Ultrastructure of Vascular Permeability in Urticaria

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ABSTRACT: **Background:** Few studies have addressed the ultrastructure of vascular permeability in urticaria.

Objectives: To describe the types of endothelial cell organelles involved in vascular permeability in drug-induced acute urticaria (DIAU).

Methods: Seven patients with DIAU were enrolled in the study. Biopsies of urticarial lesions and apparently normal skin were performed. The 14 collected fragments were processed with immunogold electron microscopy using single stains for tryptase and factor XIIIa (FXIIIa) and double immunogold labeling for both tryptase and FXIIIa.

Results: Some sections demonstrated mast cells in the degranulation process, in both anaphylactic and piecemeal degranulation. After double immunogold staining, 10 nm (FXIIIa) and 15 nm (tryptase) gold particles were both present, covering the granules in the mast cells, indicating that both tryptase and FXIIIa were localized within the granules of these cells. Interestingly, we found strong evidence of the presence of caveolae and vesico-vacuolar organelles (VVOs) in the endothelial cells of the biopsies. In addition to these findings, we were able to demonstrate the presence of tryptase and FXIIIa in the endothelial cells, in urticarial lesions and in apparently normal skin.

Conclusions: VVOs are present in the endothelial cells of post-capillary venules in DIAU. This is the first report on the expression of FXIIIa and tryptase in the cytoplasm of endothelial cells in urticaria.

IMAJ 2013; 15: 239–243

KEY WORDS: urticaria, drug adverse reaction, ultrastructure, vascular permeability, caveola

The clinical effects of urticaria are due to local vasodilatation, increased blood flow, and vascular permeability [1]. Lymphatic drainage has a modulating influence on the degree of local edema [1]. The extent of urticaria that develops depends on the severity of capillary leakage and the efficiency of its clearance by the lymphatic system [1]. The main sites of increased vascular permeability are post-capillary lesions [2,3]. It is likely that histamine is the main mediator of this process via the H1 and H2 receptors, but its perpetuation is attributed to additional mediators represented by prostaglandin D2 and leukotriene C4, which are vasodilators that also increase vascular permeability [4,5]. In addition to these findings, vascular endothelial growth factor also increases the permeability of human endothelial cells in vitro [5,6]. Acute vascular hyperpermeability is the rapid increase in vascular permeability that occurs when the microvasculature is acutely exposed to a number of vascular permeabilizing factors, e.g., VEGF-A, histamine, serotonin, and platelet-activating factor [6]. Since some of these agents (e.g., histamine, serotonin, and VEGF-A) are normally stored in tissue mast cells [7-9] they can be released during mast cell degranulation.

More recently, a structure, called a vesico-vacuolar organelle, was discovered in the venular endothelium, offering an alternative to the transendothelial cell route for plasma extravasation in response to permeability factors. VVOs are grape-like clusters consisting of hundreds of uncoated cytoplasmic vesicles and vacuoles, which together comprise an organelle that crosses the venular endothelial cytoplasm from the lumen to the albumen [6]. The purpose of this study was to verify the presence of VVOs in the endothelial cells of biopsies taken from patients with drug-induced acute urticaria.

PATIENTS AND METHODS

Seven female patients with DIAU were enrolled in this study (mean age 38.7 years):

- Case 1: 42 years old, Caucasian, symptomatic for 4 weeks, associated with potassium diclophenac
- Case 2: age 55, Afro-American, symptomatic for 3 weeks, associated with acetylsalicylic acid, dexchlorpheniramine, phenylephrine and caffeine
- Case 3: age 35, Caucasian, symptomatic for 2 weeks, associated with acetylsalicylic acid, dexchlorpheniramine, phenylephrine and caffeine
- Case 4: age 9, Caucasian, symptomatic for 8 days, associated with potassium diclophenac
- Case 5: age 29, Caucasian, symptomatic for 3 days, associated with ferrous sulphate

VEGF = vascular endothelial growth factor
VVO = vesico-vacuolar organelle
DIAU = drug-induced acute urticaria

