

Original article

Osteopontin is expressed and functional in human eosinophils

Background: Eosinophils are critically involved in allergic inflammation and tissue remodeling. Osteopontin (OPN) is a glycoprotein molecule which exhibits pro-fibrogenic and pro-angiogenic properties and has recently also been implicated in allergic diseases. In this study, we investigated the expression and function of OPN in human eosinophils.

Methods: Osteopontin mRNA (RT-PCR) and protein (immunofluorescence) expression in peripheral blood eosinophils from atopic human subjects were evaluated. Soluble OPN release was determined in resting and activated eosinophils. The contribution of OPN to eosinophil-induced angiogenesis was determined using the chick embryo chorio-allantoic membrane (CAM) assay and OPN-induced eosinophil chemotaxis was determined (ChemoTx System microplate wells). Finally, OPN expression in bronchoalveolar lavage (BAL) fluids from mild asthmatic and normal control subjects was determined.

Results: Osteopontin is expressed in human eosinophils and is increased following GM-CSF and IL-5 activation. Eosinophil-derived OPN contributes to eosinophil-induced angiogenesis. Recombinant OPN promotes eosinophil chemotaxis *in vitro* and this effect is mediated by $\alpha_4\beta_1$ integrin binding. Soluble OPN is increased in the bronchoalveolar lavage fluid from mild asthmatic subjects and correlates with eosinophil counts.

Conclusions: We therefore conclude that OPN is likely to contribute to the process of angiogenesis observed in the airways in asthma.

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Tissue eosinophilia is a common feature of allergic airway diseases such as rhinitis, nasal polyposis and bronchial asthma (1). The infiltration and subsequent activation of eosinophils in the airways result in the secretion of specific granule proteins, particularly cationic proteins (major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin), synthesis and release of lipid mediators, inflammatory cytokines, chemokines and growth factors (2). Through these mediators, eosinophils contribute to the perpetuation and amplification of airway inflammation as well as to the development of some features of airway remodeling such as sub-epithelial fibrosis, increased smooth muscle mass and blood vessel formation (3).

Osteopontin is a recently described Th1 cytokine implicated in cell-mediated immunity and tissue repair (4–6). It is a phosphorylated acidic RGD-containing glycoprotein with cell-adhesive and chemotactic activities (6). This molecule exists both as an immobilized extracellular matrix component in mineralized tissues and as a

soluble molecule in body fluids (6). In the lung, OPN is highly expressed by epithelial cells after injury, by alveolar and interstitial macrophages, T lymphocytes and endothelial cells (7). By acting through binding to cell surface integrin receptors and certain variants of the hyaluronic acid receptor CD44_{V6-V9} (8), OPN can mediate interactions between cells and extracellular matrix components, recruit leukocytes to the inflammatory site and promote type-1 cytokine expression in macrophages and lymphocytes. Osteopontin was originally described as playing a role in bone calcification but has subsequently been demonstrated to play a role in cardiovascular disease and tumor progression and metastasis (9–11). In this context, OPN has been shown to be pro-angiogenic. Furthermore, studies in a murine model of bleomycin-induced lung fibrosis and in human idiopathic pulmonary fibrosis have identified OPN as an important cytokine in lung fibrosis, including migration and proliferation of lung fibroblasts, collagen deposition (12) and angiogenesis. Although OPN is mainly involved in type-1-driven

processes, its contribution in Th2-mediated diseases has been recently addressed. Increased OPN has been detected in the tear fluids of patients with allergic ocular diseases (13) and in lung biopsies from asthmatic individuals (14). Increased expression of OPN in inflammatory cells underneath the subepithelial membrane of asthmatic airways suggests that eosinophils might be a potential cellular source of this mediator (15). In fact, Plager et al. (16) were the first to show that differentiating eosinophils expressed OPN mRNA.

In this study, we show that OPN is expressed and functional in human eosinophils and acts as a chemoattractant for eosinophils *in vitro*. Eosinophil derived OPN induces angiogenesis in a CAM model, its expression is increased in the Bronchoalveolar lavage (BAL) fluid from mild asthmatic subjects and this expression correlates with the number of BAL eosinophils. Our data thus suggest that OPN is produced by eosinophils and is likely to have functional relevance in allergic airway inflammation and airway remodeling in asthma.

