The role of endohelium and vascular system in hereditary angioedema

Doctoral thesis

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1. Introduction

1.1. Hereditary angioedema due to C1-INH deficiency

Hereditary angioedema (HAE) caused by C1-INH deficiency (C1-INH-HAE) is a rare disease of autosomal dominant inheritance, resulting from mutations in the SERPING1 gene on chromosome 11. There are two C1-INH-HAE types: in type I the concentration of C1-INH and also the C1-INH activity is decreased, in type II the concentration of C1-INH is normal (or even increased) but the activity is decreased caused by the fuctionally inactive mutant protein. Eightyfive percent of the patients has C1-INH-HAE type I and 15 % belongs to C1-INH-HAE type II.

The characteristic sympthomes of C1-INH-HAE are recurrent, non-itchy edemas on skin and submucosa accompained by pain, nausea, vomiting and diarrhea. The edematous swelling can appear on the skin of the face, lips, neck, trunk, abdomen, external genitals, or extremities. Abdominal edemas can lead to causless surgery, whereas untreated upper airway edemas can cause asphyxiation. Hypovolemic shock may also be a consequence of the edema formation, it is quite rare, but also needs instant medical intervention. However non life-threatening edemas last approximately 2 to 5 days before resolving spontaneously.

Interestingly, the clinical manifestation of C1-INH-HAE differs even among patients with the same mutations. These differences appeare even in the onset of the first symptoms, in the attack frequency, in the localization and severity of edemas, in the absence or presence of prodromal symptoms and in the necessary of the long therm prophylaxis. The trigger factors can also differ between individual patients and between distinct attacks of the same patient. Lots of trigger factors are known (e.g.: use of estrogen-containing oral contraceptives, foodstuffs, mental stress, pregnancy, infection, physical exertion, menstruation, mechanical trauma, fatigue/exhaustion, weather changes), nonetheless in the high percentage of the attacks the trigger is unidentifiable.

1.2. The function of C1-INH, and its role in the pathomechanism of the C1-INH-HAE

C1-INH belongs to the superfamily of serpins (serin-protease inhibitors). Serpins inhibit serine proteases by an irreversible suicide substrate mechanism when target proteases attack the ‘‘fake’’ substrate conformation of the serpin leading to the formation of a covalent complex between protease and serpin, respectively (‘‘deadly handshake’’).

C1-INH inhibits the serine proteases from the complement-, coagulation-fibrinolytic- and contact system. During the action of these cascades, several proinflammatory-, vasoactive- or permeability enhancing factors are released, and their concentration is even higher in the absence of C1-INH. These factors are anaphylatoxins from the complement cascade (C3a, C4a, C5a), bradykinin the product of the increased enzymatic activity from the coagulation contact- and fibrinolitic systems. In C1-INH-HAE, the elevated bradykinin level is behind the increased vascular permeability and edema formation.

Several cell types produce C1-INH (e.g.: monocytes, microglias, fibroblasts) but most of the C1-INH production occurs in the liver. Endothelial cells are also considered as C1-INH producers but the evidences are not obvious.

1.3. The endothelial cells

Endothelium is a metabolically active cell network playing an important role in many physiological functions. They participate in the control of vasomotor tone, trafficking of cells and nutrients, permeability, adaptive and innate immunity, and neovascularisation as well as in the maintenance of hemostasis. Endothelial cells are typically flat, their thickness varies between 0,1 and 1 μm but their shape and size vary across the vascular tree.

The healthy endothelium is highly active and adaptive system and able to answer to several stimuli. The endothelium is analogous to an input–output

device reacting to the several incoming signal and tries to support the homeostatic balance. Many signaling pathways integrate at the level of endothelium without malfunctioning, however, substantial impacts may lead to imbalance of the system. This occur in C1-INH-HAE when the over-production of BK impairs the permselectivity.

According to the literature, the liver is the main producer of C1-INH. Although endothelial cells may also produce it, this was barely investigated. Since endothelial cells play important role in the pathomechanism of C1-INH-HAE, it would be important to clarify whether they produce C1-INH, and if yes, the C1-INH production of endothelial is comparable with that of the liver.

Investigation of the endothelial function is currently used only in research. The simplest/easiest way to detect markers from serum or plasma of the patients. Such endothelial specific markers are: the concentration and the collagen binding capacity of von Willebrand factor that plays a role in coagulation; the vasoactive peptide, endothelin-1 and the soluble form of E-selectin adhesion molecule.

