The importance of lectin pathway activation in hereditary angioedema

Doctoral thesis

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1. Introduction

1.1. The complement lectin pathway

In human, soluble pattern recognition molecules (such as mannose-binding lectin (MBL), ficolin-1, ficolin-2, ficolin-3, and colletin-11) acting in concert with MBL-associated serine proteases (MASP-1, and MASP-2) activate the lectin pathway of the complement system. According to preliminary studies, MASP-3 as well as the non-enzymatic proteins MAP-1, and sMAP are also involved in the regulation of this activation.

The binding of MBL/ficolins to the carbohydrate molecules (mannose, N-acetyl-glucosamine, fucose) present on the surface of bacteria, viruses, and other microorganisms, as well as of dead host cells activates MASP-2, which cleaves the complement components C4 and C2 to produce C3-convertase (C4bC2a). The latter cleaves the C3 protein into C3a and C3b fragments. C3b, by binding to the C3-convertase attached to the surface of the activator modifies the enzyme specificity of the latter, and this creates the classical C5-convertase (C4b2a3b). In this situation, C3b serves as a binding site for C5, which is cleaved by the C5-convertase into C5a and C5b. The former is a soluble, small molecular weight anaphylatoxin; the larger fragment, C5b forms a complex with the complement proteins C6, C7, and C8 (additional cleavage steps do not occur beyond C6). The final step is the binding of the C9 complement proteins, which then polymerize and induce a conformational change. This yields the terminal complement complex (TCC), which spans the entire thickness of the lipid bilayer membrane of the target cells, causing the rapid osmolysis of the latter.

1.2 Hereditary angioedema resulting from the deficiency of the C1-inhibitor

This condition is a form of hereditary angioedema (HAE-C1-INH) caused by the heterozygous deficiency of the C1-inhibitor (C1-INH), which regulates the coagulation, fibrinolytic, and contact systems of the blood, as well as the classical and lectin pathways of the complement system. This abnormality

follows autosomal dominant inheritance, and its aetiology involves mutation of the *C1-INH (SERPING1)* gene, resulting in functional C1-INH deficiency. The characteristic manifestations of the disease include recurrent, non-inflammatory angioedema in the subcutis or the submucosa, often without any prodromal signs. The frequency and severity of these attacks are unpredictable, in both any given patient, and considering the affected members of a family – this may be related either to genetic or to environmental causes. Viral and bacterial infections (e.g. by *Helicobacter pylori*), endocrine effects, mechanical trauma, emotional stress, and certain medicinal products (such as ACE inhibitors, oestrogen-containing contraceptives) contribute to the onset and increase the severity of the oedematous episodes.

1.3 The relationship between the complement system and HAE-C1-INH

The role of the classical complement activation pathway in HAE-C1-INH is well documented, whereas publications on the activation of the MBL-mediated lectin pathway have started to appear only in recent years. Previous studies have shown lower levels of C1-INH, C4, and MASP-2, as well as reduced total activity of the MBL-lectin pathway. However, only little is known yet about the levels of ficolins, associated proteases, and serine-protease-like proteins in this disorder.

We assume that the low activity of the entire lectin pathway may result from the reduced level of MASP-2 and/or low C4 concentration characteristic of HAE-C1-INH. The latter is a diagnostic feature of HAE-C1-INH, and may be consequent to the uncontrolled, spontaneous activation of the classical and/or the lectin pathways in the lack of functional C1-INH. The latter involves the unregulated activation of the C1 complex, as well as of MASP-1 and MASP-2, leading to the enhanced cleavage of C4. In the absence of functional C1-INH, both the classical and the lectin pathway may undergo spontaneous activation (i.e. without induction by any activating agent), which may become excessive through influences by the specific activators of the individual pathways. Only

speculations exist about the cause of the low MASP-2 level seen in patients with HAE-C1-INH. Considering the low incidence of MASP-2 deficiency (occurring in an estimated 0,3% of the population), reduced MASP-2 concentration is much more likely to result from the uncontrolled consumptive mechanisms driven by MBL and ficolins.

