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Effects of *Parietaria judaica* pollen extract on human microvascular endothelial cells ☆

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ABSTRACT

Pollinosis from *Parietaria judaica* is one of the main causes of allergy in the Mediterranean area. The present study is designed to assess if *P. judaica* pollens contain bioactive compounds able to elicit a functional response in endothelial cells.

We have demonstrated that addition of pollen extract to human lung microvascular endothelial cells (HMVEC-L) induces a modification of cell morphology, actin cytoskeletal rearrangements and an increase in endothelial cell permeability. We further showed that the treatment of endothelial cells with pollen extract causes an increase of E-selectin and VCAM-1 protein levels as well as an increase of IL-8 production. The stimulation of cell–cell adhesion molecules was paralleled by a dose-dependent increase of adhesion of polymorphonuclear cells (PMNs) to HMVEC-L monolayer. Our results suggest for the first time that pollen affect directly endothelial cells (EC) modulating critical functions related to the inflammatory response.

Parietaria judaica (*P.j*), a weed belonging to the Urticaceae family, is commonly found in urban areas and its pollen is one of the main causes of allergy in the Mediterranean area. The small dimensions of *P. judaica* pollens (13–17 μm) allow their easy access in the high respiratory tract where they contact the epithelial cells. Studies on pollen behavior showed that humidity and temperature conditions similar to the respiratory system cause the granules to break and lose cellular content. Pollens contain a great number of compounds, some of them behave as allergens but others have specific enzymatic activities. For example, eicosanoid-like substances have been found in birch, grass, and mugwort pollen [1]. Pollen extracts contain proteolytic activities which can alter the epithelial integrity contributing to the sensitization and pathogenesis of allergy [2]. In *P. judaica*, Cortes et al. have identified a 98-kDa aminopeptidase that was able to degrade bioactive peptides and to cause cellular detachment through the cleavage of E-cadherin and occludin [3]. Following the disruption of the epithelial barrier, a variety of proteins and/or bioactive compounds contained in the pollen grains may transverse the epithelial sheet and come in contact with stroma cells including fibroblasts and endothelial cells. This interaction may affect different processes involved in inflammatory response that accompany allergy development.

Endothelial cells play a key role in the pathophysiology of allergy [4]. Cell adhesion between endothelium and inflammatory cells is an important early step during allergy development. Increased expression of cell adhesion molecules such as selectins, ICAM-1 and VCAM-1 on EC is responsible for the initial tethering and rolling phase and later stable adhesion of leukocytes [5]. At the same time, the ability of leukocytes to migrate through the endothelial barrier depends by the disruption of EC junctions including components of the adherens junctions (VE Cadherin complex) and tight junctions (occludin, claudins, junctional adhesion molecules [JAM]). The present study is designed to assess if *P. judaica* pollens contain bioactive compounds able to elicit a functional response in endothelial cells. We have demonstrated that addition of pollen extract to HMVEC-L induces a modification of cell morphology accompanied by cytoskeletal rearrangements, an alteration of monolayer integrity with loss and delocalization of adhesion molecules belonging to EC adherens junctions, and an increase in E-selectin and VCAM-1 as well of IL-8, an inflammatory mediator. These findings suggest for the first time that pollen components may affect directly endothelial cells modulating critical EC functions related to the inflammatory response.

Methods

Cell culture, reagents, and treatments. Human lung microvascular endothelial cells were obtained from Lonza (Clonetics, Verviers, Belgium) and grown according to supplier's information. All other reagents were purchased from Sigma (St. Louis, MO, USA), if not

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otherwise cited. Aprotinin, pepstatin, EDTA, and bestatin had been previously tested in cell vitality studies and used as inhibitors of different classes of proteases.