1.4. Vasoactive peptides

Regulation of the blood pressure is a very complex process consists of the local-, neural- and hormonal regulation systems. The neural regulation acts when fast intervention is required, whereas the hormonal regulation acts mainly in "emergency" state. Endothelial cells and the vasoactive substance released from endothelial cells (vasodilating: e.g: NO, PGI_2, vasoconstricting e.g: ET-1) and also the kallikrein-kinin system and are part of the local regulatory system. Bradykinin (BK) released e.g. during tissue trauma, acts as a vasodilator and also enhances the endothelial permeability. Additionally, neuropeptides (substance P, CGRP, neurokinin A) released from the afferent polymodal neurons during inflammation, and histamine produced by mast cells as well as anaphyilatoxins of complement system also enhances endothelial permeability and vasodilation. Since several part of the local regulatory system (endothelial

cells, kallikrein-kinin system) is affected in C1-INH-HAE, the question arises: other plasma factors involved in blood pressure- and/or vascular permeability regulation, are also affected in C1-INH-HAE?

Such factors may be the adrenomedullin (ADM), which is a vasodilator peptide produced also by endothelial cells and acts as an anti-apoptotic factor for them, the arginine vasopressin (AVP), as a vasoconstrictor and the regulator of water reabsorption in the kidney and the water homeostasis of the body, and atrial natriuretic peptide (ANP), which is also a vasodilatator and has several effects on the endothelial permeability depending on the anatomical location. ANP decreases the edema formation in the lung and in the brain, whereas increases the permeability in other organs.

2. Objective

Without questioning the central role of BK in C1-INH-HAE pathomechanism but considering the diversity of attack frequency, severity and localization a question arises: is there any other factor beside BK that influences the edema formation?

Because the endothelial status is barely investigated in C1-INH-HAE, our goal was to investigate:

- Whether there is any alteration in the endothelial function during C1-INH-HAE attacks, and danazol treatment and smoking have any effect on the levels of endothelial markers?

Endothelial cell function and reaction to external stimuli differ within the body, so we asked:

- Whether there is any similarity amongst several attacks of the same patients regarding the endothelial marker levels?

Besides BK increases the permeability, it also has a strong vasodilating effect. A couple of factors produced by endothelial cells can act against this effect. Hence, we would like to investigate such factors, which: a) are small peptide with vasoactive activity, similarly to BK, b) are produced by endothelial cells and/or affect the endothelial cells, c) act on blood pressure and permeability, d) can be measured in a simple way. Therefore we chose four small vasoactive peptides, endothelin-1, adrenomedullin, arginin vasopressin and atrial natriuretic peptid, and asked the following questions:

- Is there any difference between the vasoactive peptide levels of C1-INH-HAE patients in inter attack period and of control subject?
- Is there any alteration during attack in C1-INH-HAE patients? Is there any correlation between the four vasoactive peptide levels or between vasoactive peptid levels and other vasoregulation affecting factors (e.g: smoking, BMI, age, gender)?

To resolve the differences in individual attacks, we also asked:

- Is there any characteristic pattern in the change of the vasoactive peptide levels in several attacks of the same patients ?

Endothelial C1-INH production were barely investigated and these investigation were made in the field so far were not proper in all detail so we planned to investiget:

- Whether HUVECs produce C1-INH?

All these questions concern novel aspects of the C1-INH-HAE pathomechanism: the role of the endothelial cells and the vasoregulation.

3. Methods

3.1. Study subjects

We studied blood samples obtained between 2006 and 2010, during symptom-free periods of 100 C1-INH-HAE patients followed up at the National Angioedema Centre (from 47 family, 43 males, 57 females, median age: 35,5 years, C1-INH-HAE type I: 89 patients, C1-INH-HAE type II: 11 pateints). During follow-up, the clinical and laboratory findings accumulated from the subjects have been recorded in a database, as well as serum-, plasma-, and DNA banks have been established using the blood samples. In 18 of the 100 patients, blood sampling could be undertaken during oedematous attacks (in all 46 case), before the administration of therapeutic C1-INH concentrate. The age- and sex-matched control group comprised 111 healthy adults (49 males, 62 females, median age: 34,0 years).

3.2. Laboratory methods

Serum and plasma samples were stored at minus 80 °C until processing. C4 levels were measured with radial immunodiffusion, whereas commercial, as well as in-house ELISA-based methods were used to determine the C1-INH concentrations, C1-INH activity, the levels of VWF:Ag, VWF:CBA and soluble E-selectin. The MRproANP, MRproADM, CTproAVP and CTproET-1 levels were measured by a TR-FRET sandwich method (BRAHMS Kryptor). We investigated the proANP peptide clevage with a substrate-cleavage method.

