

Original article

The role of eosinophil major basic protein in angiogenesis

Background: Eosinophil-derived major basic protein (MBP) plays an active role in allergic inflammation and tissue remodelling. However, its role in angiogenesis has not been established as yet. Therefore our objective was to investigate whether MBP exhibits any direct pro-angiogenic effects.

Methods: Rat aortic endothelial cells and human umbilical vascular endothelial cells were cultured with different concentrations of MBP and their viability (Trypan blue exclusion test), proliferation (thymidine incorporation) and capillary-like structure formation (matrigel assay) were investigated *in vitro*. The angiogenic activity of MBP was then tested *in vivo* using the chick chorio allantoic membrane (CAM) assay.

Results: Subcytotoxic concentrations of MBP induce endothelial cell proliferation and enhance the pro-mitogenic effect of vascular endothelial growth factor (VEGF), but do not affect their VEGF release. MBP promotes capillarogenesis by endothelial cells seeded on matrigel and sprouting formation in the CAM assay. Furthermore, we have shown that the pro-angiogenic effect of MBP is not due to its cationic charge since stimulation of the CAMs with the synthetic polycation, poly-L-arginine does not induce any angiogenic effects.

Conclusions: These data demonstrate that MBP has pro-angiogenic effects *in vitro* and *in vivo*, providing a novel mechanism whereby MBP can participate in tissue inflammation and remodelling in atopic diseases.

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Infiltration and subsequent activation of eosinophils in the inflamed tissue is a common feature of allergic diseases such as rhinitis, bronchial asthma and atopic dermatitis (1, 2). Eosinophils have been historically thought to cause tissue damage mostly through the release of granule-associated cationic proteins major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (3–5). More recently, however, a tissue healing role has been ascribed to eosinophils that is attributed to their ability to produce pro-fibrogenic cytokines, growth factors and metalloproteinases (6, 7) and to induce angiogenesis partially through their preformed vascular endothelial growth factor (VEGF) (8, 9).

MBP, localized in the core of the eosinophil secondary granules and comprising more than 50% of the granule protein, has been assumed to be the principal toxic molecule released by eosinophils during allergic inflammation, parasitic infection and certain cancers (3, 4, 10).

Many of the biological properties of MBP have been attributed to the strong positive charge of the molecule and its high arginine content (11), even though its cationic nature does not fully explain its activity (12). The cell agonist activities of subcytotoxic concentrations of MBP recently described in various cell types (11, 13–18), support the hypothesis that MBP might play immunoregulatory roles in eosinophil-associated diseases. The deposition of MBP in the inflamed tissue during airway allergic diseases might contribute to the amplification and perpetuation of inflammation by inducing the release of superoxide anion and lysozyme by neutrophils (13), EDN and interleukin-8 (IL-8) by eosinophils (15) and histamine and prostaglandin (PG) D₂ by mast cells (11, 16). In addition to its role in inflammation, MBP might contribute to airway remodelling through activation of resident cells, such as fibroblasts (17) and epithelial cells (18). It has been shown that MBP acts synergistically with IL-1 and transforming growth factor-beta (TGF- β) to increase the synthesis of the pro-fibrogenic cytokines IL-6, IL-11

and LIF-1 in lung fibroblasts (17) and stimulates bronchial epithelium to synthesize remodelling factors, such as endotelin-1, TGF- α , TGF- β , platelet-derived growth factor-beta (PDGF- β), metalloproteinase-9, fibronectin and tenascin (18). Although all these mediators might promote angiogenesis by acting on endothelial cells and/or on ECM (8), the possibility that MBP may exert direct pro-angiogenic effects has not been investigated as yet.

In this study, we provide evidence for the first time that eosinophil-derived MBP is pro-angiogenic by stimulating endothelial cells proliferation *in vitro* and angiogenesis in matrigel and in the CAM assay. Furthermore, we have shown that the synthetic polycation, poly-L-arginine does not induce angiogenesis *in vivo* indicating that the pro-angiogenic effect of MBP is not due to its cationic charge. Taken together, our findings provide a novel mechanism whereby MBP can participate in the inflammatory and remodelling processes of eosinophil-associated diseases.

Methods

Purification of human eosinophil MBP

MBP was purified from eosinophils obtained from patients with marked eosinophilia as described (19, 20). The purified protein was stored at -70°C , and samples were thawed immediately before use. MBP was judged pure by its banding pattern on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after staining with Coomassie brilliant blue R. Protein concentrations were determined using the appropriate E277 values.