DOBÓ *et al* have shown that MASP-1 and MASP-2 both can cleave high molecular weight kininogen similar to plasma kallikrein; however, only MASP-1, but not MASP-2 can release bradykinin. In view of these findings, it appears reasonable to assume that owing to the impaired regulation by C1-INH, the auto-activation of MASP-1 may contribute to the elevation of bradykinin level in HAE-C1-INH. This may occur even in the lack of the activators of the contact systems, through mechanisms not dependent on coagulation factor XII (FXII), or kallikrein. As both MASP-1 and MASP-2 may undergo activation during infections (which are common inducers of oedematous episodes), this FXII- and kallikrein-independent pathway of bradykinin production may have an important role in a proportion of the attacks typical of HAE-C1-INH. C1-INH is a potent, endogenous inhibitor of both the MASP-1, and of the MASP-2 serine proteases, which can activate the lectin pathway through complex formation with MBL and ficolins. In view of the foregoing, we aimed to study the processes involved in the lectin pathway in a disorder, in which functional C1-INH has an essential role.

2. Objective

We intended to answer the following questions by studying a well-characterized population of patients with HAE-C1-INH.

2.1 How does reduced C1-INH activity (typical of HAE-C1-INH) influence the levels of early, as well as of common, native components of the classical and of the lectin pathway?

So far, only two studies have investigated the role of the MBL pathway in HAE-C1-INH; information on the role of the ficolin-lectin pathway in this disorder is yet limited. Based on earlier studies, we presumed that ficolins (being present in a quantity exceeding that of MBL) might mediate an uncontrolled complement activation, which may markedly increase the consumption of C1-INH in patients with HAE-C1-INH. In order to test this assumption, we studied the levels of the components of the classical and lectin pathways both in symptom-free patients, and in healthy controls.

2.2 What is the effect of the deficiency of functional C1-INH on the levels of complexes produced in the classical, lectin, alternative, and terminal pathways?

According to current knowledge, C1-INH is the most important inhibitor of complement (e.g. of the C1-complex, MBL-MASP-2, ficolin-3/MASP-2) complexes within the classical and lectin-induced pathways of the complement system. Therefore, we presumed that the reduced concentration of functional C1-INH influences the levels of complexes generated by the complement cascade. Therefore, our studies focused on analysing the levels of the complexes C1rC1sC1-INH (classical pathway), ficolin-3/MASP-2 (ficolin-lectin pathway), C3bBbP (alternative pathway), and SC5b-9 (terminal pathway) in hereditary angioedema resulting from C1-INH deficiency.

2.3 Does the deficiency of functional C1-inhibitor has any influence on the activity of the early MBL-lectin, or of the ficolin-lectin pathways in symptom-free HAE-C1-INH patients?

Our work group has showed that both the concentration of MASP-2 and the total activity of the MBL-lectin pathway, but not MBL level decrease in HAE-C1-INH patients. Therefore, we studied the deposition of C4 and of TCC – mediated by the MBL/MASP-2 complex, and by ficolin-3, respectively – in HAE-C1-INH patients, and in healthy controls.

2.4 Is there any relationship between the levels of MBL-lectin, or ficolin-lectin pathway components, and the severity of HAE-C1-INH?

According to the findings of an earlier study, the activation level of the MBL-lectin pathway does not influence the severity of symptoms; however, the role of ficolin-2 and ficolin-3 – both being present in larger quantities than MBL – has not yet been studied in HAE-C1-INH. Thus, we aimed to analyse the potential correlation between the components of the ficolin-lectin pathway and the severity of HAE-C1-INH.

2.5 Do the levels of ficolin-lectin pathway parameters change during the edematous attacks of HAE-C1-INH?

The activation of ficolin-bound MASP-1 and MASP-2 may contribute to the consumptive depletion of functional C1-INH present in a low quantity. This would lead to the uncontrolled activation of C1-INH-regulated plasma systems, resulting in oedema formation. According to earlier studies, MASP-1 is involved in the cleavage of high molecular weight kininogen to bradykinin – this observation also suggests a potential role for the lectin pathway in the pathomechanism of HAE-C1-INH. In order to ascertain this role, we studied the levels of the proteins of the ficolin-lectin pathway, as well as the activation of the latter in of HAE-C1-INH patients, both during symptom-free periods, and during oedematous attacks.

3. Methods

3.1. Study subjects

We studied blood samples obtained between 2004 and 2010, during symptom-free periods from 138 HAE-C1-INH patients followed-up at the National Angioedema Centre (60 males, 78 females, median age: 35,3 years). During follow-up, the clinical and laboratory findings accumulated from the subjects have been recorded in a database, as well as a serum, plasma, and DNA bank has been established using the blood samples. In 35 of the 138 patients, blood sampling could be undertaken during oedematous attacks, before the administration of C1-INH concentrate. One hundred and twelve blood samples were analysed altogether; including those obtained during subcutaneous (n=41), upper airway (n=18), and abdominal episodes (n=53) of acute oedema.