HUVEC cells were prepared from human umbilical vein and were used before the 4th passage. qPCR technique was used to detect the C1-INH mRNA (LightCycler). We used Western blot technique to show C1-INH protein from HUVEC lysates. Affinity purified rabbit anti human C1-INH antibody was used in Western blot.

3.3. Statistical analysis

The statistical analysis was undertaken using the Prism for Windows application (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics Version 20 (IBM Corp.). All statistical tests were two-tailed, and the results were considered statistically significant when p-value was <0,05.

4. Results

4.1. Characterization of the patients and control subjects

We measured the vasoactive peptides leves (ANP, ADM, AVP and ET-1) from plasma sample of 100 C1-INH HAE patients from inter attack phasae and of 111 control subjects. Furthermore we measured the levels of vasoactive peptides and endothelial markers (VWF:Ag, VWF:CBA, sE-sel) from the 46 during attack plasma sample of 18 C1-INH-HAE patients.

4.2. Demonstration of endothelial activation during C1-INH-HAE attacks

We detected endothelial activation by measuring the alteration of the four endothelial specific marker (VWF:Ag, VWF:CBA, sE-sel and ET-1) levels during attacks in C1-INH-HAE patients. During attacks, the level of endothelial markers was significantly increased, which indicates endothelial cell activation.

We also demonstarated that smoking and regular danazol intake affect the changes in the levels of endothelial markers. We found that patients exhibit similar endothelial marker patterns during their attacks, whereas there are differences between the patients regarding changes in endothelial markers.

4.3. Comparing the level of vasoactive peptides from C1-INH-HAE patients and control subjects, and from inter-attack phase and during-attack phase of C1-INH-HAE patients

There was no difference in AVP, ADM and ET-1 levels between inter attack samples of C1-INH-HAE patients and samples of control subjects. Furthermore, we assessed that the presence of cardiovascular risk factors influence the AVP levels, both in patients and controls. In case of ADM and ET-1, the interaction of cardiovascular risks and C1-INH-HAE together affected the peptide levels both in patients and controls. We found in both groups the known correlation between ADM and ages, ANP and ages, ADM and BMI, ANP and gender and also AVP and gender.

We found that ANP levels were significantly lower in inter attack phase of C1-INH-HAE patients than in control subjects.

During attacks, there was a significant increase in the levels of AVP, ADM and ET-1 mostly in patients without cardiovascular risk factors. Moreover, in case of AVP and ADM, we also found a characteristics pattern in the changes of their levels attributed to individual patients, similarly to ET-1 and other endothelial cell markers. There was no alteration in the levels of ANP during attacks.

We found that the levels of vasoactive peptides in inter attack phase of C1-INH-HAE patients and of control subjects, as well as the changes of peptide levels during attacks correlated.

The low ANP level was presumably caused by an increased serin-protease activity due to C1-INH deficiency, which leads to a faster turnover of the proANP protein which is detected by BRAHMS Kryptro method. To confirm this hypotesis, we designed a peptide substrate that contains the potential serin-protease cleavage site of the proANP protein. We found substrate cleavage activity in some samples of patients, and purified serin proteases (trombin, C1s, MASP-1, XII factor) also cleaved the substrate. This substrat cleavage activity was significantly inhibited by C1-INH in most cases.

4.4. Detection of C1-INH in endothelial cells

We demnostrated that HUVEC cells produced both C1-INH mRNA and protein. But we could not detect the C1-INH protein release from the cells so far.

5. Conclusions

1) We found increased levels of VWF:Ag, VWF:CBA, sE-sel and ET-1 during edematous attacks in C1-INH-HAE patients (6.1.1). It suggests the activation of endothelial cells, which can be a response to the to the increased levels of the permeability enhancing factors. Endothelial cell function can also be influenced by other factors, such as smoking or danazol intake. The endothelial cell activation showed an individual pattern, it seems more specific for the pateint than for the certain attacks (6.1.1).

2) In inter attack phase, there was no alteration in the levels of AVP, ADM and ET-1 vasoactive peptides levels of C1-INH-HAE patients and of control subjects (6.1.2). However, during attacks, the levels of these 3 vasoactive peptides increased significantly (6.1.2). The changes in vasoactive peptide levels also showed an individual pattern. The presence of cardiovascular risk factors may moderate the presumable compensatory reaction of vasoactive peptides. The increased levels of vasoactive peptides and endothelial cell factors may well be involved in the termination of edematous attacks by the restitution of the normal vascular permeability, during spontaneus attack termination (6.1.1 and 6.1.2).

