Investigation of MASP-1-induced proinflammatory response and especially adhesion features in endothelial cells

Doctoral Thesis

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1. INTRODUCTION

The complement system functions based on the cascade like action of serine proteases, which is an important part of natural immunity; however, through its many functions, represents a link between innate and adaptive immunity. Depending on the triggering agent, the complement system can be activated in three ways: the classical, the lectin, and the alternative pathways. These pathways result in C3 activation as the central phase of the reaction and can lead to the formation of opsonins, anaphylatoxins and membrane attack complexes (MAC). One of the serine proteases of the complement lectin pathway, the mannan-binding lectin-associated serine protease 1 (MASP-1) functions far beyond the activation of the complement system. Activated MASP-1 is able to directly activate endothelial cells, shifting their phenotype towards a pro-inflammatory direction. Furthermore, endothelial cells are not only responsive to inflammatory cytokines and various microbial macromolecules, but anaphylatoxins produced during complement system activation also lead to pro-inflammatory phenotype.

Endothelial cells are highly versatile in their function, they can regulate the transport of soluble metabolites for tissues, the

barrier function, the vascular tone, and the growth of new blood vessels; they form thrombo-resistant and anti-coagulant surface, and activate or inactivate many vasoactive hormones. In addition, they are involved in the regulation of immunological processes by affecting almost every member of the humoral and cellular immune system. This regulation is mainly accomplished by their cytokine-, chemokine production, lipid mediators, NO production, and surface adhesion molecule pattern. Due to their anatomical localization, they are directly exposed to the effects of complement system activation products, therefore they express high amount of various complement component specific receptors as well as complement regulatory proteins on their surface. The complement system plays a key role in inflammatory processes, molecules released during its activation have an important effect on the role of endothelial cells in the regulation of inflammation.

Inflammation is an essential defense mechanism in the body, in which endothelial cells and leukocytes play a critical role. Endothelial cells produce anti- and proinflammatory mediators, which are involved in the regulation of inflammatory processes. The endothelial cells respond to microbial macromolecules (e.g. LPS, fMLP) and pro-inflammatory cytokines (e.g. IL-1β, TNFα),

the expression pattern of their adhesion molecules are altered, their permeability is elevated and the production of pro-inflammatory cytokines and chemokines is increased, allowing leukocytes to migrate to the site of injury or infection.
The subject of my dissertation is the transcriptomic assessment of the role of MASP-1 in inflammation and to investigate the role of MASP-1 induced endothelial cells in inflammation, focusing on adhesion features.

2. OBJECTIVES

Knowing that MASP-1 shifts the phenotype of endothelial cells in a proinflammatory direction, which results in increased neutrophil adhesion to endothelial cells by increasing expression of the E-selectin adhesion molecule, we wanted to examine the details of this intercellular adhesion by developing a method, which reliably quantifies cell adhesion between endothelial cells and neutrophil granulocytes. In the light of that, in the first part of my work we wanted to answer the following questions:

- Can increased endothelial cell-neutrophil granulocyte adhesion be explained by MASP-1-induced E-selectin expression?

- What kind of adhesion forces are acting between endothelial cells and neutrophil granulocytes, measured by a new computer controlled micropipette method?
- How strong is the MASP-1 induced increase in adhesion, compared to known endothelial cell activators?
- Which signaling pathways play a role in MASP-1-induced increased adhesion?

Since the regulation of inflammation extends far beyond the cytokine and adhesion molecules we have investigated so far, we wondered whether MASP-1 could alter other inflammatory parameters of endothelial cells. Therefore, the second part of my work is focused on the transcriptomic study of the proinflammatory effect of MASP-1 on endothelial cells.

Thus, in the second part, we asked the followings:

- What percentage of genes are involved in inflammatory processes and which categories of genes are affected by MASP-1?
- Which signaling pathways play a major role in the background of MASP-1 regulated inflammation-related genes?

- How similar is the effect of MASP-1 to that of other known activators?

3. METHODS

3.1. Preparation and culturing of HUVECs; PLB-985 neutrophil model cells and MASP-1 protein used in our experiments

Our measurements were performed on human umbilical vein endothelial cells (HUVECs). Cells were harvested from the vein by collagenase digestion, then plated in gelatin-precoated cell culture flasks. The culture was kept in previously optimized medium until confluency under continuous microscopic control and used up to the third passage during our experiments.

We used recombinant MASP-1 protein expressed in bacterial expression system.

For our adhesion assays, to avoid the heterogeneity of primary cells, we used a DMSO-differentiated PLB-985 (dPLB-985) acute myeloid leukemia cell line, which is a well-known and suitable model of neutrophils.

