MASP-1, a serine protease of complement activates endothelial cells

Statements of the Ph.D. thesis

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Introduction

Several experimental data and theoretical considerations indicates that the ancient coagulation cascade and immune system once belonged to one major proteolytic cascade system. The modern serine proteases have diverged during evolution from this ancient system. Several interconnections of these major proteolytic cascades have been described.

These systems are able to activate inflammation related cells by two different mechanisms: indirectly by releasing peptid fragments (e.g. anaphylatoxins and fibrinopeptides) during the serine-protease cascade activation, or directly. Thrombin - the central component of coagulation - is able to trigger direct cell activation by cleaving protease activated receptors (PARs) on the surface of cells. In addition to this trombin is able to release peptide fragments which also contribute to the inflammatory reaction indirectly.

The complement system is a part of the innate immune system, since it can recognize, label, and eliminate invading pathogens as well as altered host cells. Activation of the complement system can induce and enhance inflammatory reaction. It is well known that activated complement proteases can trigger inflammatory reaction by liberating anaphylatoxins and chemoattractants (e.g. C3a, C5a). These peptide fragments act through G-protein coupled receptors (GPCRs) on the target cells (i.e. leukocytes, endothelial cells). These cells control the inflammatory reaction and clearance of invading pathogens by releasing cytokines and chemokines that attract the effector cells of the immune system.

Mannose-binding lectin-associated serine protease-1 (MASP-1) is the most abundant protease of the lectin pathway, but its exact physiological role had not been fully clarified at the beginning of my work. Since then it has been shown that MASP-1 plays a role in the complement system since it contributes to the initiation of the lectin pathway. MASP-1 is also able to cleave substrates outside of the complement system. It activates factor XIII, which is responsible for crosslinking the fibres of fibrin clot. MASP-1 is also able to cleave fibrinogen β chains resulting fibrinopeptide A and B. These peptides have proinflammatory activity. The crystal structure of the catalytic region of MASP-1 explains its promiscuity: the substrate specificity of MASP-1 is wider compared to other complement proteases. My aim was to investigate the role of MASP-1 in inflammatory reactions.

Aims

During injury we are facing two major challenges: the first is the loss of body fluid, while the other is the infection caused by invading pathogenes.

In the case of damage of blood vessels the coagulation cascade is activated and the fibrin clot is formed which is able to prevent the loss of body fluid. Components of the coagulation cascade also induce inflammatory reactions facilitating wound healing and attract immune cells to the site of injury to destroy the invading pathogens. The coagulation cascade is able to stimulate the cellular elements and induce inflammation by two different manner: by the fragments produced as a byproduct during the activation of the cascade (e. g. bradykinin, fibrinopeptides) in an indirect manner; or by direct cleavage of PARs by thrombin.

In the case of the pathogen invasion – as part of the innate immune system – the complement system is activated. The complement system in addition to immune complexes is able to recognize conserved structures on the surface of pathogens. The complement system - after recognition - labels and eliminates pathogens and altered host structures, and similar to coagulation, induces inflammatory reactions. The fragments that are generated during the activation of the cascade, indirectly activate immune cells and endothelial cells. We hypothesized that the complement system – similar to the coagulation cascade - is not only able to induce inflammation through proteolytic fragments, but also in a direct manner.

MASP-1 was the first member that was discovered among the proteases of the complement lectin pathway; however, its physiological function is unclear. It has a wide substrate specificity compared to the other proteases of complement and these substrates are connected to coagulation and inflammation. The same attributes are true for thrombin – which addition to the known role within coagulation- activates endothelial cells through PARs. The crystal structure of MASP-1 and other evidences refer that MASP-1 is a thrombin-like protease. The similarity between coagulation and complement as well as the evolutionary conserved properties of MASP-1 prompted us to hypothesize that MASP-1 is able to directly activate endothelial cells by cleaving PARs. During my doctoral work I was searching for the answer for the following questions:

- Does MASP-1 have a proinflammatory role similar to thrombin, and is it able to directly activate cells?
- What primary signal transduction pathways are triggered after the activation of endothelial cells?

- Is the proteolytic activity of MASP-1 and the activation of zymogene MASP-1 necessary for cell stimulation?
- What is the mechanism of endothelial cell activation: which receptor triggers the signal transduction pathways?
- Is the full length MASP-1, in complex with its pattern recognition molecule (PRM) able to activate endothelial cells?

We also employed proteomic methods for discovering new substrates of MASP-1. We discovered that MASP-1 is able to contribute to the production of bradykinin. My aim was to investigate whether the bradykinin release is also triggered by MBL-MASP complex from human blood and not only by recombinant MASP-1 fragment.

