EFFECTS OF SERINE PROTEASES ON ENDOTHELIAL PERMEABILITY

PhD thesis

Márta Lídia Nagy-Debreczeni

Doctoral School of Theoretical and

Translational Medicine

Semmelweis University

Supervisor: László Cervenak, Ph.D.

Official reviewers: Viktória Jeney, Ph.D.

Andrea Varga, Ph.D.

Head of the Complex Examination Committee:

Zoltán Benyó, M.D., Ph.D., D.Sc.

Members of the Complex Examination Committee:

Noémi Sándor, Ph.D.

Hargita Hegyesi, Ph.D.

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1. INTRODUCTION

Endothelial cells (ECs) constitute the innermost layer of vessel walls and play key roles in several physiological processes, but most importantly, they control both the quality and the quantity of cells and molecules that can cross the endothelial barrier and get to other tissues by a process called permeability regulation. Due to their special position, ECs are directly exposed to, and are immediately affected by the components of blood plasma, therefore it is crucial to understand the mechanisms by which active plasma components modify EC behavior.

Vital processes hemostasis and innate immunity are controlled by plasma enzyme cascades in which soluble serine proteases play an indispensable role. Thrombin, a well-described plasma serine protease, can activate ECs to increase endothelial permeability, however, information is very limited on the cellular effects of other plasma serine proteases. Mannan-binding lectin-associated serine protease 1 (MASP-1) is a key serine protease of complement lectin pathway activation that shows enzymatic similarities with thrombin and is also a natural target of C1-inhibitor, a master regulator of the plasma enzyme cascades. The genetic or acquired deficiency of C1-inhibitor underlies the pathomechanism of some life-threatening edematous conditions, such as hereditary angioedema (HAE), sepsis or Covid-19, therefore it is highly relevant to know whether and how MASP-1 can directly increase endothelial permeability.

As C1-inhibitor controls the activity of numerous other plasma serine proteases yet uninvestigated in cellular systems, there was also a need for a high-throughput assay suitable for the detection of complex

endothelial responses and thereby the screening of plasma serine proteases on endothelial monolayers.

2. OBJECTIVES

We have set the following objectives for our studies:

1) **Investigation of the potential permeability increasing effect of MASP-1 and the underlying molecular mechanisms.**

2) **Development of a label-free screening method for the real-time investigation of endothelial cell behavior.**

3) **Investigation of the effects of plasma serine proteases on the physical properties of endothelial monolayers using the newly developed label-free assay.**

3. RESULTS

3.1 Effect of MASP-1 on endothelial permeability

The potential effect of MASP-1 on endothelial permeability would be of high relevance in edematous conditions characterized by C1-inhibitor deficiency. To clarify the role of MASP-1 we aimed to take a complex approach.

3.1.1 Effect of rMASP-1 treatment on the impedance of the endothelial monolayer

To test whether rMASP-1 has any effect on the barrier function of the endothelial monolayer, we performed real-time impedance measurements using the xCELLigence system. Treating human umbilical vein endothelial cells (HUVECs) with recombinant MASP-1 (rMASP-1) resulted in a prominent decrease of monolayer impedance, similarly to the effects of the well-known permeability increasing agonist, thrombin.

3.1.2 Effect of rMASP-1 treatment on endothelial permeability

For the permeability measurements, we used a modified version of the recently developed XPerT technique (a simple and robust method suitable for the detection of paracellular transport) which we optimized to be suitable for measurements with HUVECs. In these permeability tests, rMASP-1 treatment resulted in significant and dose-dependent increase in endothelial permeability, comparable to the effect of thrombin. To assess the inhibitability of the rMASP-1 triggered hyperpermeability, we used its natural inhibitor C1-inhibitor and also the highly specific, artificial MASP-1 inhibitor, SGMI-1. Both inhibitors could completely block the permeability increasing effect of rMASP-1.

3.1.3 Signaling of the rMASP-1 induced permeability change

We have shown earlier that MASP-1 is able to cleave cell surface protease activated receptors PAR-1, PAR-2 and PAR-4 on HUVECs. Since intracellular Ca^{2+} mobilization is a crucial step of the PAR-mediated permeability increase, we investigated the role of each PAR using PAR

agonists and antagonists. Treating HUVECs with PAR-1 agonist resulted in a considerably large Ca^{2+} release, the PAR-4 agonist had only a mild effect, while the PAR-2 agonist failed to induce any response. Accordingly, only the PAR-1 antagonist pretreatment could significantly dampen the rMASP-1 triggered calcium flux.