- Case 6: age 32, Afro-American, symptomatic for 7 days, associated with sodium dipyrone, ranitidine, hyoscine, dimenhydrinate, pyridoxine, ascorbic acid and chlorpheniramine
- Case 7: age 49, Afro-American, symptomatic for 4 weeks, associated with sodium dipyrone and potassium diclophenac

All patients are followed at the Dermatology Outpatient Clinic of the Hospital das Clínicas, São Paulo University, and School of Medicine, Brazil. This study was approved by the Research Ethics Committee of the Sao Paulo University, School of Medicine, and Hospital das Clínicas, and all patients signed an informed consent.

INCLUSION AND EXCLUSION CRITERIA

The patients with DIAU who were enrolled had taken medication between 1 and 14 days prior to the onset of urticaria and had experienced remission after discontinuation of the suspected agent. The patients were followed for at least 3 months after the urticaria resolved. Detailed histories of the patients' eating habits, occupational exposure to allergens, signs or symptoms of active infectious disease, and comorbidities were also investigated, whenever applicable, to rule out other possible causes of urticaria. The diagnosis of DIAU was made according to the criteria established by Moore et al. [10]. For safety and ethical reasons, provocation tests did not seem justified because in all cases the suspected drug was easily avoided or changed to another non-cross-reactive medication.

We excluded from this study pregnant women, individuals younger than 18 years old, and those presenting with any concurrent systemic diseases (cardiac, respiratory, neurological, infectious, or metabolic).

TECHNIQUES

Skin fragments from non-sun-exposed areas of the trunk were obtained using a 6 mm punch under local anesthesia of the urticarial lesion and the perilesional skin (at least 5.0 cm from the biopsied urticaria lesion). The 14 collected fragments were processed for immunoelectron microscopy and fixed in 0.2% glutaraldehyde (Sigma®, No. G-5882, Sigma-Aldrich Corp., St. Louis, MO, USA)/4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), including LR White Resin (London Resin Company Ltd., UK). The sections were examined under transmission electron microscope (JEOL 1010, JEOL Ltd, Tokyo, Japan) in the Department of Pathology of the São Paulo University School of Medicine, and representative photographs were obtained.

IMMUNOELECTRON MICROSCOPY

For immunostaining of factor XIIIa (FXIIIa), the screens containing the sections were incubated with 0.05 M Tris buffered saline at pH 7.2 twice for 5 minutes, and then incubated in TBS with 3% bovine serum albumin at pH 7.2 for 5 minutes. Subsequently, the TBS was drained on Whatman filter paper and the screens were incubated for 30 minutes at room temperature with normal goat serum (dilution 1:30). After the NGS was drained without washing, the screens were incubated at 1:100 dilution with primary polyclonal antibody anti-factor XIII (Calbiochem, USA) in 0.05 M TBS at pH 7.2 plus 3% BSA overnight at 4°C. They were then washed in 0.05 M TBS with 1% BSA plus 0.1% Tween 20 four times for 5 minutes.

Next, the screen sections were incubated in TBS with 1% BSA (pH 8.2) for 30 minutes at room temperature. The screens were then incubated with the secondary antibody, goat anti-rabbit immunoglobulin G gold, labeled with colloidal gold particles (diameter 10 nm, Electron Microscopy Sciences, USA), which was diluted to 1:20 in TBS buffer with 1% BSA at pH 8.2 for 1 hour at room temperature. The screens were then washed in TBS with 0.05% BSA and 0.1% Tween 20 (pH 7.2) four times for 5 minutes, and in TBS containing 0.1% Tween 20 (pH 7.2) twice for 5 minutes. Finally, the screens were washed three times in distilled water for 2 minutes and the sections were fixed in 2% aqueous glutaraldehyde for 10 minutes. The contrast was performed with uranyl acetate and lead citrate, and the grids were observed.