Pollen extract. Parietaria judaica pollen (Allergon, Ängelholm, Sweden) was defatted with diethyl ether, extracted by magnetic stirring in PBS for 20 h at 4 °C, extensively dialysed against water and lyophilized. Pollen extract was tested for LPS contamination according a commercial kit (Pro-Q Emerald Lipopolysaccharide kit, Invitrogen, Carlsbad, CA, USA).

Remodelling of the cell cytoskeleton. Analyses of actin cytoskeleton was performed as previously described [6]. Briefly, HMVEC-L cells were seeded onto coverslips previously coated with collagen (Calbiochem, Darmstadt, Germany) and incubated with pollen extract or with BSA control. After treatment, cells were fixed in paraformaldehyde and stained with rhodamine-conjugated phalloidin (1:1000) (Invitrogen) and finally examined with a fluorescence microscope (Olympus BX50).

Western blot. Total protein cell lysates were obtained and analyzed by SDS–PAGE followed by Western blotting as previously described [7]. Equal amount of proteins were separated by a 8% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Little Chalfont, UK). The membrane was probed overnight at 4 °C with specific primary antibodies against VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or E-selectin (Abcam, Cambridge, UK) and immunocomplexes were detected by the enhanced chemiluminescence detection system (Super Signal, Pierce, Rockford, IL, USA).

Immunofluorescence. HMVEC-L cells were plated at 5×10^4 cells/well on coverslips previously coated with collagen and confluent monolayers were treated with pollen extract for 6 h to study claudin-5 and VE Cadherin protein distribution and expression level. After incubation with pollen or BSA control, cells were fixed in 3.7% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 for 3 min. Incubation with VE Cadherin (1:100; Santa Cruz Biotechnology), or claudin-5 antibodies (1:100; Santa Cruz Biotechnology) was performed in PBS 1% BSA overnight at 4 °C. Cells were stained with FITC-conjugated anti-mouse antibodies (1:100; Invitrogen) to detect VE Cadherin or Texas Red-conjugated anti-rabbit antibodies (1:100; Invitrogen) to detect claudin-5. Immunostained cells were analyzed by confocal microscopy (Olympus 1X70 with Melles Griot laser system).

RNA extraction and real-time PCR. HMVEC-L were grown to confluence in 6-well plates and incubated for different times with indicated stimuli. E-selectin, IL-8, VCAM-1 transcript levels were measured by *Taq*Man real-time quantitative polymerase chain reaction (RQ-PCR) as previously described [7]. Primers were ordered from Applied Biosystem and relative RNA levels were calculated using $\Delta\Delta C_t$ method with RNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal controls.

ELISA. To study IL-8 release by HMVEC-L, aliquots of conditioned medium (CM) from cells were collected after 6 and 12 h stimulation with pollen extract or with vehicle control. As positive control, cells were treated for 6 h with 10 ng/ml of TNFα. CM aliquots were centrifuged to remove cellular debris and IL-8 protein levels were quantified using ELISA kit (R&D Systems, Minneapolis, USA).

Permeability assays. The transendothelial permeability of HMVEC-L cell monolayers was measured using Transwell polycarbonate insert filters (Corning, Little Chalfont, UK pore size 3 μm). HMVEC-L were grown at confluence and transferred into starvation medium containing 1% BSA in DMEM without phenol red. FITC-dextran (500 kDa, Sigma) was added apically and monolayers were then either left untreated (control) or stimulated with pollen extract for 6 h. Samples were removed from the lower chamber for fluorescence measurements. Fluorescence was measured using a Jasco V-570Spectrophotometer.

Polymorphonuclear granulocytes isolation and adhesion assay. Human polymorphonuclear granulocytes (PMNs) were obtained from atopic volunteers by means of density gradient centrifugation of total blood with Ficoll-Paque Premium. After incubation of HMVEC-L monolayer for 6 h with 100 and 250 μg/ml of pollen extract, adhesion of PMN was performed as previously described [8].

Statistics. Data were expressed as means ± SEM of the indicated number of experiments. Statistical analysis was performed by using a paired samples *t* test. Differences were considered to be significant when *p* values were smaller than 0.05.