Materials and methods

Human peripheral blood eosinophil purification and cell culture

Eosinophils were obtained from the peripheral blood of atopic donors (blood eosinophil levels 5–10%) by MACS-negative immunomagnetic separation as described previously (17). Informed consent was obtained from all volunteers according to the guidelines established by the Hadassah–Hebrew University Human Experimentation Helsinki Committee. The cells were collected at a purity of 98–100% (Kimura staining) and at a viability of >98% (Trypan blue staining) (Sigma Chemicals, St. Louis, MO, USA).

For activation, freshly isolated eosinophils were cultured in 96 well U shape bottom tissue culture plates (Nalge Nunc International, Roskilde, Denmark) ($1.5 \times 10^5/0.2$ ml/well) in RPMI-1640 (Biological Industries, Beit Haemek, Israel) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Biological Industries) in the presence of different concentrations of recombinant human GM-CSF (0–20 ng/ml) or IL-5 (0–20 ng/ml) (R&D Systems, Minneapolis, MN, USA). After 15 h of incubation, the cells were centrifuged (150 g, 5 min, 4°C) and their supernatants were liquated and stored at –80°C until use.

Confocal laser microscopy

Cytospins (5 min, 1000 × g) of freshly isolated eosinophils (1×10^5 cells) were fixed in 4% of paraformaldehyde (PFA), permeabilized in 0.2% Triton 100× and incubated with mouse monoclonal anti-human OPN (1/50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) together with goat anti-human eosinophil peroxidase (EPO) polyclonal antibodies (1/50, Santa Cruz) or with irrelevant isotype-matched IgG antibodies (1 h, RT). Binding was detected using FITC-conjugated rabbit anti-goat and Cy5-conjugated goat anti-mouse fluorescent secondary antibodies (1/200) for EPO and OPN, respectively. Cells were examined using a Zeiss 410 laser scanning confocal microscope attached to a Zeiss Axiovert 135 M inverted microscope with 63×/1.2 C-Apochromat water immersion lens (Carl Zeiss, Thornwood, NY, USA).

RNA extraction and RT-PCR analysis

Total RNA (15 µg) from fresh eosinophils was extracted using standard procedures. The cDNA was amplified using primers for human OPN (Fr: 5'-CGC CGA CCA AGG AAA ACT C-3', Rv: 5'-GGG TAC TGG ATG TCA GGT CTG C-3', 569 bp). Beta-actin cDNA probe was used as control. PCR products were visualized by ethidium bromide-stained gel electrophoresis.

Chorio-allantoic membrane assay

The contribution of OPN to eosinophil-induced angiogenesis *in vivo* was studied using the CAM assay as previously described (17). Chorio-allantoic membranes were examined daily until day 12 and photographed *in vivo* with a stereomicroscope SR equipped with a Zeiss Camera System MC63. In some experiments, two observers in a double-blind fashion (at 50× magnification) counted blood vessels entering the sponge within the focal plane of the CAM.

Eosinophil chemotaxis

Eosinophil chemotaxis was measured using a microwell dual chamber system (ChemoTx chamber: filter pore size 5 µm, 6 mm diameter wells; Neuro Probe Inc. Gaithersburg, MD, USA). Different concentrations of rOPN (0.05–5 µg/ml) (Sigma Chemicals) or rhEotaxin/CCL11 (10 ng/ml) (R&D System) (positive control) were added to wells in the bottom chamber (300 µl) and covered with a framed filter. Then, eosinophils (1×10^4 cells/30 µl) were added on the top of the filter. In selected experiments freshly isolated eosinophils were preincubated for 30 min with an optimal concentration of a selective inhibitory compound for $\alpha_4\beta_1$ (500 nM) (BIO-1211; Glaxo Wellcome, London, UK) (18) before addition of the cells on the top of the filter. After 4 h of incubation (37°C, 5% CO₂), the cells in the lower chamber were counted using flow cytometry (FACScalibur; Beckton Dickinson, Cowley, Oxfordshire, UK). Relative cell counts were obtained by acquiring events for 60 s.

Subjects

Healthy non asthmatic subjects ($n = 25$) and mild asthmatic subjects ($n = 15$) were recruited from a research database of volunteers. Ethical approval was obtained from the Southampton and South West Hampshire joint local research ethics committee and the volunteers gave their written informed consent. Detailed clinical history and physical examination were performed on all subjects. Spirometric tests and assessment of asthma severity were in accordance with the BTS/SIGN guidelines. All subjects were tested for atopy by skin prick testing and were free from respiratory tract infections for a minimum of 4 weeks before inclusion in the study.