Endothelial cell purification and culture

Rat (Sprague–Dawley, 7-week-old) aortic endothelial cells were isolated, cultured and characterized as previously reported (21, 22).

Human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, VA, USA) were harvested from five samples following parental consent, grown in 0.1% gelatin-coated culture flasks in M199 medium supplemented with endothelial cell growth supplement (ECGS) (20 mg/ml), 20% heat-inactivated FCS and heparin (90 mg/ml). All the experiments were conducted with HUVEC from passage 2 to 7.

Endothelial cell viability

Endothelial cells ($5 \times 10^4/0.5$ ml) were cultured in 24-well plates [37°C , 5% carbon dioxide (CO_2)] in medium alone or with different concentrations of MBP (1–1000 nM) for 24 and 48 h. Endothelial cell viability was then evaluated by Trypan blue exclusion test.

Endothelial cell proliferation assay

Proliferation of sub-confluent endothelial cell cultures was assessed by ^3H -thymidine incorporation, as previously described (7). Briefly, endothelial cells ($8 \times 10^3/0.2$ ml) were cultured overnight in 96-well plates (37°C , 5% CO_2) in medium alone. The culture cells were washed twice with fresh medium and further incubated (24 h, 37°C , 5% CO_2) with different concentrations of MBP (1–1000 nM). Medium alone and rhVEGF (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) were used as negative

and positive controls, respectively. ^3H -thymidine (1 μCi per well) (NENTM Life Science Products Inc., Boston, MA, USA) was added as a final 24 h pulse and samples were processed as previously described (7). In another set of experiments, endothelial cells were incubated simultaneously with VEGF (10 ng/ml) and with different concentrations of MBP (10–1000 nM), before adding ^3H -thymidine.

Enzyme-linked immunosorbent assay for vascular endothelial growth factor

VEGF content was measured in the MBP preparation used throughout this study (100 and 1000 nM) and in the supernatants of endothelial cells treated with different concentrations of MBP (1–1000 nM) for 24 and 48 h. Measurement was performed by an enzyme-linked immunosorbent assay (ELISA; PeproTech Inc., Rocky Hill, NJ, USA) according to the manufacturer's instructions.

Capillary-like structure formation in Matrigel

96-well plates were precoated with 50 μl of growth factor reduced Matrigel matrix solution (BD Biosciences, Bedford, MA, USA) and the gel was allowed to solidify at 37°C for 1 h. Endothelial cells were then plated at a density of 8×10^3 cells/well and further incubated alone or in the presence of various concentrations of MBP (1–1000 nM). Medium alone and rhVEGF (10 ng/ml) (R&D Systems) were used as negative and positive controls, respectively. After 18 h, the 3D organization was examined under a inverted, phase-contrast photomicroscope, and the topological parameters of the capillary mesh (mesh areas, vessel length and mesh branching points) were measured by computed image analysis as previously described (23).

Chick embryo chorioallantoic membrane assay

The contribution of MBP in angiogenesis was next investigated *in vivo* using the chick embryo chorio-allantoic membrane (CAM) assay (24). Briefly, fertilized white leghorn chicken eggs (30/group) were incubated under constant humidity at 37°C . On the third day of incubation, a square window was opened in the shell after removal of 2–3 ml of albumen in order to detach the developing CAM from the shell. The window was sealed with a glass of the same dimension, and eggs were returned to the incubator. CAMs were treated after 8 days of culture with different concentrations of MBP (1–1000 nM) dissolved in 3 μl of serum-free medium and adsorbed on 1 mm^3 sterilized gelatin sponges (Gelfoam; Upjohn Company, Kalamazoo, MI, USA). Sponges containing vehicle alone were used as negative controls, while sponges containing rhVEGF (500 ng/embryo) were used as positive controls. CAMs were examined daily until day 12 and photographed *in ovo* with a stereomicroscope SR equipped with a Zeiss Camera System MC63. In some experiments, two observers in a double-blind fashion at $50\times$ magnification counted blood vessels entering the sponge within the focal plane of the CAM.

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. A probability of <0.05 was considered statistically significant.

Results

MBP is not cytotoxic for endothelial cells

Since MBP can exert some cytotoxic effect, we first evaluated whether it can affect endothelial cell viability. Endothelial cells were cultured in medium alone (control) or containing increased concentrations of MBP (1–1000 nM) for 24 and 48 h and their viability was evaluated by Trypan blue exclusion test. As shown in Fig. 1 no significant changes in cell viability were observed following MBP treatment, indicating that MBP does not exert any cytotoxic effects towards endothelial cells at all the concentrations tested.