Results

Parietaria judaica pollen extract modulates IL-8 expression and secretion by HMVEC-L

IL-8 is an important inflammatory cytokine induced by several compounds in epithelial cells. We tested the effects of the pollen extract on levels of mRNA expression and secretion of IL-8 in endothelial cells. Fig. 1A and B show that *P.j* pollen treatment of HMVEC-L caused both a dose- and time-dependent increase in IL-8 mRNA expression, respectively. Increase of mRNA production was statistically significant and reached approximately a 50-fold induction after overnight stimulation of the endothelial monolayer with 250 μg/ml of pollen extract. The increase in IL-8 mRNA was paralleled with a sixfold increase of cytokine release in the culture medium as determined by ELISA (Fig 1C and D). As negative control, equal amounts of BSA did not induce significantly mediator releases from endothelial monolayer. Addition of heated *P.j* extract to HMVEC-L significantly reduced the pollen-induced increase of IL-8 (data not shown); this data together with the negative results of Pro-Q Emerald Lipopolysaccharide kit excluded the LPS contamination of pollen.

Parietaria judaica pollen extract affects lung microvascular endothelial cell morphology

To visualize the effects of *P. judaica* pollen extract on lung microvascular endothelial cell morphology and to investigate the modification of cytoskeletal structures, we monitored the cells by rhodamine–phalloidin staining of actin. Supplementary Fig 1b shows that HMVEC-L exposed to 250 μg/ml of *P.j* extract changed their morphology appearing more elongated respect to BSA-treated control cells; the integrity of the monolayer was altered due to the separation of cell–cell contacts, however cellular detachment from the substrate was not observed at this time point.

Treatment of endothelial cells with *P.j* pollen extract caused a derangement of actin filaments that was visible as decrease of stress fibers through the cytoplasm.

Parietaria judaica pollen extract alters endothelial permeability and cell junctions

Vascular permeability is a parameter of endothelial cell function and its alterations are a defining feature of different processes including inflammation and ischemia/reperfusion. As shown in Fig. 2a, HMVEC-L confluent control monolayer did not permit the passage of FITC-dextran. However, addition of *P. judaica* pollen extract caused a dose-dependent increase in cell permeability.

To study if the alteration of tight junction components could be responsible for the increase of permeability, we determined by immunofluorescence analysis, the effects of *P.j* pollen extract on claudin-5, a protein belonging to the endothelial cell junctions.