Fibreoptic bronchoscopy

Subjects underwent fibreoptic bronchoscopy under local anesthesia as previously described (19) using an Olympus BFIT20 bronchoscope (Olympus, Tokyo, Japan). Bronchoalveolar lavage fluid was obtained and processed for cell count and cytokine measurement as previously described (19).

Bronchoalveolar lavage fluid was concentrated 10–30 fold using Amicon Centriprep YM-3 centrifugal filter devices (Millipore Ltd, Watford, UK) before quantification of OPN.

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of OPN in eosinophil supernatants and in BAL fluids were measured using an ELISA kit (Quantikine; R&D, Systems, Minneapolis, MN, USA). The lower limit of assay sensitivity was determined according to the manufacturer's instructions.

Statistical analysis

Data are expressed as Mean $\pm$ SEM of at least three independent experiments. Parametric ANOVA, followed by Tukey–Kramer multiple comparisons test, was used to compare groups. Pearson and Spearman correlation tests were used for correlating normal and nonparametric variables, respectively. Mann–Whitney Rank sum test was used as statistical method to compare OPN in BAL from normal and asthmatic subjects. A probability of <0.05 was considered statistically significant.

Results

OPN is expressed in human eosinophils

Eosinophils were purified from the peripheral blood of atopic donors and OPN mRNA and protein expression were determined. Eosinophils express OPN mRNA which was indicated by the presence of a single PCR product corresponding to the expected size 569 bp (Fig. 1A). Osteopontin protein expression is observed in the intracellular compartment. Double immunostaining for OPN (Cy5: red) and the granule-associated protein EPO (FITC: green) indicate that OPN is present in resting eosinophils and localized mainly in the cytoplasmic compartment of the cells rather than in their granules (Fig. 1B).

Quantification of OPN protein in the supernatant of peripheral blood eosinophils placed in culture wells revealed that OPN is released by resting eosinophils and that GM-CSF and IL-5 activation, significantly enhanced its secretion in a time (Fig. 2A) and concentration (Fig. 2B) dependent manner. Whereas eosinophils cultured for 15 h in medium alone released 95.6 ± 13 pg/ml of OPN ($5 \times 10^5/0.2$ ml), eosinophils treated with GM-CSF (2 ng/ml) or with IL-5 (2 ng/ml), increased their OPN release to 144.6 ± 12 pg/ml ($P < 0.01$) and to 187 ± 9 pg/ml ($P < 0.01$), respectively (Fig. 2B).

Eosinophil-derived OPN contributes to the pro-angiogenic effect of eosinophils in the CAM assay

We have previously shown that eosinophils induce vessel formation in the CAM assay (22). As it is well accepted that OPN is a pro-angiogenic factor, we tested the contribution of OPN to eosinophil-induced angiogenesis by neutralization experiments in the CAM assay. As shown in Fig. 3, neutralization of OPN in freshly isolated eosinophils partially reduced their angiogenic response (1.8-fold; $P < 0.05$). This effect