The same procedures employed for FXIIIa immunostaining were applied for the immune disclosure of tryptase. However, in this case, we used a primary antibody, i.e., monoclonal mouse anti-human mast cell tryptase (DAKO, Carpentaria, CA, USA), which was diluted to 1:200, and a secondary antibody, i.e., goat anti-mouse IgG gold, labeled with colloidal gold particles (diameter 15 nm, Electron Microscopy Sciences), which was diluted to 1:20 in TBS buffer with 1% BSA at pH 8.2 for 1 hour at room temperature. All of the solutions were filtered with a Millipore filter (pore size 0.22 μm).

The incubations for double immunostaining were performed in the same manner, adapting the procedures of Craig et al. [11]: the primary antibodies were combined in a mixture of the two antibodies diluted in TBS, plus 3% 0.05 M BSA (1:100 and 1:200, respectively, for FXIIIa and tryptase). Incubation with the secondary antibodies was performed by diluting goat anti-mouse IgG and goat anti-rabbit IgG in 0.05% TBS plus 1% BSA at a dilution of 1:20.

RESULTS

CLINICAL ASPECTS

The urticarial lesions consisted of wheals that were randomly distributed all over the skin, with lesions varying in diameter from 3 mm to 8 cm in all cases; however, the genital and

TBS = Tris buffered saline
NGS = normal goat serum
BSA = bovine serum albumin
IgG = immunoglobulin G

scalp regions were spared. The symptoms were described as "stinging," "burning" or "itching."

IMMUNOELECTRON MICROSCOPY

- **Single IMEM**

Urticarial lesions under IMEM showed slight vascular dilatation, with platelets, red blood cells or leukocytes in the vessel lumen. Mast cells, polymorphonuclear cells, lymphocytes and dendritic cells appeared close to the vessels. Few of the mast cells appeared normal, whereas the others were granularly depleted. In some areas, mast cells, macrophages, lymphocytes and satellite dendritic cells were closely associated [Figure 1]. Some of the macrophages were full with melanin. Variable numbers of eosinophils and polymorphonuclear neutrophils were observed, with lymphocytes and macrophages dominant. The epidermis and basal membrane zone were preserved. Mast cell granules in the cytoplasm and extruded mast cell granules between dermal collagen fibers showing tryptase and FXIIIa were also observed [Figures 2A, B and C].

Vesico-vacuolar organelles. We detected the presence of multiple and sometimes multiple intracytoplasmic vesicular organelles in some sections of the urticarial lesions, as well as the perilesional skin [Figure 3], and these organelles were grouped mainly around the gap junctions of the endothelial cells in the small venules. In addition to these VVOs, we observed caveolae in the capillary endothelial cells [Figure 4].

- **Double IMEM**

We detected FXIIIa by immunolabeling the cytoplasm of the endothelial cells that were grouped in the luminal cytoplasm projections [Figure 3]. We observed FXIIIa in the immunogold-labeled endothelial cells of both the perilesional skin and urticarial lesion sections under immunoelectron transmission microscopy.

After double immunogold staining, 10 nm (FXIIIa) and 15 nm (tryptase) gold particles were present, covering the granules of the mast cells [Figure 2A], indicating that both tryptase and FXIIIa were localized in the granules of these cells. The same observations were made in the sections of both the perilesional skin and the urticarial lesions in all seven cases. Extruded granules in the extracellular matrix showed the same double immunogold staining [Figures 2B and 2C]. The extruded granules were membrane-free and exhibited reduced matrix density. The contrast and delineation of the granule substructure were less than optimal after the immunogold procedure because the sections were not treated with osmium tetroxide to better visualize the gold particles. On double immunogold staining, both 10 nm (FXIIIa) and 15 nm (tryptase) gold particles were present in the endothelial cells [Figure 4].