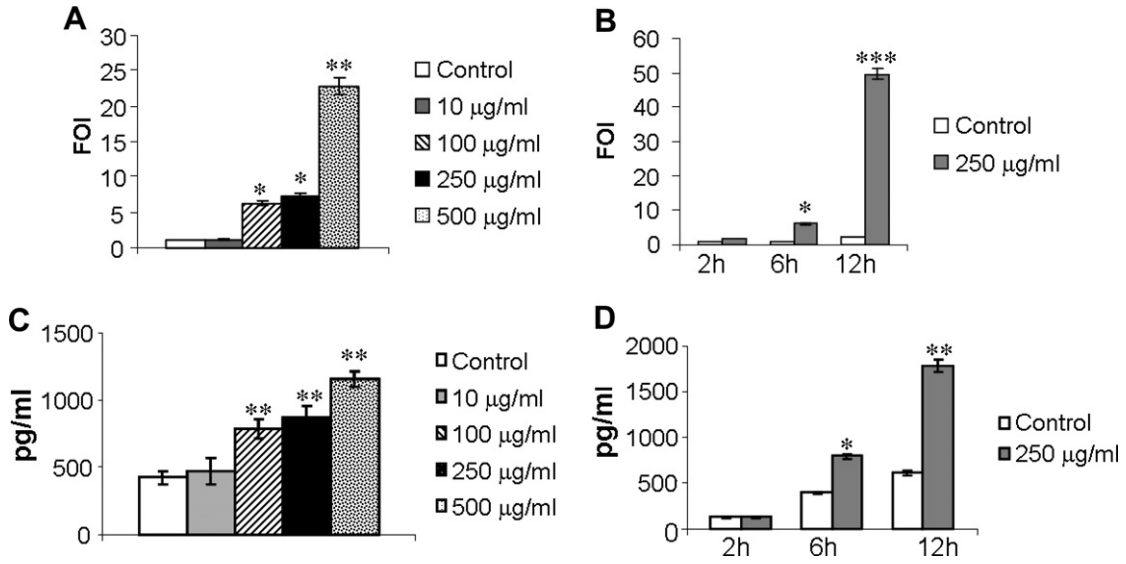

Fig. 1. IL-8 mRNA expression and protein production by HMVEC-L. *P.j* treatment of HMVEC-L causes a dose-dependent (A) and time-dependent (B) stimulation of IL-8 mRNA production. Similarly, secretion of IL-8 cytokine was induced in a dose-dependent (C) and time-dependent (D) way by addition of pollen extract. Asterisks on bars indicate statistically significant values. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.005$, $^{***}p \leqslant 0.001$.

Control cells treated with BSA for 6 h showed continuous peripheral staining for this protein (Fig. 2b). In the treated cells, the tight junction staining decreased in intensity and many areas appeared almost devoid of staining (Fig. 2c). Loss of claudin-5 from the cell