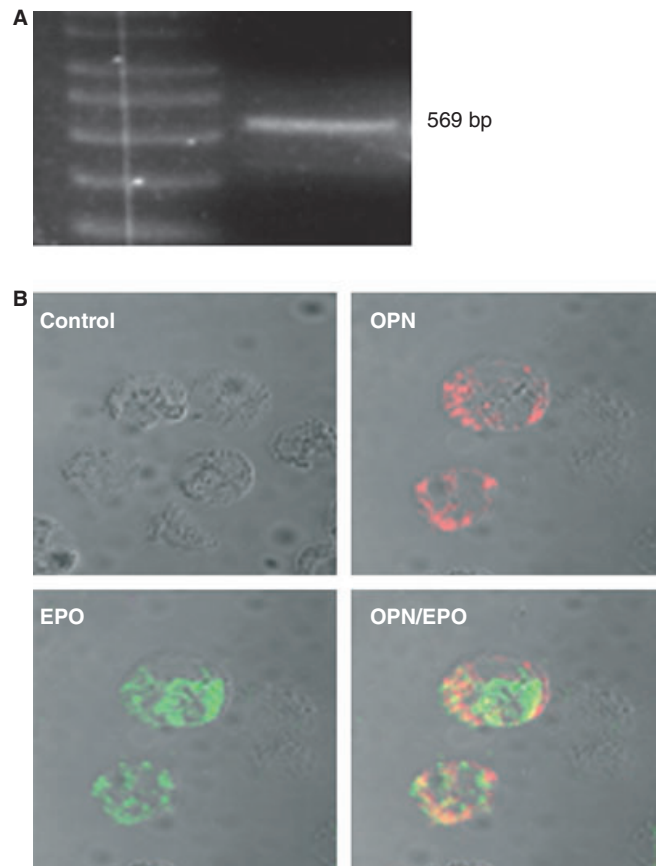


Figure 1. Expression of osteopontin (OPN) in human eosinophils. RT-PCR: 569 bp fragment amplified from mRNA of human peripheral blood eosinophils (EOS) (A) and confocal laser microscopy: double immunofluorescent staining for OPN (red) and eosinophil peroxidase (EPO) (green). Overlay (yellow) of OPN and EPO (B).

indicates that OPN is functional in eosinophils and partially contributes to their pro-angiogenic activity.

OPN induces eosinophil chemotaxis *in vitro*

As OPN is a chemotactic mediator for monocytes and macrophages, we hypothesized that OPN might contribute to the allergic airway inflammation by recruiting eosinophils into the inflamed tissue. Thus, to test our hypothesis, eosinophils were added into ChemoTx System microplate wells and the effect of OPN on the cells was investigated. As shown in Fig. 4A, OPN significantly enhanced eosinophil chemotaxis in a concentration-dependent manner with the highest effect at 500 ng/ml (4.5-fold; $P < 0.01$). This effect was similar to that induced by Eotaxin/CCL11 at 50 ng/ml, used as positive control (4.6-fold; $P < 0.01$).

To test whether the integrin $\alpha_4\beta_1$ is involved in OPN-induced chemotaxis, eosinophils were pretreated with BIO-1211, a selective antagonist for $\alpha_4\beta_1$ (23) before applying the cells into the chemotaxis plates. Pretreat-

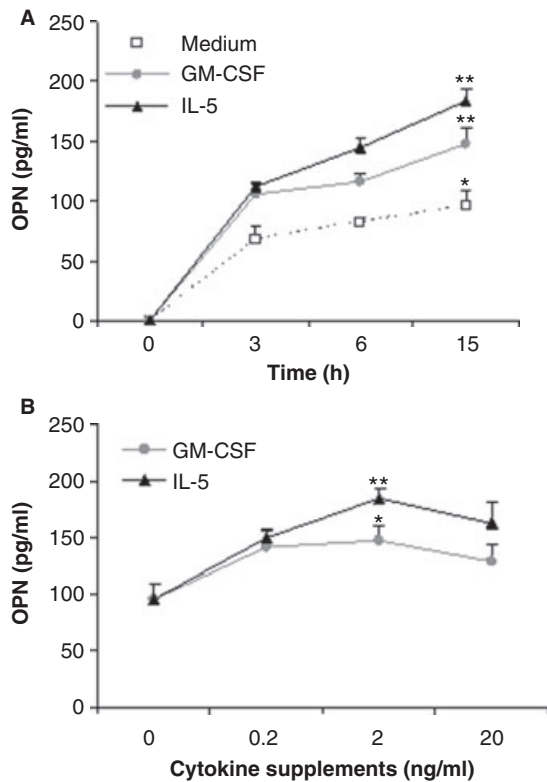


Figure 2. Effect of GM-CSF and IL-5 on osteopontin (OPN) release by eosinophils. Measurement of OPN in the supernatants of human eosinophils treated with IL-5 and GM-CSF at 2 ng/ml for different time periods (A) and with IL-5 and GM-CSF at different concentrations (0–20 ng/ml) for 15 h (B). Data are the Mean + SEM ($n = 3$). * $P < 0.05$; ** $P < 0.01$.

