 11, 14-eicosatetraenoic acid (5-oxo-ETE) (■). Data are presented as mean±SEM. a) Eosinophil CD69 expression increased in contact with endothelial cells (EC) compared to cells that have not been in contact with endothelial cells (C). Pretreatment of endothelial cells with interleukin (IL)-1β, but not IL-4 or interferon gamma (IFN-γ), further increased eosinophil CD69 expression. b) Migration through endothelial cells under the effect of 5-oxo-ETE doubled the number of CD69-positive eosinophils compared with nonmigrated cells incubated on endothelial cells in the presence of 5-oxo-ETE. #, ¶, +: $p<0.0001$ *versus* each other; §: $p=0.0037$ *versus* nonmigrated cells (n=13).

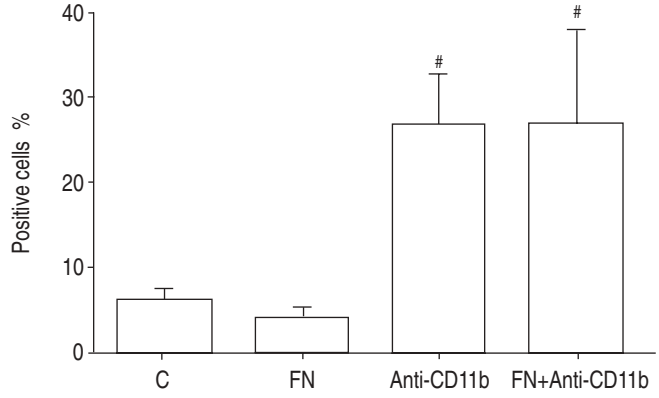

Fig. 3. – Expression of CD69 receptors of eosinophils incubated on fibronectin (FN)- and anti-CD11b antibody-coated wells. Data are presented as mean±SEM. Compared to control (C) and FN-coated wells, incubation of cells on anti-CD11b antibody-coated wells induced an increase in CD69-positive cells. #: $p<0.007$ *versus* control and FN-coated wells (n=6).

inserts (83.3±4.5%; 1.2±0.1MF), inserts coated with endothelial cells (85.5±2.6%; 1.4±0.2MF) or on endothelial cells in the presence of 5-oxo-ETE (85.5±3.8%; 1.8±0.3MF) were similar (fig. 4a). Treatment of endothelial cells with cytokines did not modify eosinophil CD35 expression: IFN-γ 87.9±1.1%, IL-4 90.1±1.4%, and IL-1β 89.5±2.3% (p=0.38). Migration of eosinophils through endothelial cells under the action of 5-oxo-ETE significantly increased CD35 expression (91.3±3.2%; 1.9±0.3MF) compared to cells incubated in inserts coated with endothelial cells and that had not migrated (p=0.0013) (fig. 4b). Cytokine pretreatment of endothelial cells did not modify the CD35 expression of migrated eosinophils.

Purified blood eosinophil expression of CD4, CD16, CD28, CD86 and HLA-DR were 15.0±3.7, 2.0±1.0, 3.5±1.3, 6.9±2.8 and 1.5±0.3%, respectively (n=4–8). Contact with and migration through endothelial cell monolayers did not modify eosinophil expression of these surface markers (data not shown).

Leukotriene C_4 release

Compared to eosinophils recovered from the upper chambers, cells of lower chambers that migrated through endothelial