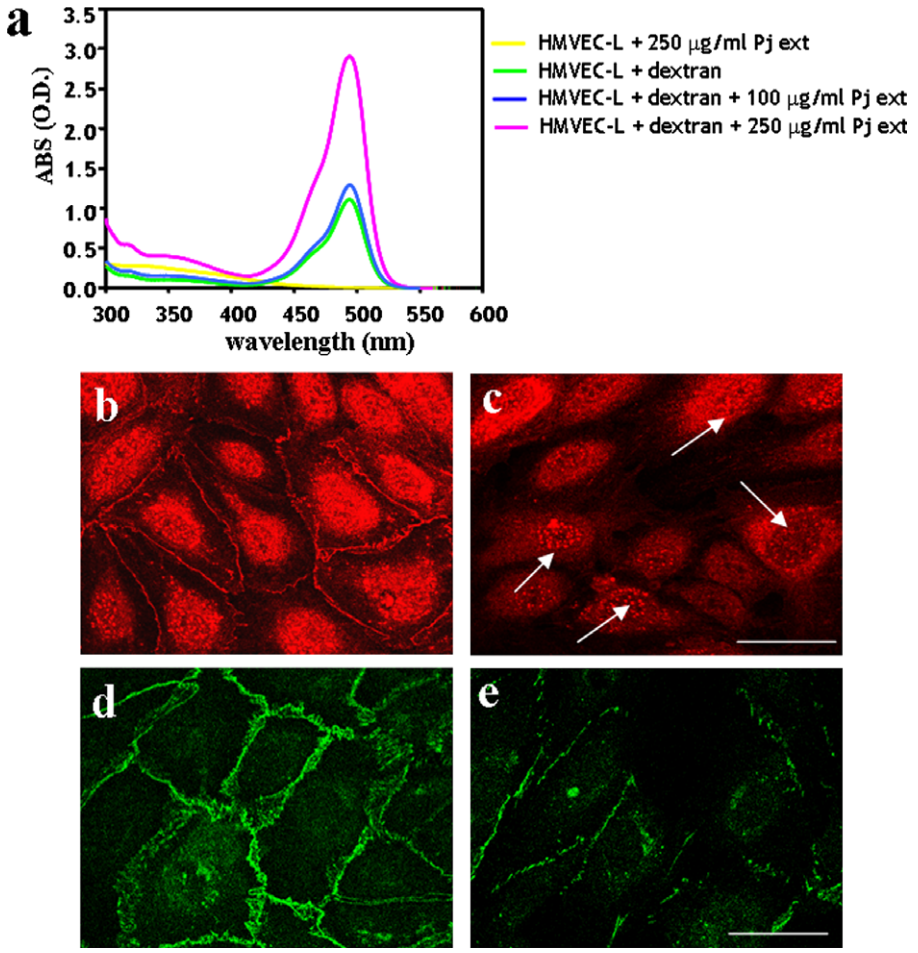

Fig. 2. Permeability measurements and adhesion molecules expression in HMVEC-L. (a) EC permeability to FITC-dextran was stimulated in a dose-dependent manner by treatment with *P.j* extract. Adhesion molecules expression was assessed by immunofluorescence. Decrease of immunostaining for claudin-5 (c) and VE Cadherin (e) expression was revealed after 6 h incubation of HMVEC with 250 μg/ml of *P.j* pollen extract compared to control cells treated with BSA (b and d, respectively). *Bar* = 10 μm. These fields are representative of three independent experiments.