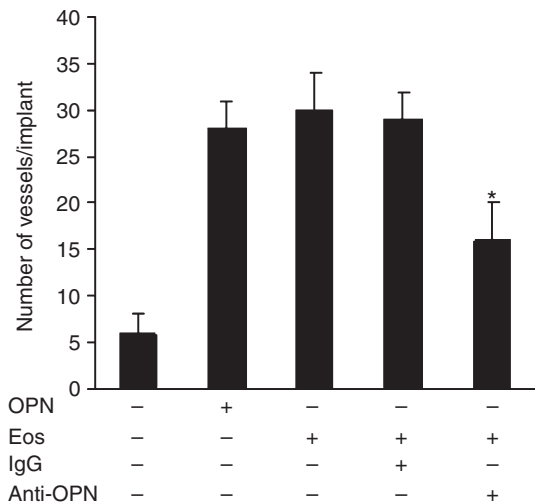


Figure 3. Contribution of osteopontin (OPN) in eosinophil-induced angiogenesis. Angiogenic response in the Allantoic Membrane CAMs treated with medium or with OPN or with eosinophils alone or together with isotype control or anti-OPN. The number of vessels was counted and expressed as Mean $\pm$ SEM ($n = 3$). * $P < 0.05$.

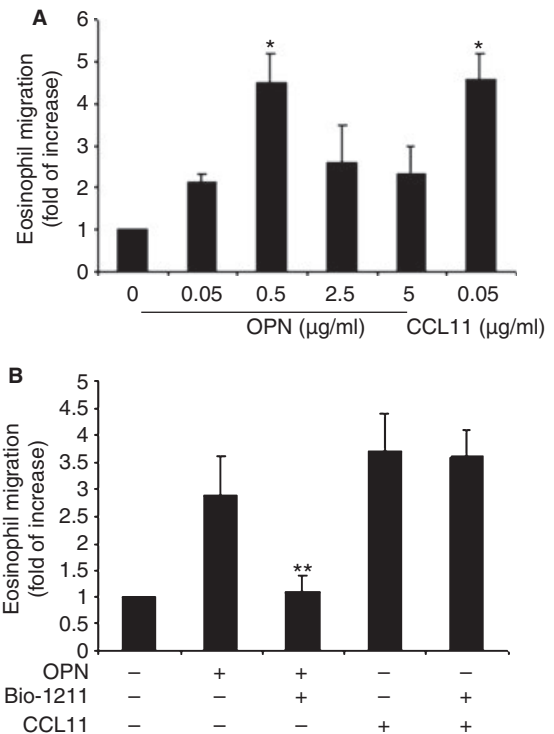


Figure 4. Effect of osteopontin (OPN) on eosinophil chemotaxis *in vitro*. Eosinophil chemotaxis induced by different concentrations of rOPN or rEotaxin/CCL11 in the ChemoTx System microplate wells after 4 h of treatment (A). Eosinophil chemotaxis induced by OPN (500 ng/ml) or Eotaxin/CCL11 (50 ng/ml) after pretreatment of the cells with BIO-1211 (500 nM) for 30 min (B). The results are expressed as fold of increase of cell chemotaxis induced by OPN or Eotaxin/CCL11 vs medium alone. Data are the Mean + SEM ($n = 3$). * $P < 0.01$.

ment with the compound resulted in a complete inhibition of eosinophil chemotaxis induced by OPN, but not that induced by Eotaxin/CCL11 (Fig. 5B), proving that OPN acts on eosinophil migration via specific binding to $\alpha_4\beta_1$.

OPN is increased in the BAL fluid from subjects with mild asthma and correlates with the eosinophil counts

To investigate the relationship between OPN and eosinophils in asthma, we first quantified the level of OPN in the BAL fluid of mild asthmatic and healthy subjects, whose characteristics are shown in Table 1.

As shown in Fig. 5, we found that the level of OPN is significantly increased in the BAL fluid of asthmatic subjects compared with those of the healthy controls ($P < 0.018$). Although the spread of data is quite large, correlation analysis in the BAL fluid of asthmatic subjects showed that OPN levels correlate with eosinophil count ($r_s = 0.6$, $P = 0.0103$), but not with the count of other inflammatory cells such as macrophages and lymphocytes. Therefore, the overlap of OPN levels between normal and asthmatic subjects probably reflects disease