The age- and sex-matched control group comprised 104 healthy adults (45 males, 59 females, median age: 52,7 years).

3.2. Laboratory methods

Serum samples were stored at minus 80 °C until processing. Antigenic C1-INH and C4 levels were measured with radial immunodiffusion, whereas commercial, as well as in-house ELISA-based methods were used to determine the concentrations of functional C1-INH, MBL, ficolin-2, ficolin-3, MASP-2, MASP-3, MAP-1, and ficolin-3/MASP-2. The levels of C1rC1sC1-INH, C4a, C4d, C3a, C3bBbP, and SC5b-9 were determined in EDTA plasma, using in-house or commercial ELISA techniques. The inducibility of the activation of the MBL/MASP-2 complex, as well as the volume of ficolin-3-mediated TCC deposition were measured with in-house ELISA methods.

3.3. Statistical analysis

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

4. Results

4.1. The effect of reduced C1-INH activity on the levels of the early and of the common components of the classical and of the lectin pathway

The levels of the early and of the common parameters of the classical and of the lectin pathway were different in HAE-C1-INH patients and in healthy controls. In conformity with the diagnostic criteria of HAE-C1-INH, the levels of antigenic and functional C1-INH were both reduced, along with that of C4, in the patients compared to the controls. Ficolin-2 and MASP-2 levels were lower, but MBL and MASP-3 concentrations were higher in HAE-C1-INH patients than in controls. There were no differences in ficolin-3, and MAP-1 levels.

We found a significant, positive correlation between ficolin-2 and MAP-1, as well as between MASP-3 and MAP-1 levels, both in patients and in controls; however, these relationships were stronger in the patients. Ficolin-3 levels showed a similar, statistically significant, positive correlation with MAP-1, MASP-2, and MASP-3 levels in both study groups. Interestingly, the correlation between ficolin-2 and MASP-3 levels was negative in the controls ($r=-0,4625$, $p<0,0010$), but positive in the patients with HAE-C1-INH ($r=0,3443$, $p=0,0008$).

4.2 The development of a new method for the measurement of the ficolin-3/MASP-2 complex

In order to create the means for the more thorough exploration of the role of the ficolin-lectin pathway, we developed in collaboration with the workgroup of Peter Garred (Rigshospitalet, University Hospital of Copenhagen, Denmark) a new, quantitative, sandwich ELISA based method for the quantitative determination of circulating ficolin-3/MASP-2 complexes (an initiator complex of the ficolin-lectin pathway). Furthermore, this method measures also the extent of the inducibility of the activation of the ficolin-3 mediated lectin pathway, regardless of any potential decrease in the levels of other components involved in the complement cascade. The method is based on a commercially available monoclonal anti-human MASP-2 antibody, which recognises the CCP1/2-SP

fragment of the MASP-2 protein. A biotinylated, anti-human, ficolin-3 antibody (FCN334*Bio) was used as the detection antibody. The level of the ficolin-3/MASP-2 complex (as measured in blood samples from 97 healthy blood donors) correlated significantly with the magnitude of ficolin-3-mediated C4 deposition ($r = 0,671$, $p<0,0001$), as well as with ficolin-3, and MASP-2 levels ($r = 0,2532$, $p=0,0124$ vs. $r= 0,4505$, $p<0,0001$).

4.3 The effect of the reduced C1-INH activity on the levels of the complexes generated during complement activation, as well as on the activity of the individual pathways

The concentration of C1rC1sC1-INH was approx. ten times higher in the samples obtained from symptom-free HAE-C1-INH patients, than in healthy controls ($p<0,0001$), whereas C3bBbP, and SC5b-9 levels were similar in the two groups. Healthy controls had significantly higher levels of the ficolin-3/MASP-2 complex than patients with HAE-C1-INH ($p=0,0005$).

We did not find any differences between the patients and the controls as regards the inducibility of the activation of the MBL/MASP-2 complex ($p=0,9325$). On the other hand, the magnitude of ficolin-3-mediated TCC deposition was significantly lower in symptom-free HAE-C1-INH patients than in healthy controls ($p<0,0001$).