IMEM = immunoelectron microscopy

Figure 1. Capillary vessel on dermal microvascular unit (apparently normal skin). Note the numerous vesicles in the flattened cytoplasm, called "caveolae." EC = endothelial cell, MC = mast cell, N = nerve (immunoelectron transmission microscopy, IMETM, original magnification, OM, x 5000)

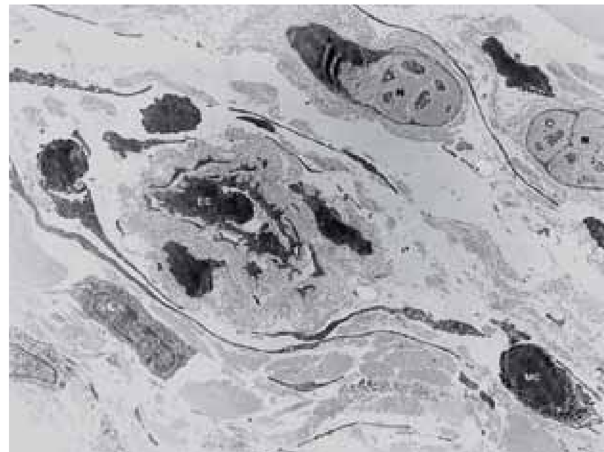
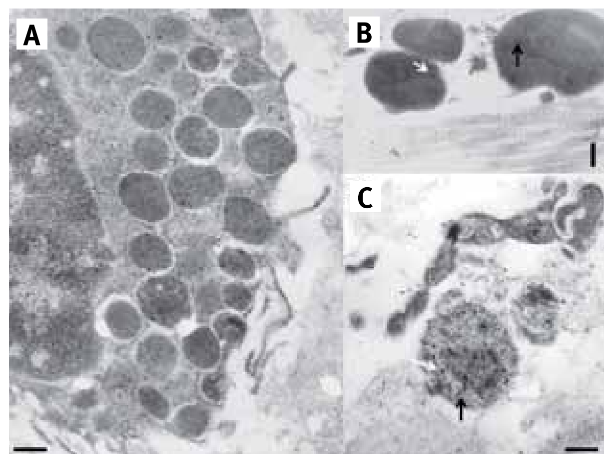


Figure 2. Mast cell granules in urticaria lesion. **[A]** Mast cell showing anaphylactic type of degranulation (double IMETM, OM x 50,000). **[B]** Extruded mast cell granules into dermis; immunolabeled tryptase (white arrow) and immunolabeled FXIIIA (black arrow) (double IMETM, OM 100.000x). **[C]** Extruded mast cell granules dissolving into the dermis; immunolabeled tryptase (white arrow) and immunolabeled FXIIIA (black arrow) (double IMETM, OM x 100,000) (black bar = 200 nm)



DISCUSSION

This study demonstrated, for the first time, the presence of VVOs and vacuoles in biopsies from patients with urticaria, in both urticarial lesions and perilesional skin.

Mast cells usually show perivascular localization, and basophils, given appropriate stimuli, rapidly migrate to the dermis via a transendothelial cell route to reach the perivascular milieu

Figure 3. Vesico-vacuolar organelles (VVOs) in urticaria lesion. **[A]** Scheme of endothelial cell with caveolae aggregation to form VVOs, which transport macromolecules of the vascular lumen to the extravascular milieu. **[B]** Endothelial cell (EC) showing numerous VVOs (white circles) (double IMETM, OM x 12,500) (black bar = 1 μm). **[C]** VVOs in endothelial cell cytoplasm (double IMETM, OM x 100,000) (black bar = 2 nm)