Methods

During my work I used numerous molecular biology methods. The experiments were performed by recombinantly expressed MASP-1 fragment (CCP1-CCP2-SP). Proteins were produced as inclusion bodies in bacterial expression system; after refolding, purification by chromatography and characterization they were used on mammalian cells. In order to understand the activation of cells in more details I produced two inactive mutant forms of the recombinant protein. The first one (R448Q) cannot be cleaved and it remains in an inactive (zymogene) form, the other (S646A) does not have proteolytic activity.

As an experimental system, we chose cultured human umbilical vein endothelial cells (HUVECs), since these comprise a primary human endothelial cell line modeling the physiological conditions. We followed intracellular Ca^{2+}-mobilization by microscopy in MASP-1-treated HUVECs loaded with a Ca^{2+} sensitive dye (Fluo-4). We detected NFκB tranlocalization by fluorescent staining whereas the p38 MAPK phosphorylation was followed by Western blot. The PAR4 expression was measured by quantitative RT-PCR on mRNA level, and on the protein level it was proven by agonist and Western blot.

The substrate specificity of MASP-1 was determined by using fluorescent labeled peptides representing the protease sensitive region of PARs. Using these analogue substrates I determined the k_{cat} and K_M kinetic constants of the MASP-1 cleavage.

Our experiments were further verified by MBL-MASP complex purified from human plasma. I developed a new purification method for MBL-MASP complex which is suitable for high quantity purification of MASP-1. During purification we determined the MBL and MASP-1 concentration by sandwich assay based on TRIFMA (time-resolved immunofluorometric assay) principle. The characterization of samples was performed by Western blot.

Scientific results and conclusions

During my doctoral work I have gained the following scientific results:

- I have showed that MASP-1, a protease of lectin pathway directly activates endothelial cells and induce proinflammatory reaction. MASP-1 is able to activate Ca^{2+}-signaling, NFκB and p38 MAPK pathways in cultured, human umbilical vein endothelial cells (HUVECs). Activation was initiated by MASP-1 only, the related protease – MASP-2 – had no such effect.
- I have investigated the mechanism of cell activation from two aspects: on one hand I have showed that the lectin pathway initiation is necessary for stimulating the endothelial cells, on the other hand I found the possible receptor of MASP-1 on the surface of endothelial cells.
- The zymogene, inactive mutant of MASP-1 (R448Q) is not able to induce proinflammatory reactions in endothelial cells. By using C1-inh a specific inhibitor of MASP-1 and the proteolytically inactive mutant of MASP-1 (S646A) I proved that the proteolytic activity of MASP-1 is neccesary for the effect of MASP-1.
- I have identified the receptor of MASP-1 on the endothelial cells. Using synthetic peptide substrates representing the protease-sensitive regions of PARs, we were able to show that PAR4 is a target of MASP-1. I also investigated the PAR expression patterns on endothelial cells by qPCR, agonists and antagonists. I showed that HUVECs express functional PAR4 and the amount of membrane-bound intact PAR4 decreases after MASP-1 treatment.
- Since MASP-1 can be found as a dimer in complex with PRMs in physiological situations, I developed a method for purification of high quantity of MBL-MASP complex, and verified that the activation of endothelial cells is also triggered by the complex purified from human plasma.
- We also identified potential new substrates of MASP-1 by proteomic approach and found that MASP-1 - in addition to kallikrein-kinin system – contributes to the production of bradykinin by a new factor XII- and kallikrein- independent mechanism. I further verified the physiological significance of bradykinin production by using MBL-MASP complex purified from human plasma.

The results I obtained during my work point out a new important connection between coagulation, kallikrein-kinin system, complement system and its role in inducing inflammatory reactions. These results unravel an unknown new connection between two evolutionary and functionally connected main cascade system of the blood.

I showed for the first time that MASP-1 is able to directly activate endothelial cells as well as able to cleave high molecular weight kininogen resulting bradykinin release. The produced bradykinin - as a vasoactive mediator in addition to the effect of MASP-1 – is able to activate endothelial cells. In this way MASP-1 is able to trigger and enhance the inflammatory reactions by two manner in endothelial cells.

It has been long known that production of biologically active peptides (anaphylatoxins: C3a and C5a) during complement activation initiates inflammation through binding to receptors on the cell surface (such as leukocytes and endothelial cells). Anaphylatoxins appear at later stages of the complement cascade activation. This newly exposed role of MASP-1 could physiologically be very important for an immediate response upon recognition of danger signals, since MASP-1 is the first component of the lectin pathway that is activated.

Publications related to the thesis

József Dobó, Balázs Major, Katalin A. Kékesi, István Szabó, **Márton Megyeri**, Krishnan Hajela, Gábor Juhász, Péter Závodszky, Péter Gál

Cleavage of Kininogen and Subsequent Bradykinin Release by the Complement Component: Mannose-Binding Lectin-Associated Serine Protease (MASP)-1

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Complement protease MASP-1 activates human endothelial cells: PAR4 activation is a link between complement and endothelial function

J. Immunol. (2009) 183: 3409-3416 IF: 5,646