MBP induces endothelial cell proliferation and enhances the pro-mitogenic effect of VEGF *in vitro*

To investigate the effect of MBP on endothelial cell proliferation, sub-confluent endothelial cells were cultured in medium alone or in medium containing increasing concentrations of MBP (1–1000 nM) for 24 h. Their proliferation was then evaluated by ^3H -Thymidine incorporation assay. As shown in Fig. 2, MBP increased significantly endothelial cell proliferation by 2.0- and 2.7-fold at 100 and 1000 nM, respectively ($P < 0.01$). VEGF (10 ng/ml), used as positive control, increased endothelial cell proliferation by 2.9-fold ($P < 0.01$). Interestingly, the addition of MBP together with VEGF resulted in a further increase in endothelial cells proliferation (3.5-fold at 1000 nM) ($P < 0.001$) (Fig. 3).

MBP does not induce VEGF release by endothelial cells

To address the possibility that the mitogenic effect of MBP could be due either to its content of VEGF or to its effect on VEGF release by endothelial cells the following experiments were performed. The MBP preparation used

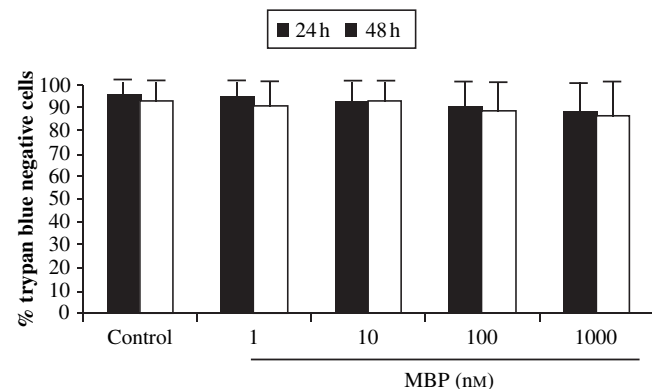


Figure 1. MBP is not cytotoxic for endothelial cells: endothelial cells were cultured in medium alone (control) or with different concentrations of MBP for 24 and 48 h. Endothelial cell viability was then evaluated by Trypan blue exclusion test. The results, expressed as % Trypan blue negative cells, are the mean $\pm$ SEM ($n = 3$).

in this study was assayed for possible 'contaminant' VEGF content by ELISA. No detectable VEGF (≤ 0 ng/ml) was found at the two higher concentrations tested (100 and 1000 nM). Furthermore, endothelial cells were cultured for 24 and 48 h in medium alone or with different concentrations of MBP (1–1000 nM) in 1% serum-reduced medium and the VEGF content was then measured in their supernatant. MBP did not induce VEGF release in endothelial cell supernatants at any concentration tested (VEGF ≤ 0 ng/ml), suggesting that the pro-angiogenic effect of MBP is not due to up-regulation of VEGF in endothelial cells.

MBP induces capillary-like structure formation in matrigel

The effect of MBP on angiogenesis *in vitro* was then studied by a Matrigel capillarogenesis assay. Endothelial cells treated with increasing concentrations of MBP spread through the Matrigel surface and aligned to form

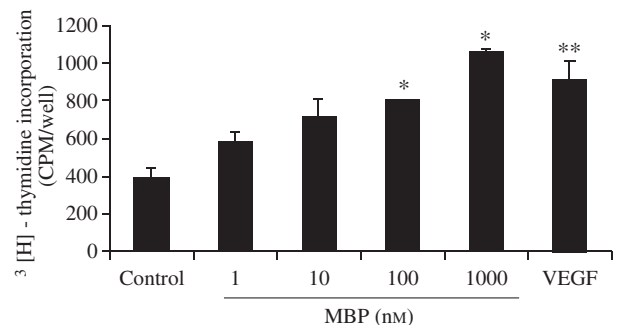


Figure 2. Endothelial cells ($8 \times 10^3/0.2$ ml/well) were incubated with medium alone (control) or with different concentrations of MBP or with VEGF (10 ng/ml) for 24 h. Endothelial cell proliferation was then evaluated by ^3H -Thymidine incorporation assay and expressed as CPM/well. Data are the mean $\pm$ SEM ($n = 3$). * $P < 0.01$, ** $P < 0.001$ vs control.