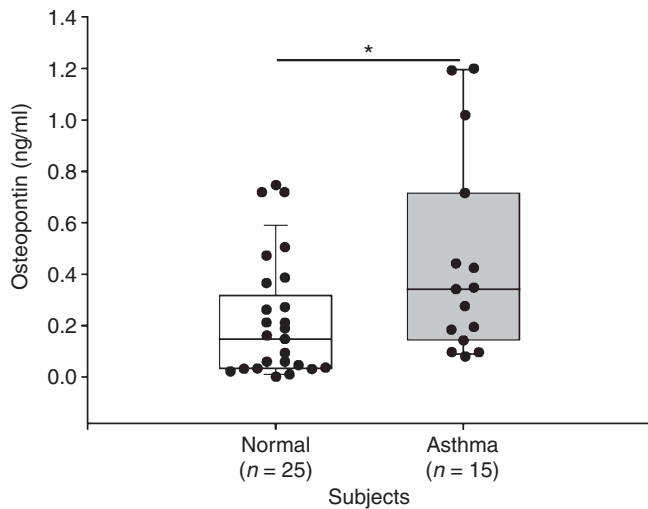


Figure 5. Osteopontin (OPN) level in the BAL fluid of asthmatic subjects and healthy controls. BAL fluid of healthy controls ($n = 25$) and mild asthmatic subjects ($n = 15$) was concentrated 10–30 fold using Amicon Centrifrep YM-3 centrifugal filter devices before measurement of OPN using Quantikine ELISA kit. The results are the median and range. Data were analysed using the Mann–Whitney U -test and significant differences are indicated.

Table 1. Characteristics of mild asthmatic and healthy subjects

	Control subjects	Subjects with Asthma
No. subjects	25	15
Sex, F/M	18/7	5/10
Age: Mean (range) years	29 (18–53)	29 (18–42)
FEV ₁ prebronchodilator, % predicted	102.6 $\pm$ 9.12	82 $\pm$ 16*
PC20, mg/ml methacholine	>16	3.5 (0.12–13.5)*

Data are presented as Mean $\pm$ SD or range. * $P < 0.05$.

F, females; M, males; PC₂₀, the concentration of methacholine that causes a 20% decline in FEV₁.

heterogeneity in terms of the presence or absence of eosinophils. Although the groups are not exactly sex-matched, we could find no obvious gender-related effects in the levels of OPN in BAL fluid.

Discussion

Eosinophils are found in abundance in the airways of asthmatic patients and have traditionally been considered the primary modulator of epithelial damage, via the release of preformed mediators such as eosinophil major basic protein (MBP) and eosinophil cationic protein (ECP) (2). However, eosinophils have also been implicated in the pathogenesis of airway remodeling observed in asthmatic airways (3, 20). They are a source of TGF β as well as several other profibrogenic molecules and cytokines (FGF-2, HB-EGF, PDGF, IL-13), matrix

metalloproteinase-9 (MMP-9) and tissue inhibitors of metalloproteins (TIMP-1 and -2) (2). Studies using anti IL-5 antibodies demonstrate that reduced eosinophil numbers in the airways of asthmatic patients are associated with reduced extra-cellular matrix deposition in the reticular basement membrane (pro-collagen III, tenascin, lumican) (21). A similar increase or reduction in remodeling is observed in IL-5 transgenic or knockout mice respectively (20, 22, 23).

Osteopontin has been shown to be proangiogenic and this effect accounts, at least in part, for its effect in promoting tumor growth and metastasis (10, 22). Elevated levels of OPN have been observed in lung cancer, breast cancer and others and OPN expression correlates with cancer stage (11). The proangiogenic effect is mediated in part via enhancing vascular endothelial growth factor (VEGF) production from tumor cells but also via enhancing VEGF-stimulated neovascularization (24). By contrast, Leali described an inhibitory effect for OPN on murine aortic endothelial cell migration and sprouting and suggested that this inhibitory effect may contribute to OPN-induced atherosclerosis (25).