Then, we tested the PAR-dependence of the permeability response induced by rMASP-1. The PAR-1 and the PAR-4 agonists could significantly increase endothelial permeability (to a similar extent as rMASP-1), while the PAR-2 agonist was found to be ineffective. However, only the PAR-1 antagonist pretreatment could prevent the permeability response triggered by rMASP-1.

Next, we investigated the role of two major protein kinases of cytoskeletal remodeling – Rho kinase (ROCK) and myosin light chain kinase (MLCK) – in the permeability increasing effect of MASP-1. The permeability of HUVECs pretreated with a synthetic ROCK inhibitor was left unchanged by rMASP-1, while the inhibitor MLCK was ineffective.

3.1.4 Effects of rMASP-1 on the actin cytoskeleton of endothelial cells

We also examined the cytoskeletal changes underlying the rMASP-1-triggered permeability change. Similarly to thrombin, rMASP-1 treatment induced a strong myosin light chain (MLC) phosphorylation and cytoskeletal rearrangement, resulting in the formation of actin stress fibers. All the above-mentioned effects of rMASP-1 could be inhibited by C1-inhibitor and SGMI-1. Again, only the PAR-1 antagonist pretreatment could significantly block the effects of rMASP-1, while antagonists of PAR-2 and

PAR-4 proved ineffective. Furthermore, the ROCK inhibitor could completely prevent rMASP-1 induced cytoskeletal events, while the inhibitor of MLCK failed to block the effect of rMASP-1.

3.1.5 Effects of rMASP-1 on the pattern of molecules important in endothelial cell adhesion

Next, we investigated the effect of rMASP-1 on the pattern of some important EC adhesion molecules. The well-organized and uninterrupted network of adhesion molecules VE-cadherin and PECAM-1, and intracellular adaptor molecule ZO-1 seen around the untreated cells was spectacularly disrupted by the rMASP-1 treatment, which resulted in the formation of paracellular gaps.

3.1.6 Effects of rMASP-1 on the expression of permeability related genes

We also wanted to investigate whether rMASP-1 can influence the expression of permeability related genes in HUVECs as a prolonged effect, therefore, we analyzed the data of our previously performed whole transcriptome microarray analysis. The 2-hour rMASP-1 treatment significantly changed the expression levels of 25 permeability-related genes. An interesting example is that rMASP-1 up-regulated the expression of B2R, the EC surface receptor for bradykinin, which is a very potent edematogenic peptide.

3.2 Investigation of the effects of plasma serine proteases on ECs using a label-free optical biosensor

Seeing the results with MASP-1, we were curious to know whether other plasma serine proteases could also directly affect the physical properties of the endothelium. Therefore, we aimed to develop an optical biosensor-based, high-throughput and label-free assay, suitable for the real-time monitoring of endothelial cell responses to screen a set of plasma serine proteases on endothelial monolayers. For the assay development, we chose the Epic BenchTop platform, an optical biosensor system that uses the resonant waveguide grating (RWG) technology. The system can in situ monitor refractive index changes in an evanescent electromagnetic field, generated in the close proximity (~150 nm) of the surface of the sensor in 96- or 384-well microplates. Being able to convert nanometer scale cellular changes to picometer shifts of the so-called resonant wavelength in a real-time and completely label-free manner, Epic BT can detect practically any type of a cellular response making the system exceptionally suitable for screening purposes.

3.2.1 Biosensor surface modification: optimization of gelatin coatings for cell seeding

The surface of the biosensor was made biocompatible with gelatin coating, which mimics the basal membrane and facilitates EC adhesion. Using several biophysical approaches, our partners optimized the gelatine concentration to create a highly hydrated and homogenous layer, thin enough for the biosensor to “see through” while also has ideal viscoelastic properties providing a stable basis for ECs.

3.2.2 Optimization of the Epic BT-based endothelial label-free biochip (EnLaB) for the label-free investigation of complex EC responses to plasma serine protease treatments

Confluent monolayers of HUVECs were successfully created on the surface of gelatin coated 384-well Epic biosensor microplates (cultured in a humidified incubator). The response curve was not affected by the addition of growth medium to the HUVEC monolayers, but when trypsin treatment was applied and ECs completely detached from the surface, it resulted in a great negative wavelength shift. Thrombin – a serine protease that triggers paracellular gap formation, but not cell detachment – induced a more moderate, but still definite negative wavelength shift.