3) In contrast to the other vasoactive peptides, the level of ANP was significantly lower in patients (even in inter attack phase) and did not change during attacks (6.1.3). We do not have an explanation yet if it is the consequence of decreased production or increased metabolism of ANP. In a substrate cleavage experiment we found serin protease activity in the sample of some pateints, which may cause the decreased ANP level, but to clarify this hypothesis, further investigation is required. Nonetheless, the lower ANP level can be a new biomarker of C1-INH-HAE. Since decreased ANP level is associated with type 2 diabetes, regular diabetes check-up of the C1-INH-HAE patients is advisable, even if the increased incidence of diabetes has not been observed in C1-INH-HAE. This latter assumption may also suggest that decreased C1-INH activity protects against metabolic diseases (e.g.: type 2

diabetes, insulin resistance) as it is observed in case of cardiovascular diseases (6.1.3).

4) We demosntrated that HUVECs produced C1-INH. C1-INH protein was demonstrated by Western blot technique, and mRNA by qPCR. Based on Westrn blot measurements, endothelial C1-INH production seems to be in a similar order of magnitude as the C1-INH production of the liver. Because this is a rough estimation to clarify the exact amount of endothelial C1-INH, further investigations are needed to clarify the C1-INH distribution amongst the endothelial cells of various tissues (particularly lung and brain endothelial cells) as well as to clarify the triggering factor for the C1-INH release from endothelial cells.

Prophylactic danazol treatment, which has several side-effects, enhances the C1-INH production of the liver. Our results suggest that stimulation of endothelial C1-INH production can be new target in therapy development.

6. List of publications

6.1. Publications related to the thesis

1) Kajdacsi E, Jani PK, Csuka D, Varga LA, Prohaszka Z, Farkas H, et al. Endothelial cell activation during edematous attacks of hereditary angioedema types I and II. J Allergy Clin Immunol. 2014;133(6):1686-91.

IF: 11,476 (2014)

2) Kajdacsi E, Jani PK, Csuka D, Varga L, Prohaszka Z, Farkas H, et al. Novel Vasoregulatory Aspects of Hereditary Angioedema: the Role of Arginine Vasopressin, Adrenomedullin and Endothelin-1. J Clin Immunol. 2016;36(2):160-70.

IF: 3,184* (2014)

3) Kajdacsi E, Varga L, Prohaszka Z, Farkas H, Cervenak L. Atrial natriuretic peptide as a novel biomarker of hereditary angioedema. Clin Immunol. 2016;165:45-6.**

A disszertációhoz kapcsolódó publikációkra vonatkozó összesített impakt faktor: **18,332.**

**The impakt factor of the given year is not available.*

*** The journal IF in 2014 is 3.672, but due to the classification of the publication it is not listed.*

6.2. Other publications

1) Sipiczki M, Kajdacsi E. Jaminaea angkorensis gen. nov., sp. nov., a novel anamorphic fungus containing an S943 nuclear small-subunit rRNA group IB

intron represents a basal branch of Microstromatales. Int J Syst Evol Microbiol. 2009;59(Pt 4):914-20.

IF: 2,113 (2009)

2) Cervenak L, Kajdacsi E, Farkas H. Reply: To PMID 24522092. J Allergy Clin Immunol. 2014;134(1):241-2

3) Jani PK, Kajdacsi E, Megyeri M, Dobo J, Doleschall Z, Futosi K, et al. MASP-1 induces a unique cytokine pattern in endothelial cells: a novel link between complement system and neutrophil granulocytes. PLoS One. 2014;9(1):e87104.

IF: 3,234 (2014)

4) Megyeri M, Jani PK, Kajdacsi E, Dobo J, Schwaner E, Major B, Rigo J Jr, Zavodszky P, Thiel S, Cervenak L, Gal P. Serum MASP-1 in complex with MBL activates endothelial cells. MOLECULAR IMMUNOLOGY 59:(1) pp. 39-45. (2014)

IF: 2,973(2014)

5) Farkas H, Veszeli N, Kajdacsi E, Cervenak L, Varga L. "Nuts and Bolts" of Laboratory Evaluation of Angioedema. Clin Rev Allergy Immunol. 2016.

IF: 5,463* (2014)

6) Jani PK, Schwaner E, Kajdacsi E, Debreczeni ML, Ungai-Salánki R, Dobó J, Doleschall Z, Rigó J Jr, Geiszt M, Szabó B, Gál P, Cervenak L. Complement MASP-1 enhances adhesion between endothelial cells and neutrophils by up-regulating Eselectin expression. Mol Immunol. 2016

IF: 2,973* (2014)

A disszertáció témájától független publikációkra vonatkozó összesített impakt faktor: **16,756.**

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