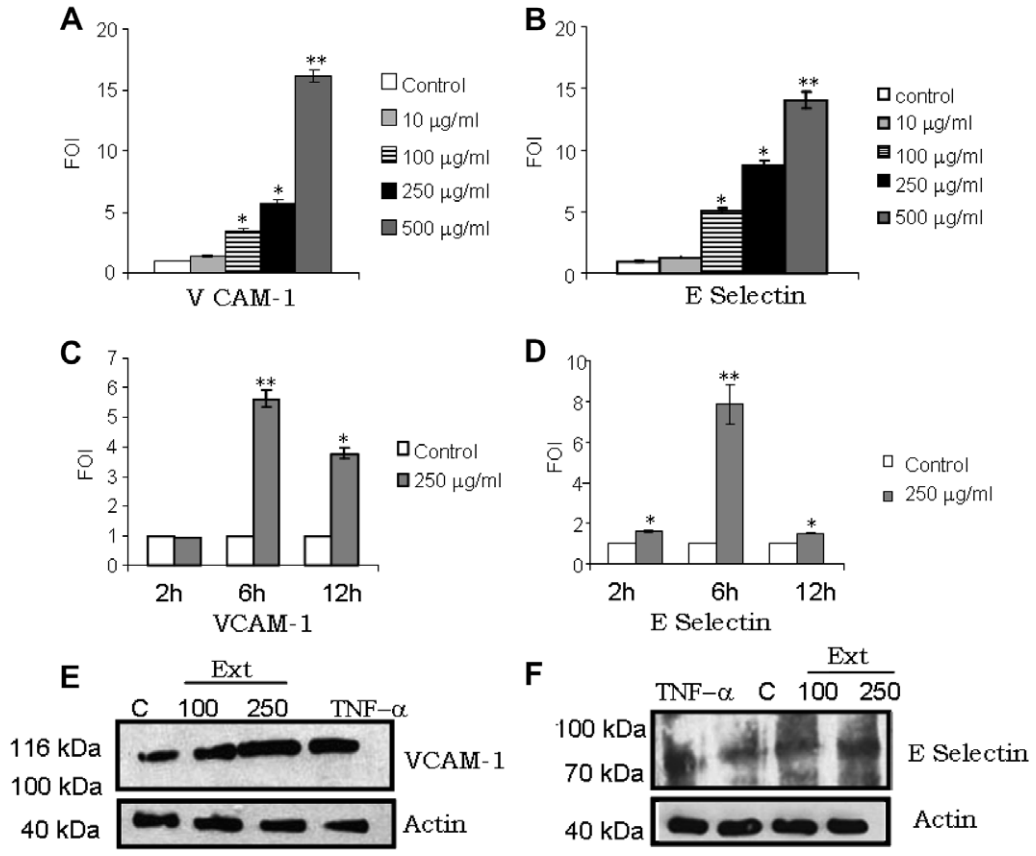

Fig. 3. E-selectin and VCAM-1 mRNA expression and protein production by HMVEC-L. Cells were incubated with *P.j* pollen extract. (A) dose-dependent increase in VCAM-1 mRNA expression; (B) dose-dependent increase in E-selectin mRNA expression; (C) time-dependent increase in VCAM-1 mRNA expression; (D) time-dependent increase in E-selectin mRNA expression. Values are representative of three independent experiments. (E) Representative blot of *P.j* pollen-induced VCAM-1 level (upper panel); (F) representative blot of *P.j* pollen-induced E-selectin level (upper panel). Actin was used as control for loading (E and F lower panels). $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.005$.

membrane was accompanied by the appearance of a granular or punctate staining in the cytoplasm (Fig. 2c, arrows).

VE Cadherin, a transmembrane adhesion molecule belonging to other junction types, was also disrupted in cell–cell location and decreased in total quantity after a 6 h treatment with *P.j* pollen extract (Fig. 2d and e). Overall these results indicate that pollen treatment reduced intercellular adhesion, as a biological consequence of loss of tight junction (claudin-5) and zonulae adherens (VE Cadherin) components; focal degradation of endothelial junctions may be responsible of the increase in vascular permeability and following edema that are observed in allergic reactions.

Parietaria judaica pollen extract up-regulates cell–cell adhesion molecules expression

E-selectin and VCAM-1 are expressed by endothelial cells following activation by cytokines released during the inflammatory process [9].

To determine whether *P. judaica* pollen extract may affect cell–cell adhesion molecule mRNA expression, HMVEC-L were treated with *P.j* extract or vehicle control. As shown in Fig. 3A and B, addition to endothelial monolayer of scalar doses of *P.j* extract caused a dose-dependent and statistical significant increase of VCAM-1 and E-selectin mRNA expression, respectively. We found a time-dependent stimulation of gene expression when 250 μg/ml of pollen extract was tested at different time points. More specifically, pollen stimulation for 6 h caused, respectively, a 5- and 7-fold increase of VCAM-1 and E-selectin mRNA expression (Fig. 3C and D). *P. judaica* pollen extract did not modulate mRNA expression levels of P-selectin and COX-2 in HMVEC-L suggesting a specificity of its action (data not shown). Fig. 4e and f shows that incubation of EC with increasing doses of *P.j* extract induced a dose-dependent augment, respectively, of VCAM-1 and E-selectin protein levels as assayed by immunoblotting.

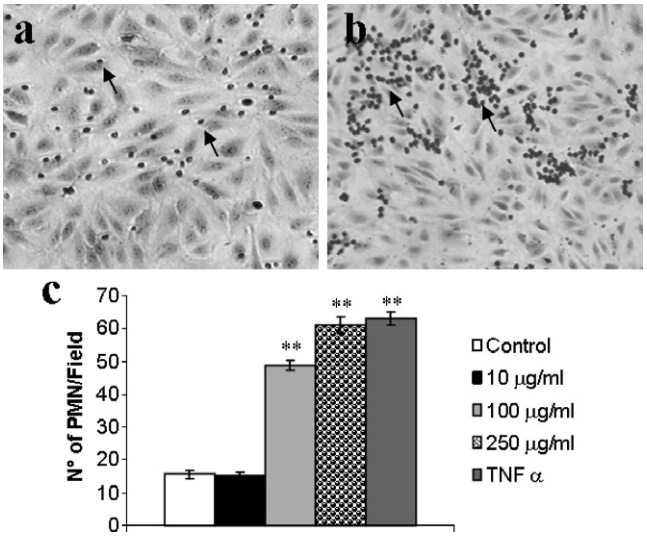

Fig. 4. Adhesion of PMN to EC monolayers following treatment with *P.j* extract. Cells observed at contrast phase microscope. (a) BSA-treated control cells; (b) PMNs adhesion after 6 h from the addition of 250 μg/ml *P.j* pollen extract. (c) Dose-dependent increase of PMN adhered to HMVEC-L monolayer. $^{**}p \leqslant 0.005$.