Eosinophils have also been shown to have pro-angiogenic effects and release VEGF (17) as well as bFGF, TNF α , GM-CSF, NGF, IL-8 and eotaxin (26). We have recently shown that MBP contributes to the eosinophil-induced angiogenesis (27). Eosinophils enhance endothelial cell proliferation and induce a strong angiogenic response in rat aorta rings and CAM assay (17). This is partially inhibited (55%) by neutralizing anti-VEGF and anti-MBP antibodies. Moreover, eosinophils also enhance VEGF mRNA production in endothelial cells and increase expression of VEGF receptors on endothelial cells (17). Levels of VEGF correlate with eosinophil numbers and ECP levels in induced sputum of asthmatics (28). We found that human eosinophils produce OPN. Using neutralizing antibodies and the CAM assay, we demonstrated that eosinophil-derived OPN contributes to eosinophil-induced angiogenesis. We also observed that a combination of anti-OPN and anti-VEGF antibodies caused a further reduction of eosinophil-induced angiogenesis in the CAMs assay (data not shown), indicating a synergistic activity of the two factors. The interaction between OPN and VEGF has been also shown *in vitro* on endothelial cells. For example, VEGF is able to induce OPN release by endothelial cells and to render these cells more sensitive to OPN via the up-regulation of its receptor $\alpha v\beta 3$ (29). Osteopontin in turn induces VEGF production from tumor cells (11). Thus, the pro-angiogenic effect of eosinophils is a consequence of the release and interplay of several mediators including VEGF, OPN and others.

We found that GM-CSF and IL-5, but not IL-3 and Eotaxin/CCL11 were able to enhance the spontaneous release of OPN by eosinophils *in vitro*, thus demonstrating a link between OPN, eosinophils and the allergic inflammatory milieu. Our data are in accordance with the

findings of Horiuchi (30) who showed that GM-CSF and IL-5 stimulate eosinophils to release VEGF. GM-CSF also has pro-angiogenic effects mediated by direct proliferative stimulation of endothelial cells (31).

Eosinophils are not only a source of pro-angiogenic mediators, but can also be a target for them. The VEGF receptors VEGFR-1 and -2 are both present on eosinophils and VEGF promotes eosinophil degranulation and migration *in vitro* mainly by VEGFR1 binding (26, 32). Osteopontin is a chemoattractant for several inflammatory cells such as neutrophils, monocytes, macrophages and a sub-population of lymphocytes. In this study, we found that OPN promotes eosinophil chemotaxis *in vitro* and this effect is similar to that induced by Eotaxin/CCL11 the main chemoattractant for eosinophils. Osteopontin has been shown to be chemotactic by binding to integrin receptors, including $\alpha_v\beta_3$ and $\alpha_4\beta_1$ or via different CD44 isoforms (8). Among these adhesion molecules, $\alpha_4\beta_1$ has been demonstrated to be highly expressed on eosinophils and to be primarily responsible for eosinophil trafficking in the airways (33). By using a selective antagonist for $\alpha_4\beta_1$, we have also demonstrated that OPN-induced eosinophil chemotaxis *in vitro* is due to $\alpha_4\beta_1$ binding.

In this study, we found that soluble OPN is increased in the BAL fluid from mild asthmatic subjects and correlates with eosinophil counts. In addition, OPN correlated with GM-CSF and eotaxin-1. This is somewhat counter-intuitive as OPN has been considered a 'Th-1' type cytokine. Osteopontin differentially favors expression of IL-12 and induces maturation of dendritic cells to a Th-1 promoting phenotype (4, 5). Osteopontin has also been implicated in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (34). There is, however, evidence that OPN may play a role in allergic inflammation. Osteopontin has been detected in tear fluid from patients with allergic conjunctivitis (13). Endobronchial

biopsies from asthmatic patients show increased immunostaining for OPN (14, 15). Staining is prominent in the epithelial layer as well as in eosinophils, macrophages and lymphocytes present in the submucosal inflammatory infiltrate. Xanthou et al. found that OPN has a bi-modal immunomodulatory effect in a murine model of allergen-induced airway inflammation, a Th2- enhancing effect during antigenic sensitization, and a Th2- repressor effect during antigen challenge (14). This effect is mediated by inhibition of migration of plasmacytoid and conventional dendritic cells, respectively. In their model, administration of recombinant OPN during antigen challenge protected mice from allergic disease. Nagasaka et al. found that mast cells produce OPN and that OPN induces chemotaxis and degranulation of these cells (35). Osteopontin expression is also enhanced in a murine model of chronic allergen-induced airway remodeling suggesting that it plays a role not only in inflammation but also in the development of structural changes in the airways (15).

Although eosinophils are only one of several cell-types to produce OPN and this molecule accounts for only part of the proangiogenic effect of eosinophils, our findings provide further evidence for the multifaceted role of eosinophils in airway remodeling and for the interactions between eosinophils and angiogenic mediators at site of inflammation. Thus, eosinophils not only contribute to the inflammatory process but also to the development of vascular remodeling during bronchial asthma.

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