4.4 Relationships between lectin pathway parameters and the severity of HAE-C1-INH

We checked the existence of any relationship between the levels of the individual parameters of the lectin pathway, and of the markers of the severity of HAE-C1-INH. Functional, as well as antigenic C1-INH and C4 levels exhibited the closest, significant correlation with the mean annual number of subcutaneous/abdominal/upper airway oedematous episodes, mean annual attack number, and the mean cumulative dose of C1-INH administered over a year. Ficolin-2 levels exhibited a significant, negative correlation with the number of

abdominal attacks occurring – as well as with the cumulative dose of C1-INH concentrate administered – over a year. Ficolin-3 concentration exhibited a significant, negative correlation with mean annual attack number, the incidence of subcutaneous or upper airway attacks, as well as with the mean cumulative consumption of C1-INH concentrate. Moreover, the levels of the C1rC1sC1-INH complex, as well as the magnitude of ficolin-3-mediated TCC deposition were in positive correlation with all severity markers of HAE-C1-INH.

4.5 Changes in the levels of the parameters of the ficolin-lectin pathway, as well as of the activation products during acute edematous episodes of HAE-C1-INH

Remarkably, the activity of functional C1-INH increased by approx. 50% ($p<0,0009$) during attacks compared to levels measured in the same patients during symptom-free periods, whereas antigenic C1-INH level did not change. Comparing the samples from the same patients revealed a moderate elevation of the levels of the ficolin-3/MASP-2 complex vs. those measured in symtpom-free periods ($p=0,0224$). Ficolin-3-mediated TCC deposition, by contrast decreased markedly during the attacks compared with symptom-free periods ($p=0,0002$).

Although the levels of the parameters reflecting the activation of the classical pathway (C1rC1sC1-INH, C4a, and C4d) were similar during symptom-free periods and during edematous attacks, their levels were significantly different, compared with healthy controls. Furthermore, we observed reduced activation of the complement cascade beyond the C3 cleavage step, in samples obtained during attacks, as evidenced by reduced C3a and C3bBbP levels.

5. Conclusions

1) Our study confirmed the assumption that the classical and the lectin pathways undergo permanent (auto)activation in hereditary angioedema due to the deficiency of the functional C1-inhibitor, even during symptom-free periods. This uncontrolled, continuous activation of the complement may result in lower ficolin-2, MASP-2, and C4 levels than in healthy individuals. On the other hand, the concentration of MASP-3 – another potential, regulatory factor – was elevated in HAE-C1-INH patients. Based on the correlations found among the parameters of the lectin pathway, ficolin-mediated complement activation may be responsible for lower MASP-2 level.

2) As regards the classical and of the lectin pathway in symptom-free HAE-C1-INH patients, the level of C1rC1sC1-INH complex was elevated, whereas that of the ficolin-3/MASP-2 complex – for the determination of which we introduced a new method – was lower than in healthy controls. As the levels of C3bBbP and of SC5b-9 were not higher in symptom-free HAE-C1-INH patients than in controls, complement activation in the lack of C1-INH does not appear to extend beyond the C3 component of the cascade.

3) The inducibility of MBL-MASP-2 activation – for the determination of which we introduced a new method – was similar in symptom-free HAE-C1-INH patients and in healthy controls. However, the extent of ficolin-3-mediated TCC-deposition was significantly lower in symptom-free patients, and this appears to be a consequence of reduced MASP-2 and C4 levels.

4) Our study demonstrated for the first time that the levels of ficolin-lectin pathway parameters correlate with the severity of HAE-C1-INH, whereas there is no relationship between the components of the MBL-lectin pathway and the markers of disease severity. Symptom severity in HAE-C1-INH was

inversely correlated with ficolin-2 and ficolin-3 levels. Therefore, as shown by our results, lower ficolin-2 and ficolin-3 levels are associated with the occurrence of more frequent and severe oedematous episodes. Further studies are necessary to decide, whether low ficolin-2 and ficolin-3 levels represent the underlying cause or rather, a consequence in the pathomechanism of HAE-C1-INH.

5) Our study investigated for the first time the course of complement activation during oedematous attacks in paired blood samples from patients with HAE-C1-INH. We showed that acute oedema formation may be accompanied by the activation of the ficolin-lectin pathway, as indicated by the elevation of the levels of the ficolin-3/MASP-2 complex, as well as by reduced ficolin-3-mediated TCC deposition. Remarkably, functional C1-INH level increased during the oedematous attacks by approx. 50% in comparison to the levels seen during symptom-free periods, and in significant correlation with the level of the ficolin-3/MASP-2 complex. Further studies are necessary to ascertain whether the activation of the ficolin-lectin pathway is the cause of oedematous attacks, or it is just a consequence.

6. List of publications

6.1. Publications related to the thesis

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