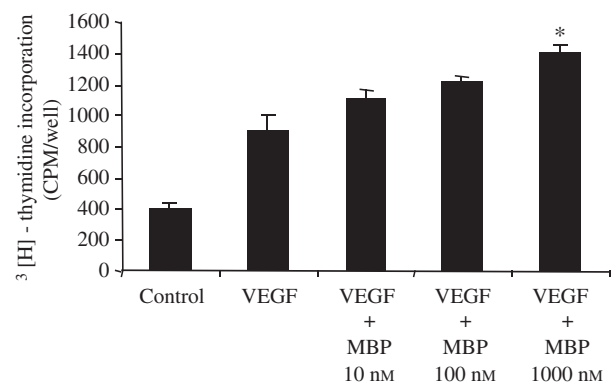


Figure 3. Endothelial cells ($8 \times 10^3/0.2$ ml/well) were incubated with medium alone (control) or with VEGF alone or together with MBP for 24 h. Endothelial cell proliferation was then evaluated by ^3H -Thymidine incorporation assay and expressed as CPM/well. Data are the mean $\pm$ SEM ($n = 3$). * $P < 0.01$ vs control.

branching with a tendency to form tubes with multicentric junctions (Fig. 4A). Quantification analysis has shown that treatment of endothelial cells with MBP increased vessel length (Fig. 4B) and branching points (Fig. 4C) in a dose-dependent manner with the maximum effect reached at 1000 nM (vessel length: 2.4-fold, $P < 0.01$; branching point numbers: 2.8-fold, $P < 0.01$). The angiogenic effects induced by VEGF used as positive control were quantitatively higher than

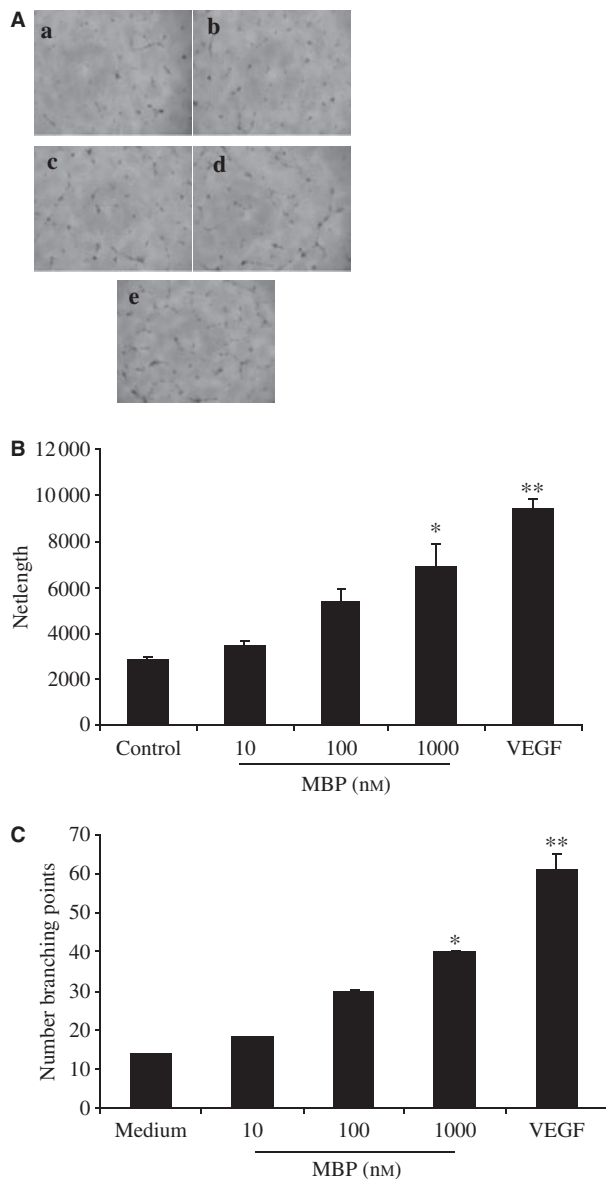


Figure 4. (A) Endothelial cells (8×10^3 /well) were plated on matrigel solution ($50 \times \mu\text{l}$ /well) and cultured for 18 h with medium alone (control) (a) or with the following concentrations of MBP: 10 nM (b), 100 nM (c) and 1000 nM (d) or with VEGF (10 ng/ml) (e). Quantification of topological parameters of the capillary meshes: vessel length (B) and mesh branching points (C) were measured by computed image analysis. Data are the mean $\pm$ SEM ($n = 3$). * $P < 0.01$, ** $P < 0.001$ vs control.

those induced by MBP (vessel length: 3.2-fold, $P < 0.001$; branching point numbers: 4.3-fold, $P < 0.001$).