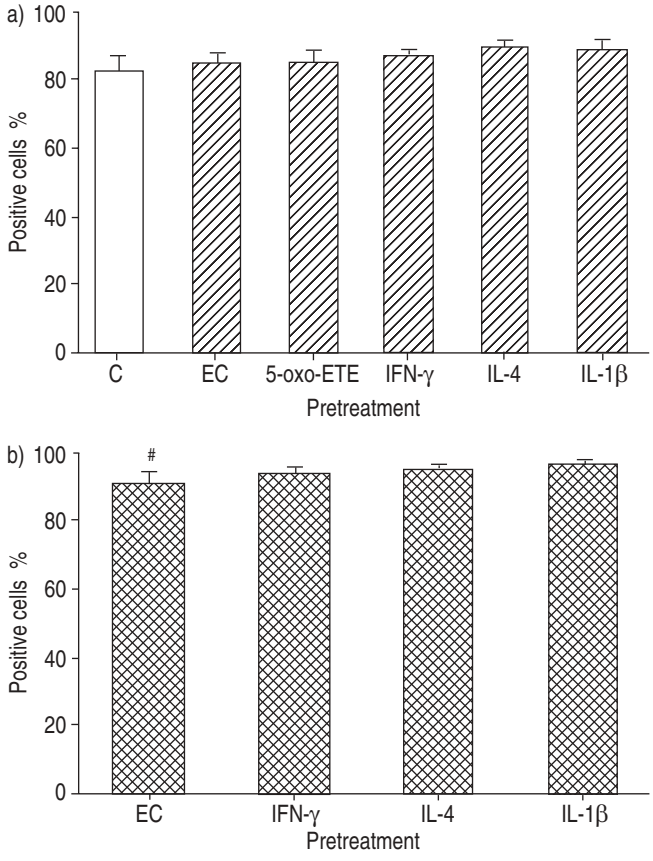

Fig. 4. – Expression of CD35 receptors on a) eosinophils incubated on inserts or culture plates coated (▨) or not (□) with endothelial cells and b) cells that migrated through inserts coated with endothelial cells (EC) under the effect of 5-oxo-6, 8, 11, 14-eicosatetraenoic acid (5-oxo-ETE) (■). Data are presented as mean±SEM. a) CD35 expression of eosinophils incubated on inserts coated or not with endothelial cells were similar (n=8). b) Migration of eosinophils through inserts coated with endothelial cells under the effect of 5-oxo-ETE significantly increased CD35 expression compared to nonmigrated cells incubated on endothelial cells in the presence of 5-oxo-ETE. EC: untreated endothelial cells. #: $p=0.0013$ *versus* nonmigrated cells (n=13). C: eosinophils incubated on uncoated cells; IFN-γ: interferon gamma; IL: interleukin.

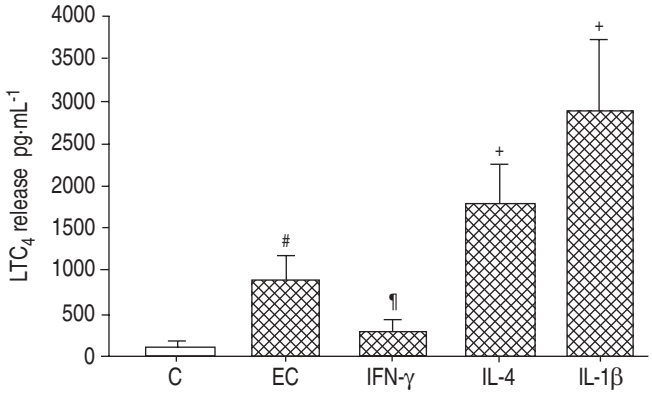

Fig. 5.–Release of leukotriene (LT) C_4 by eosinophils under migration conditions. Data are presented as mean±SEM. Release of LTC_4 by eosinophils that had migrated across inserts coated with endothelial cells (EC) under the effect of 5-oxo-6, 8, 11, 14-eicosatetraenoic acid (■) was increased compared to eosinophils incubated on uncoated inserts (C; □) (p=0.0055). Eosinophils that migrated through interleukin (IL)-4- or IL-1β-pretreated endothelial cells released more LTC_4 than eosinophils that migrated through untreated or interferon gamma (IFN-γ)-pretreated endothelial cells. #, ¶, +: p<0.0006 *versus* each other (n=10).

cells under the action of 5-oxo-ETE released more than eight times more LTC_4 (103.9±30.2 and 872.4±292.5 pg·mL^{-1}, respectively; p=0.0055) (fig. 5). Migration through IFN-γ-pretreated endothelial cells decreased eosinophil LTC_4 release (293.7±116.9 pg·mL^{-1}; p=0.0006). In contrast, migration through IL-4- and IL-1β-treated endothelial cells further increased eosinophil LTC_4 release (1,789.1±457.3 and 2,895.1±831.4 pg·mL^{-1}, respectively; p=0.0006). Eosinophils incubated in endothelial cell-coated and uncoated culture plates released similar amounts of LTC_4 (32.4±12.7 and 82.9±28.2 pg·mL^{-1}, respectively; p=0.2, n=6). Moreover, under these conditions, addition of 5-oxo-ETE did not amplify the PAF/C5a-induced LTC_4 release (32.4±12.4; n=3).

Discussion

Eosinophil emigration from blood to tissue and migration through endothelium may contribute to the observed activation status of tissue eosinophils compared to their blood counterparts. This study shows that *in vitro* contact with endothelial cells increases eosinophil CD69 expression and that eosinophil migration through an endothelial cell monolayer further increases CD69 expression and also augments CD35 expression and LTC_4 release. Moreover, the endothelial cell effects on eosinophils are modulated by cytokines, IL-1β or IL-4 amplifying them and IFN-γ decreasing them.

In the present study, endothelial cell pretreatment with IL-4 or IL-1β but not IFN-γ facilitated the passage of eosinophils across endothelial cell monolayers. Since IL-4 and IL-1β upregulate ICAM-1 and VCAM-1 on endothelial cells [30, 31], these ligands may increase adhesion of eosinophils to endothelial cells *via* Mac-1 (a β_2 integrin) and VLA-4, respectively, and, consequently, may promote eosinophil migration.

5-oxo-ETE, a potent chemoattractant [32], promoted the migration of most of the eosinophils. IFN-γ partially inhibits 5-oxo-ETE-induced eosinophil migration. The mechanism of this inhibition remains undefined. IFN-γ has no known effect on the expression of adherence molecules such as VCAM-1 but increases the expression of ICAM-1 on airway epithelium [33].