3.2. Measurement of differentiated PLB-985 cells adhesion to HUVECs

To assess the adhesion of dPLB-985 cells adhered to HUVEC cells, a wash-off method was used, whereas a computer controlled micropipette method was used to quantify the adhesion forces.

3.2.1. Wash-off method with multichannel pipette

Fluorescent dye-labeled PLB-985 or dPLB-985 cell suspension was co-incubated with HUVECs, which had previously been treated with rMASP-1 or other activators or left untreated. Then, total number of dPLB-985 cells was determined with a fluorescence plate reader. Thereafter, the plate was washed twice vigorously with a multichannel pipette, and the fluorescence of bound cells was monitored again. The number of bound cells normalized with the initial number of cells was calculated after three independent measurements.

3.2.2. Computer controlled micropipette method

A computer controlled micropipette was used to quantify the adhesion force between the variously pre-treated HUVECs and dPLB-985 cells. Attached dPLB-985 cells were scanned and

recognized using the CellSorter software. The adhesion force was measured with a glass micropipette. The micropipette was automatically positioned to 100 selected cells one by one, in order to try to pick them up. Repeating this cycle (i.e. the positioning and pick-up process) each time on the same path with an increased vacuum, the adhesion force of cells could be precisely measured. The number of the cells was counted before and after each cycle, and the ratio of adherent to originally selected cells was calculated.

3.3. Microarray analysis

Agilent Two-color Microarray Based Gene Expression Analysis was used for our gene expression studies. Equal amounts of Cy3-labeled (untreated) and Cy5-labeled (treated) cRNA from samples were simultaneously co-hybridized onto the arrayed oligonucleotides on the same G3 Human Gene Expression 8 × 60 K v2 Microarrayslide. The hybridized microarrays were then washed according to manufacturer's instructions and scanned. We used fold change (FC) values generated by the software from the array data as the ratio of Lowess normalized, background subtracted Cy5/Cy3 signals if they passed the built-in QC analysis of the software. To confirm

gene expression changes measured by the microarray, quantitative gene expression was analyzed using quantitative real-time PCR (qPCR).

4. RESULTS

4.1. Measurement of PLB-985 and dPLB-985 cell adhesion to E-selectin coated plates

In our adhesion assays, we demonstrated that MASP-1-induced HUVEC cells have increased the expression of E-selectin adhesion molecules, which leads to the adhesion of dPLB cells. To further confirm by functional test that differentiated PLB-985 cells are good models of neutrophil granulocytes and to test their ability to adhere to HUVEC cells, we assessed their adhesive capacity to a plate, previously coated with recombinant E-selectin. The adhesion of dPLB985 cells to the coated plate was dose-dependent, and treatment with E-selectin in a concentration as low as 5 g/mL resulted in significant adhesion compared to the uncoated controls. We did not observe similar dose-dependent adhesion of non-differentiated PLB cells to E-selectin coated plates.

4.2. Assessing dPLB-985 cell adhesion to HUVECs

4.2.1. Time dependence of E-selectin expression induced by rMASP-1

To assess the influence of rMASP-1 kinetics on the expression of adhesion molecules at protein level, kinetic measurements were performed. rMASP-1 induced significant expression of E-selectin as early as at 3 h, but the maximum effect was observed at 6 h. E-selectin expression declined until 24 h post treatment.

4.2.2. Measurement of adhesion between dPLB-985 cells and endothelial cells by wash-off method

In our work, a wash-off method with multichannel pipette was used to investigate the adhesion between dPLB-985 cells and endothelial cells. Adhesion pattern of dPLB-985 cells binding to HUVEC cells is very similar to that of E-selectin expression kinetics. We observed that rMASP-1 treatment increased dPLB-985 cell adhesion to HUVECs at 6 h, but not at 24 h. Both the kinetics and the volume of this effect were similar for rMASP-1 and for thrombin. In contrast, TNFalpha induced enhanced adhesion to HUVECs at 6 h, but adherence was even greater by 24 h. The adhesion between dPLB-985 cells and

rMASP-1-induced HUVECs could be reduced to the level of untreated HUVECs by pre-incubating the dPLB-985 cells with soluble recombinant E-selectin.

4.2.3. Analysis of signaling pathways in MASP-1 induced adhesion

Only the p38-MAPK inhibitor was able to block this adhesion, whereas JNK, NFκB, and ERK 1/2 pathway inhibitors had no blocking effect.

4.2.4. Measurement of the adhesion force between dPLB-985 cells and endothelial cells with a computer controlled micropipette method

We used a computer controlled micropipette method to quantify the adhesion force between dPLB-985 cells and rMASP-1-treated HUVECs. The attached dPLB-985 cells were probed (i.e., by trying to pick them up) with the automated micropipette. The hydrodynamic lifting force, acting on the targeted single cells, was increased in each subsequent cycle of the measurement. More dPLB-985 cells stayed attached to rMASP-1-treated endothelial cells than to non-treated controls at higher detaching forces. We observed the same effect after

thrombin treatment. Induction with TNFalpha for 24 h, used as positive control, was the most potent in enhancing adhesion.