The effect of thrombin was further investigated in 6 different concentrations and was found to be dose dependent. According to these results, EnLaB appeared to be suitable for testing the effects of other plasma serine proteases on ECs.

3.2.3 Investigating the effects of plasma serine proteases on endothelial monolayers using the EnLaB system

After fine-tuning the system with thrombin, we screened other target plasma serine proteases of C1-inhibitor which can be potential players in the pathomechanism of C1-inhibitor deficiencies but have not yet been investigated thoroughly on endothelial monolayers, such as MASP-2, kallikrein, C1s, C1r and FXII. We also included MASP-3, a serine protease that cannot be blocked by C1-inhibitor.

From the tested recombinant or plasma derived enzymes, rMASP-3, rC1s, and rFXII did not alter EC behavior, but rMASP-2, rC1r and kallikrein treatment resulted in characteristic wavelength shift curves significantly different from the medium control. A negative wavelength shift was induced by rMASP-2 and kallikrein, while rC1r had an opposite effect and caused a positive signal. The effect of all three proteases was dose dependent. Covalent complex formation between rMASP-2, kallikrein and their natural inhibitor, C1-inhibitor completely blocked the effects of these proteases. However, surprisingly, the rC1r retained its cellular effects when applied in complex with C1-inhibitor, although PAGE tests verified that the covalent complex was enzymatically inactive.

3.2.3 Validation of the new hits found with EnLaB using well-established assays

To validate our results obtained using EnLaB, we performed intracellular Ca^{2+} mobilization assays and permeability tests. Treating HUVECs with rMASP-2 and kallikrein induced a Ca^{2+} response similarly to thrombin (which was used as a positive control). The proteases showed no such effects if applied in complex with C1-inhibitor. On the other hand, rC1r could not trigger the mobilization of intracellular Ca^{2+}. We chose rMASP-3 to represent the proteases that did not alter the wavelength shift curve in the Epic BT system, and consistently with those results, it failed to trigger a Ca^{2+} signal. In the permeability tests, rMASP-2, rC1r and kallikrein were all able to increase paracellular permeability by the triggering of adhesion molecule VE-cadherin disappearance from adherent junctions leading to the disruption of EC-EC

adhesion. In accordance with the Epic BT results, rMASP-2 and kallikrein could be blocked by C1-inhibitor, while the effect of rC1r was again found to be insensitive to its natural inhibitor; moreover, the rC1r/C1-inhibitor complex induced an even greater monolayer permeability than rC1r alone. Another interesting observation was that rC1r (applied alone or in complex with C1-inhibitor) caused the total, homogenous loss of VE-cadherin from the surface of the cells, while the other proteases triggered the disappearance of this adhesion molecule only in the close proximity of the paracellular gaps. We repeated these experiments also with the plasma derived, full-length form of C1r (pC1r), which behaved similarly to the recombinant version in every respect.

4. CONCLUSIONS

During my doctoral studies, I have contributed to the improvement of a permeability test and the development of an optical biosensor based cellular assay. With these novel approaches, we revealed hitherto unknown cellular functions of plasma serine proteases MASP-1, MASP-2, C1r and kallikrein. All these proteases were found to disrupt the endothelial barrier by the disintegration of adhesion junctions, which lead to an increased monolayer permeability. In the case of MASP-1, we have successfully identified some of the key players in the most important steps of the molecular mechanism of permeability induction. The directions are also clear for future studies, as the exact molecular events mediating the endothelial effects of MASP-2, C1r and kallikrein are waiting to be explored.

Our findings also have pathophysiological implications. Normally, all the newly identified permeability increasing serine proteases are controlled by the same serpin, C1-inhibitor, therefore in HAE, where C1-inhibitor is deficient, these proteases may contribute to the onset, the worsening or the sustainment of edematous attacks. Moreover, these serine proteases may also play a role in other life-threatening edematous conditions such as sepsis, ischemia-reperfusion injury or Covid-19, and once their exact role is clarified, they can potentially become targets of future drug development. Finally, we have also successfully worked out a very promising, easy-to-use, label-free assay, EnLaB that can later be used for the high-throughput screening of other subsets of plasma components or even drug candidates on endothelial as well as epithelial monolayers.

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