Induction of CD69 expression on eosinophils has been reported as an indication of eosinophil activation [34]. The function of CD69 on eosinophils and other cell types is not entirely understood, although engagement of this marker leads to eosinophil apoptosis *in vitro* and cytokine release in other cell types [35]. CD69 may be a coreceptor for eosinophil activation, or, as for platelets and monocytes, facilitate mediator release or degranulation [9, 36, 37]. CD69 is barely detectable on freshly isolated eosinophils but is rapidly expressed after stimulation with numerous cytokines [9, 38–40]. CD69 is also present on bronchoalveolar lavage eosinophils [8, 9, 34]. The present data and the recent report of YAMAMOTO *et al.* [16] show that CD69 expression is increased by contact with and further increased after migration through endothelial cells. The induction of CD69 expression is also observed on eosinophils incubated with antibodies directed against CD11b and inhibited by an anti-ICAM-1 antibody, suggesting that binding of eosinophils to endothelial cells *via* ICAM-1/β_2 integrin interaction is probably involved in endothelial cell-mediated eosinophil activation. The greater expression of CD69 on migrated eosinophils is probably due to the effect of migration through the endothelial cell monolayer, given that CD69 expression on eosinophils cocultured with endothelial cells in the presence of 5-oxo-ETE was not increased compared to that on eosinophils in contact with endothelial cells. YAMAMOTO *et al.* [16] showed that migration across IL-1β-pretreated endothelial cells stimulated eosinophil expression of CD69, CD54 and HLA-DR. The present study failed to show upregulation of CD69 and HLA-DR expression on eosinophils that had migrated across IL-1β-treated endothelial cells compared to those that had migrated through unstimulated endothelial cells. One possible explanation for this discrepancy is the different eosinophil populations studied. YAMAMOTO *et al.* [16] analysed a small population of eosinophils (<15% of total eosinophils placed on the endothelial cell monolayer) that migrated spontaneously through IL-1β-pretreated endothelial cells, whereas the present study analysed eosinophils that migrated through endothelial cells under the action of 5-oxo-ETE (>80% of total eosinophils). Moreover, YAMAMOTO *et al.* [16] measured MF on a linear scale, which greatly increases the sensitivity but also significantly decreases the specificity of measurements.

In the present study, CD35 was highly expressed on blood eosinophils. As shown by WALKER *et al.* [27], this expression was not modified by contact with, but increased slightly after migration through, endothelial cells. Complement receptors such as CD35 are known to play a role in the activation and adhesion of neutrophils and eosinophils, and, during asthmatic reaction, expression of these receptors is elevated on circulating granulocytes [41]. In contrast, other receptors, CD4, CD16, CD28, CD86 and HLA-DR, were barely expressed by blood eosinophils and their expression was not upregulated by migration through endothelial cells. These receptors are involved in interactions of eosinophils with mediators and other cells and are upregulated on tissue eosinophils [42]. The present results suggest that eosinophil migration through endothelial cells is not involved in this upregulation.

The present study shows that passage of eosinophils through endothelial cells modulates LTC_4 release. MUNOZ *et al.* [43] reported that incubation on IL-1β-treated endothelial cells but not endothelial cells alone augmented LTC_4 release from stimulated eosinophils and that this augmentation was related to the process of eosinophil binding at the endothelial surface [43]. Herein, the effect of incubation on cytokine-treated endothelial cells was not evaluated, but it was shown that migration of eosinophils through IL-1β- or IL-4-treated endothelial cells further increased LTC_4 release. It is likely that part of this increase is mediated *via* upregulation of adhesion molecule expression on endothelial cells [30, 31]. The inhibitory effect of endothelial cell pretreatment with IFN-γ is important (fig. 5) and could also

be mediated *via* modulation of adhesion molecule expression on endothelial cells. Moreover, the amount of LTC_4 produced by eosinophils *in vivo* depends on its level of activation and eosinophil exposure to cytokines and mediators [10]. Thus, during migration, exposure to cytokine(s)/mediator(s) expressed by endothelial cells might be involved in the modulation of LTC_4 release observed after migration of eosinophils through endothelial cells. Previous reports showed that IL-1 and IL-4 increased GM-CSF production by endothelial cells, whereas IFN-γ decreased it [44–46].

Since blood eosinophils from asthmatic subjects are activated compared to normal eosinophils [1–5], the present authors postulated that asthmatic eosinophils might respond differently to migration through an endothelial cell monolayer. However, no difference was observed between normal and asthmatic blood eosinophils for the measured parameters. This may be related to the clinical state of the patients, given that the subjects recruited in the present study had very mild asthma. Blood eosinophils from subjects with more severe asthma might respond differently to interactions with endothelial cells.

In conclusion, the present study investigated many cell surface markers and release of leukotriene C_4, a potent mediator. The data suggest that, *in vivo*, endothelial cells modulate specific eosinophil cell-surface marker expression and function, thus modifying eosinophil phenotype, and that these effects are themselves modulated by various cytokines and, at least in part, mediated *via* adhesion molecules. Therefore, evaluation of the activation status of eosinophils following their passage from blood to tissue and the increase in their capacity to interact with cells and extracellular matrix components is a very important step in understanding the role of these cells in tissue homeostasis.

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