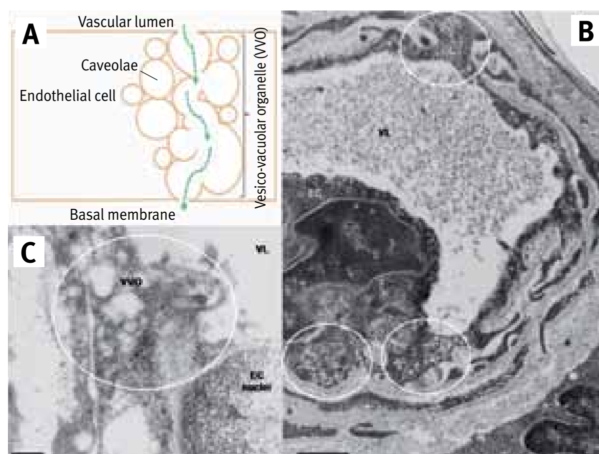
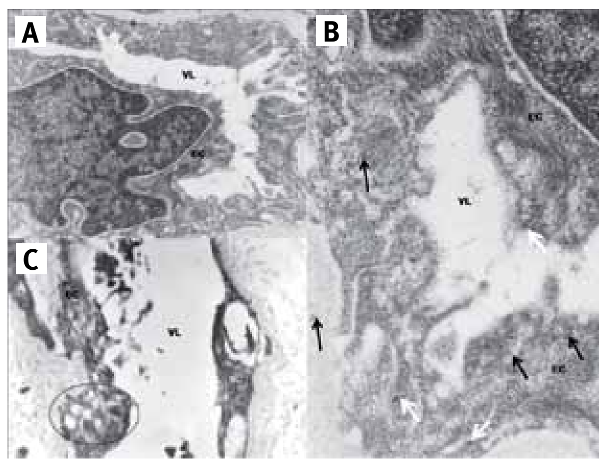


Figure 4. [A] Endothelial cell of capillary vessel in urticaria lesion (double IMETM, OM x 50,000). **[B]** Detail of [A], endothelial cell of capillary vessel; immunolabeled tryptase (white arrow) and immunolabeled FXIIIA (black arrow) (double IMETM, OM 50.000x). **[C]** Cytoplasm of endothelial cell (EC) showing caveolae (black dot circle) (double IMETM, OM x 3500).



[11,12]. VVOs are large collections of vesicles and vacuoles focally distributed in the venular endothelial cytoplasm, and they often show parajunctional localization [12]. In standard electron transmission microscopic sections (~70–80 nm) the number of VVOs can vary from several to hundreds [12,13]. Connections to luminal, abluminal and lateral plasma membranes can be found in these single sections, and in some cases these sections are interconnected as complex transcellular structures [11]. Dvorak and co-authors [13] demonstrated that in transgenic mice that overexpress interleukin-4 in inflamed eyelid tissue, the tissues contain increased numbers of mast cells that exhibit piecemeal degranulation; the authors showed that the micro-vessels also had prominent VVOs. Moreover, they showed that the binding of histamine to the stomata of VVOs in inflammatory eye disease most likely indicated the presence of histamine receptors in this location, which bind histamine, leading to increased permeability and the induction of macromolecular leakage [13].

We observed VVOs in the urticarial biopsies of patients with DIAU and therefore propose that urticaria could provide an in vivo model for studying VVOs in cutaneous inflammation. The VVOs could be present in the small post-capillary venules of patients with urticaria, contributing to the extensive increased vascular permeability observed in urticarial lesions, similar to the transport mechanism of proteins and plasma to the vascular lumen and into the extracellular matrix of the dermis.

Plasma-protein extravasation into normal tissues occurs because the capillary endothelium contains a large number of small vesicles (~70 nm in diameter), which today are termed caveolae [6]. The majority of caveolae are connected to the luminal and abluminal plasma membranes by means of stomata, which generally are closed diaphragms [6]. Nagy et al. [6] postulated that the caveolae cross the capillary endothelium carrying a cargo of plasma fluids and proteins. We were able to demonstrate the presence of caveolae in the capillary endothelium cells of patients with DIAU, in both urticarial lesions and perilesional skin. These findings in both urticaria lesions and perilesional skin might indicate that at the microvasculature level of the skin, the skin appears to be in a state of generalized reactivity due to bioactive mediators, with lesions clinically appearing and disappearing in various areas of the body during the disease evolution. To date, endothelial transcytosis via caveolae is the most studied and best-documented system of transcellular transport [14]. To the best of our knowledge, prior to this study the presence of caveolae in the capillary endothelium cells of patients with urticaria has not been reported.