MBP stimulates blood vessel sprouts in the chick embryo chorioallantoic membrane assay

To test the effect of MBP on angiogenesis *in vivo*, different concentrations of MBP (1–1000 nM) were added on top of the chick embryo CAMs system and new vessels formation was evaluated (Fig. 5). Medium alone (control) and VEGF (500 ng/ml) were used as negative and positive controls, respectively. MBP, at all the concentrations tested, induced a potent angiogenic response quantitatively and qualitatively similar to that induced by VEGF. MBP at 100 nM increased new vessel formation by 4.6-fold vs control ($P < 0.001$) and this effect was comparable to the effect induced by VEGF (5.3-fold; $P < 0.001$) (A). The angiogenic responses caused by MBP and VEGF were typically characterized by the presence of allantoic vessels spreading radially towards the sponge in a spoked wheel pattern, while in the CAM incubated with medium alone no vascular reaction was detectable around the sponge (B).

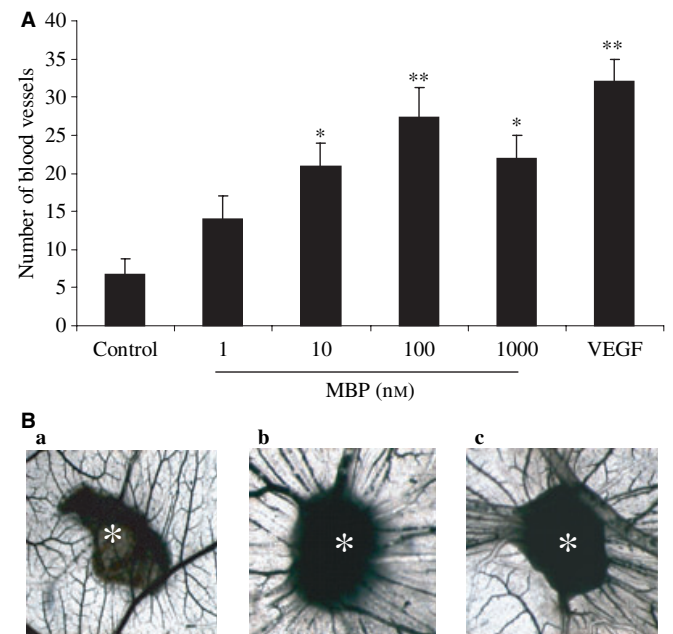


Figure 5. (A) Medium alone (control) or different concentrations of MBP or VEGF were delivered on the top of the CAMs, using a gelatine sponge implant and the number of vessels was counted. (B) In the membrane treated with medium alone (control) (a) no vascular reaction was detected. The gelatin sponge treated with MBP (b) is surrounded by allantoic vessels that develop radially towards the implant (asterisk) in a 'spoked-wheel' pattern. This angiogenic response was similar to that observed in the membrane treated with VEGF (c) used as positive control. Data are the mean $\pm$ SEM ($n = 3$). * $P < 0.01$, ** $P < 0.001$ vs control.

To assess the role of the positive charge of MBP on its pro-angiogenic effect, poly-L-arginine at the same concentrations used for MBP were added on the top of the CAM. This treatment did not induce any vascular reaction, indicating that the pro-angiogenic effect of MBP is charge independent (data not shown).

Discussion

We have shown that MBP is a novel defined eosinophil derived pro-angiogenic factor. The production of VEGF and of other pro-angiogenic factors, such as basic fibroblast growth factor (b-FGF), tumour necrosis factor alpha (TNF α), granulocyte macrophage colony stimulating factor (GM-CSF), nerve growth factor (NGF), IL-8 and eotaxin, has suggested a strong link between eosinophils and angiogenesis (8, 9, 25). We have recently shown that eosinophil sonicates and supernatants display direct pro-angiogenic effects promoting endothelial cells proliferation, inducing their VEGF production and rendering these cells more sensitive to VEGF via the up-regulation of its specific surface receptor (8). Moreover, eosinophils induce new vessel formation in the aorta rings and in the CAM assays (8). The use of neutralizing antibodies demonstrated that these effects are mediated principally but not uniquely by the eosinophil-derived VEGF suggesting the presence of other angiogenic factors among the eosinophil preformed mediators. An intriguing candidate was MBP being the most abundant cationic protein in eosinophil granules as well as involved in a wide range of activities.