PMNs-binding assays to HMVEC-L

A hallmark feature of the inflammatory reaction accompanying allergy is the infiltration of PMNs into the airway tissues. We tested the ability of PMNs to adhere to an endothelial monolayer to investigate functional effects of the observed increase of E-selectin and VCAM-1 expression in HMVEC-L treated with pollen extract. Fig. 4b shows the increase of adhesion of granulocytes (arrows) to HMVEC-L after a 6 h treatment with 250 μg/ml of pollen extract. Pollen-stimulated adhesion of PMNs to endothelial cells was comparable to that achieved when granulocytes were plated on TNF-α stimulated HMVEC-L (Fig. 4c).

Proteases inhibitors block the effect of pollen extract on IL-8 and E-selectin expression and revert the pollen-mediated modification in cell morphology

To determine whether the effect of pollen extract on IL-8 and E-selectin expression could be blocked by protease inhibitors, HMVEC-L were treated with *P.j* extract in the presence of aprotinin, bestatin, pepstatin or EDTA. As shown in supplementary Fig. 2, panel A, all compounds except for aprotinin effectively inhibited the *P.j*-mediated stimulation of E-selectin and IL-8 mRNA production. Similarly, inclusion of protease inhibitors during treatment of HMVEC-L with *P.j* pollen resulted in a marked inhibition of morphological changes and cytoskeletal reorganization of endothelial cells (supplementary Fig. 2, panel B). These results suggest that a proteolytic activity in *P. judaica* pollen is required to induce the activated phenotype of endothelial cells.

Discussion

A common event in the inflammatory reaction accompanying the development of allergy is the alteration of endothelial cell component. Endothelial dysfunction is associated with endothelial cell activation, a phenotype of inflammatory tissue that supports leukocyte adhesion and increases vascular permeability via upregulation of surface cell adhesion molecules (e.g. E-selectin and VCAM-1) and the release of cytokines (e.g. IL-8) [10].

Several studies have demonstrated that pollen extracts can alter airways epithelial integrity [11]. We reasoned that if this occurs it is also possible that pollen components might come in contact with cells found in the stromal compartment such as endothelial cells. The main finding of this study is the pollen-mediated involvement of EC in inflammatory allergy. Because the specific response of EC to pollen extracts is still not known, we have used human lung microvascular endothelial cells to investigate the mechanism by which pollen contents could affect endothelial cell function. HMVEC-L may provide a suitable model since these cells retain morphological and functional characteristics of normal human microvascular endothelia.

Proinflammatory cytokines, including IL-8, play a major role in triggering and sustaining the inflammatory response, as found in severe asthma [12]. IL-8 acts on surrounding cells, such as neutrophils, T lymphocytes, and eosinophils and promotes the migration of these cells through endothelial layers.

Initial studies were performed to determine whether addition of *P. judaica* pollen extracts to HMVEC-L monolayer influenced the production and release of interleukin-8. Our results show that *P.j* pollen extracts caused a time- and dose-dependent increase of IL-8 mRNA expression as well as of IL-8 protein levels secreted by endothelial cells. Several studies have shown the production of IL-8 and other cytokines by airway epithelial cells [13] or from mast cells following exposure to bioreactive molecules, including some that are allergenic. In turn, these mediators determine the accumulation of inflammatory cells, such as eosinophils and neutrophils, to perpetuate the chronic allergic inflammation of the airways. In most cases the stimulation of cytokine release was dependent upon proteolytic activities of the molecules added to the cells. Proteases from dust mites and fungi, are able to activate NF-κB, a transcription factor important for the production of IL-8 by epithelial cells [14].