In addition to caveolae, in physiological states, water and lipophilic solutes (e.g., gases such as O_2 and CO_2) are able to diffuse through endothelial cells; they also pass readily through inter-endothelial cell junctions and through endothelial fenestrae [6]. Fenestrae are markedly narrow zones (diameter 70–150 nm) of microvascular endothelium that can be induced by VEGF-A; they are especially numerous in specialized vascular beds which supply tissues that secrete hormones [6].

Recently, Asero and colleagues [15] found significantly elevated fragment F1+2 and D-dimer plasma levels, well-known markers of coagulation and fibrinolysis activation, in a selected group of patients with severe exacerbation of chronic urticaria. This finding showed that in this subset the coagulation cascade may be activated to promote the production of fibrin as a result of the generation of thrombin. Thrombin could be involved in chronic urticaria, not only because it increases vascular per-

meability but because it can induce mast cell degranulation, a phenomenon that is crucial to the pathophysiology of chronic urticaria [16-18]. The presence of FXIIIa+ in endothelial cells was detected by Schaumburg-Lever et al. [19] in cases of dermatofibroma, basal cell carcinoma and malignant mastocytosis under immunoelectron transmission microscopy but not in immunohistochemistry studies for the same cases. In the present study we observed similar features in the endothelial cells of patients with DIAU. To date, FXIIIa (protransglutaminase) has been attributed some functions, in addition to fibrin stabilization, in the coagulation cascade. Transglutaminases form a large family of intracellular and extracellular enzymes that catalyze the $Ca2^{+}$-dependent post-translational modification of proteins [20]. The A subunit of coagulation, FXIII, has been identified in the cytoplasm of platelets, megakaryocytes, and monocytes and macrophages [20]. Over the last three decades it has become evident that the role of FXIII is not restricted solely to the area of hemostasis, and like other transglutaminases it also promotes the determination of cell shape by cross-linking the actin cytoskeleton with its related proteins by promoting cell adhesion and stabilizing extracellular matrices [21,22].

In this way, the presence of FXIIIa in the endothelial cells of patients with DIAU could be involved in at least two pathophysiologies of urticaria: a) stabilizing fibrin that is extravasated from the vascular lumen, which generates thrombin and thus participates in homeostasis in these events, as previously postulated by Asero et al. [15]; and b) linking actin to the endothelial cell cytoskeleton, possibly playing a role in the opening of the diaphragms of VVO vesicles and vacuoles.

Itoh and team [23] studied the physiology and pathophysiology of tryptase and proteinase-activated receptor-2 in the vascular endothelial barrier function of cultured endothelial cells from human pulmonary artery and bovine aorta samples. These authors concluded that the release of mast cell tryptase and the subsequent activation of endothelial PAR-2 caused gap formation between endothelial cells in acute lung injury induced by radiographic contrast medium.

In summary, we found that endothelial cells in DIAU are involved in vascular hyperpermeability through the vesico-vacuolar organelles. Further study will characterize the possible participation of histamine, thrombin, tryptase and VEGF-A in this process. In addition to these findings, FXIIIa (protransglutaminase) was present in the endothelial cells in our patients, suggesting its possible role in the opening of VVO diaphragms. Tryptase was found in the endothelial cells and could be a contributing factor in endothelial activation and microvascular leakage.

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PAR-2 = proteinase-activated receptor-2