In the present work, we first evaluated the effect of MBP on endothelial cell viability. We found that at the used concentration it is not cytotoxic for endothelial cells but rather that it stimulated EC proliferation in a concentration-dependent manner at nM concentrations.

In a previous work in which guinea pig eosinophil derived MBP was used at a similar concentration a decrease in EC growth was observed (26). This can be explained by the difference in the source of both MBP and the EC. In our study MBP was from human eosinophils and EC from rat aorta or HUVEC while in the previous work MBP was from guinea pig eosinophils and EC from swine were used. Moreover, different assays used for the growth evaluation can also be a source of substantially different results (27).

We found that MBP caused a concentration-dependent increase in endothelial cell proliferation and that it enhanced the pro-mitogenic effect of VEGF.

In our previous work eosinophil sonicates were found to affect angiogenesis by increasing the VEGF released from the same EC (8). Therefore, we first ruled out a 'contaminating' presence of VEGF in the MBP preparation, that was found to be undetectable. Thereafter we evaluated whether the observed effect of MBP on EC could be mediated by VEGF release. We found that MBP

does not induce VEGF release by EC which strongly indicates a direct effect of MBP on EC. Furthermore we observed that MBP enhances the pro-mitogenic effect of VEGF *in vitro*, suggesting the presence of an additional pathway for the induction of angiogenesis.

Proliferation of endothelial cells is an early and crucial event in angiogenesis, providing the necessary number of cells for a new vessel to be made (28). Subsequent to this step, the new outgrowth of endothelial cells must reorganize into a three-dimensional tubular structure. Therefore, the effects of MBP in a tube-like formation model, which has been extensively applied for the detection of pro-angiogenic stimuli, were evaluated. MBP induced capillary-like structure formation in matrigel. This was evaluated in this assay with two parameters for angiogenesis: the net length and the number of branching points. Both parameters showed a concentration-dependent stimulation of capillary-like formation.

To confirm the *in vivo* relevance of the pro-angiogenic role of the eosinophils we adopted the model of chick embryo CAM, an extra-embryonic membrane, which serves as a gas exchange surface whose function is supported by an extensive capillary network (24). In the CAM vasculature, the interactions between the extracellular matrix and the cellular components lead to a complete angiogenic process between the chorionic epithelium and the mesodermal blood vessels (29). Also in this system, we found that MBP significantly increased blood vessel sprouting, being 100 nM the most effective concentration with an effect similar to the one of VEGF.

MBP has a very strong positive charge and, as a result, binds avidly to negative charged surfaces. It has previously been demonstrated that eosinophil cationic proteins increase airway responsiveness dependent on charge interactions (30). Therefore to assess the contribution of the positive charge of MBP on its pro-angiogenic effect, poly-L-arginine, a synthetic polycation, was added in the CAM assay. This treatment did not induce any vascular reaction, indicating that the pro-angiogenic effect of MBP is not due to its cationic charge. We recently found that MBP acts as a mast cell activator *in vitro* in part by binding to integrin β_1 on the mast cell surface (Bachelet I and Levi-Schaffer F, unpublished data). We therefore hypothesize that the pro-angiogenic effects of MBP could be mediated by its binding to a defined receptor on EC, possibly to β_1 integrin. Interestingly β_1 integrin has been found to be important in angiogenesis as it is involved in EC proliferation and migration (31).

For years MBP has been known as one of the major causes for eosinophil-mediated tissue damage, being highly cytotoxic. Together with this it has been accepted that at low concentrations it can have an activation effect on both structural and inflammatory cells. For example MBP can activate the synthesis of remodeling factors by airway epithelial cells (18) and together the IL-1 and TGF β increases IL-6 type cytokine production in human

lung fibroblasts (17). Moreover MBP can activate histamine release from basophils and mast cells in a noncytotoxic fashion (11).

We have therefore defined a new activating role of MBP: a pro-angiogenic effect. Interestingly in previous studies we found that MBP is a potent inhibitor of heparanase activity (32). Heparanase, by degrading heparan sulphate (HS), is a facilitator of cell migration. It also releases angiogenic factors and accessory fragments of HS from the microenvironment and induces an angiogenic response *in vivo*. Consequently, we must speculate that the ability of the eosinophils to be pro- or anti-angiogenic *in vivo* depends on the specific microenvironment of the disease such as the presence

of pro- or anti-angiogenic factors produced by other cells.

In conclusion eosinophils must be taken in consideration when investigating angiogenesis linked to allergy and to several chronic inflammatory diseases characterized by tissue hypereosinophilia.

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