Our study, using a range of proteases inhibitors, confirms the presence of a proteolytic activity that can induce IL-8 expression by endothelial cells. In particular, bestatin, an aminopeptidase inhibitor showed the strongest activity in reducing the pollen-stimulated expression of IL-8 mRNA. This result is in accordance with recent data demonstrating the presence of a novel aminopeptidase in *P. judaica* pollen [3]. By zymographic analyses we have identified, in addition to the aminopeptidase found by Cortes and colleagues, another lytic activity that could be responsible of our observation (Alessandro et al., manuscript in preparation).

The prominent pathophysiologic features of allergic asthma are airway edema and inflammation, with an increased microvascular permeability of bronchial circulation and airway epithelium and movement of inflammatory cells into the lumen of the airway [15]. A more permeable airway endothelium and epithelium would in turn favor further insult from repeated allergen exposure. Loss of endothelial tight junction molecules, reorganization of the endothelial cytoskeleton and alteration of cell shape are responsible of dysfunctions of barrier properties leading to microvascular leakage. Interestingly, Schraufstatter and colleagues have shown that stimulation of microvascular endothelial cells with IL-8 leads to cell retraction and gap formation between adjacent cells; the effect of the cytokine on cytoskeletal rearrangement was caused by receptor-mediated activation of Rho and Rac signaling pathways [16]. Our study demonstrate that exposure of endothelial cells to *P.j* pollen extract causes a reorganization of actin filaments accompanied with a change of cell morphology and increases of *in vitro* endothelial monolayer permeability. These effects could be reversed by incubating the cells with proteases inhibitors thus stressing the possibility of the involvement of proteolytic activity in pollen-mediated EC activation.

Alteration of cellular adhesion structures may explain the morphologic changes (cellular elongation) and the increase in monolayer permeability induced by the *P. judaica* aqueous extract. We have observed, by immunofluorescence analyses, that treatment of EC with *P.j* extract determined an evident reduction of protein levels of tight junction (claudin-5) and zonulae adherens (VE Cadherin) components. Several studies, using *in vitro* models of epithelial barrier, have evidenced that allergens or components of pollen contained enzymatic activity able to degrade cell–cell adhesion molecules thus facilitating allergen delivery [3,17]. A similar scenario may be conceived at the endothelial cell surface where proteases released by pollen can trigger protease-activated receptors (PAR) signaling leading to modification of endothelial functions.

Although the role of PMNs in the pathogenesis of allergic disease remains undefined, studies have reported evidence linking the presence of PMNs with airway damage and dysfunction [18]. A number of studies have evidenced that PMNs express many cytokine and chemokine receptors such as IL-4, GM-CSF, and IL-8, as well as IgE receptors, indicating the ability of these cells to respond to the stimuli characterizing the allergic process [19]. The recruitment of PMNs into tissues is a multistep process that involves selectin-mediated tethering, followed by integrin mediated firm adhesion, transendothelial migration, and directed motility toward the inflammatory stimulus. E-selectin and VCAM-1 are expressed at low levels by normal endothelium but are dramatically increased after stimulation by inflammatory mediators. Our results demonstrated that *in vitro* activation of HMVEC-L monolayers by

P.j pollen under conditions that promote permeability, increases E-selectin and VCAM-1 expression and enhanced PMNs adhesion.

The expression of E-selectin was inhibited by treating the *P.j* extract with pepstatin, EDTA and bestatin while aprotinin was ineffective, suggesting that protease activity partially mediate these effects. These data are consistent with the observation that PAR-induced signaling in endothelial cells may upregulate E-selectin and cell adhesion molecules thus playing a proinflammatory role [20]. The receptors and signal transduction pathways involved in these mechanisms are currently under investigations in our laboratory. In conclusion, we show for the first time that endothelial component of airway unit may be directly altered by pollen contents toward an activated inflammatory status. A more in depth characterization of the pollen constituents responsible of this phenomenon as well of the underlying molecular mechanisms may offer new therapeutic approaches to treat the inflammatory response accompanying allergic reaction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.118.

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