References

1. Sabroe RA, Greaves MW. What is urticaria? Anatomical, physiological, and histological considerations and classification. In: Greaves MW, Kaplan AP, eds. Urticaria and Angioedema. New York: Marcel Dekker Inc., 2004: 1-50.
2. Majno G, Gilmore V, Leventhal M. On the mechanism of vascular leakage caused by histamine-type mediators: a microscopic study in vivo. *Circ Res* 1967; 21: 833-47.
3. Camp RD, Coutts AA, Greaves MW, Kay AB, Walport MJ. Responses of human skin to intradermal injection of leukotrienes C4, D4 and B4. *Br J Pharmacol* 1983; 80: 497-502.
4. Soter NA, Lewis RA, Corey EJ, Austen KF. Local effects of synthetic leukotrienes (LTC4, LTD4, LTE4, and LTB4) in human skin. *J Invest Dermatol* 1983; 80: 115-19.
5. Hippenstiel S, Krüll M, Ikemann A, Risau W, Clauss M, Suttorp N. VEGF induces hyperpermeability by a direct action on endothelial cells. *Am J Physiol* 1998; 274: L678-84.
6. Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF. Vascular permeability, vascular hyperpermeability and angiogenesis. *Angiogenesis* 2008; 11: 109-19.
7. Boesiger J, Tsai M, Maurer M, et al. Mast cells can secrete vascular permeability factor/vascular endothelial cell growth factor and exhibit enhanced release after immunoglobulin E dependent upregulation of Fc epsilon receptor I expression. *J Exp Med* 1998; 188: 1135-45.
8. Galli SJ. Mast cells and basophils. *Curr Opin Hematol* 2000; 7: 32-9.
9. Galli SJ. The Paul Kallos Memorial Lecture. The mast cell: a versatile effector cell for a challenging world. *Int Arch Allergy Immunol* 1997; 113: 14-22.
10. Moore N, Biour M, Paux G, et al. Adverse drug reaction monitoring: doing it the French way. *Lancet* 1985; ii: 1056-8.
11. Craig SS, Schechter NM, Schwartz LB. Ultrastructural analysis of human T and TC mast cells identified by immunoelectron microscopy. *Lab Invest* 1988; 58: 682-9.
12. Dvorak AM. Mast cell-derived mediators of enhanced microvascular permeability, vascular permeability factor/vascular endothelial growth factor, histamine, and serotonin, cause leakage of macromolecules through a new endothelial cell permeability organelle, the vesiculo-vacuolar organelle. In: Dvorak AM, ed. Ultrastructure of Mast Cells and Basophils. Basel: Karger AG, 2005: 185-204.
13. Dvorak AM, Kohn S, Morgan ES, Fox P, Nagy JA, Dvorak HF. The vesiculovacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. *J Leukoc Biol* 1996; 59: 100-15.
14. Predescu SA, Predescu DN, Malik AB. Molecular determinants of endothelial transcytosis and their role in endothelial permeability. *Am J Physiol Lung Cell Mol Physiol* 2007; 293: L823-42.
15. Asero R, Tedeschi A, Riboldi P, Griffini S, Bonanni E, Cugno M. Severe chronic urticaria is associated with elevated plasma levels of D-dimer. *Allergy* 2008; 63: 176-80.
16. Schaeffer RC, Gong F, Bitrick MS, Smith TL. Thrombin and bradykinin initiate discrete endothelial solute permeability mechanisms. *Am J Physiol* 1993; 264: 1798-809.
17. Cirino G, Cicala C, Bucci MR, Sorrentino L, Maranganore JM, Stone SR. Thrombin functions as an inflammatory mediator through activation of its receptors. *J Exp Med* 1996; 183: 821-7.
18. Razin E, Marx G. Thrombin-induced degranulation of cultured bone marrow derived mast cells. *J Immunol* 1984; 133: 3282-5.
19. Schaumburg-Lever G, Gehring B, Kaiserling E. Ultrastructural localization of factor XIIIa. *J Cutan Pathol* 1994; 21: 129-34.
20. Esposito C, Caputo I. Mammalian transglutaminases identification of substrates as a key to physiological function and physiopathological relevance. *FEBS J* 2005; 272: 615-31.
21. Savary A, Szucs S, Balogh I, et al. Possible role of factor XIII subunit A in Fc gamma and complement receptor-mediated phagocytosis. *Cell Immunol* 2004; 228: 81-90.
22. Majno G, Shea JA, Leventhal M. Endothelial contraction induced by histamine-type mediators: an electron microscopic study. *J Cell Biol* 1969; 42: 647-2.
23. Itoh Y, Sendo T, Oishi R. Physiology and pathophysiology of proteinase-activated receptors (PARs): role of tryptase/PAR-2 in vascular endothelial barrier function. *J Pharmacol Sci* 2005; 97: 14-19.