4.3. Identification of rMASP-1-induced inflammation related genes.

We utilized Agilent microarray to analyze the effects of rMASP-1 in HUVECs, on a set of 884 IR genes. Gene Set Enrichment Analysis showed an overall activation of inflammation-related genes in response to rMASP-1. rMASP-1 treatment up- and down-regulated 19 and 11 IR genes, respectively.

4.4. Biological function and signaling pathway analysis of rMASP-1-induced inflammation-related genes.

rMASP-1 was found to regulate a diverse range of IR genes, including adhesion molecules, cytokines and growth factors, and genes involved in signal transduction. We have previously described that MASP-1 induced E-Selectin, IL-6 and IL-8 expression in HUVECs, which predominantly requires the involvement of p38-MAPK and NFκB signaling pathways. Thus, we assessed the involvement of these pathways in the regulation of IR genes. Both p38-MAPK inhibitor and NFκB inhibitor

efficiently suppressed the effect of rMASP-1. p38-MAPK inhibitor suppressed the 83% of the IR genes regulated by MASP-1, whereas NFκB inhibitor could block the 40% of these IR genes.

4.5. Comparing the effects of rMASP-1 and other pro-inflammatory factors on the IR gene expression in HUVECs.

The signaling pathways, cytokines and adhesion molecules induced by the most investigated factors that provide pro-inflammatory signals to endothelial cells (such as TNFα, thrombin, histamine, or LPS) are well characterized. Therefore, we aimed to assess whether MASP-1 can induce similar pattern of gene expression as the above-mentioned factors. The majority of the rMASP-1 altered 30 IR genes (19 up- and 11 down-regulated) were co-regulated by TNFα, thrombin, histamine, and/or LPS (10, 13, 12, 15 up-regulated IR genes and 1, 4, 2, 8 down-regulated IR genes, respectively). Interestingly, we found considerably more rMASP-1 up-regulated genes, which were co-induced by all the other activators (7/19), than down-regulated genes (1/11).

5. CONCLUSIONS

The dPLB-985 cells could bind to E-selectin, whereas non-differentiated PLB-985 could not, which proves that differentiation involves significant changes in adhesion features. This change in adhesion molecules supports the adherence of dPLB-985 cells to HUVECs, highlighting the crucial role of MASP-1-induced E-selectin in the adhesion of endothelial cells and neutrophils.

We demonstrated the importance of the p38-MAPK pathway in the background of E-selectin mediated adhesion between HUVECs and dPLB-985 cells.

The strength of adhesion between MASP-1-activated endothelial cells and dPLB-985 cells was quantified using a computer controlled micropipette method, and found to be similar to that of thrombin-treated HUVECs.

The results of the MASP-1 induced inflammatory pattern and the adhesion force measurements suggest, that MASP-1 activated via the complement lectin pathway, in addition to cleaving certain elements of the complement system, also plays an important role in the involvement of neutrophil granulocytes in the antimicrobial response through endothelial cells. This relationship between the

complement system and neutrophil granulocytes may be important in synchronization of the first line defense mechanisms, the antimicrobial neutrophil response, and the complement mediated immune response. To investigate how and which inflammation-related genes expression is altered by MASP-1 in endothelial cells, we utilized microarray gene expression technique. By Gene Set Enrichment Analysis we found that our IR gene set is more intensively regulated by rMASP-1 than the non-IR genes. This regulation affects all processes of inflammation, regarding the function of genes, MASP-1 altered IR genes cover all sub-processes of inflammation. We have shown that the proportion of chemokine genes among the genes regulated by MASP-1 is high, which also confirms the relationship between the complement system and the neutrophil granulocytes, resulting in the recruitment of neutrophil granulocytes and thus their adhesion and transmigration. In MASP-1 induced endothelial cells, the activation of p38-MAPK and NFκB pathways, as well as expression of adhesion molecules and cytokines, results in the formation of a characteristic inflammatory phenotype of endothelial cells. Transcriptomic analysis also confirmed that the p38-MAPK and NFκB pathways directly contribute to the inflammatory activation of endothelial

cells. The effect of MASP-1 on IR genes overlaps to some extent with the effect of the best known endothelial cell activators (TNFα, thrombin, histamine and LPS). By regulating different sets of genes, besides a general pattern of inflammatory responses, MASP-1 may accomplish unique features in some inflammatory processes.

The profile of MASP-1 suggests that MASP-1 is an inflammation triggering enzyme with partially unique characteristics.

6. LIST OF PERSONAL PUBLICATIONS

6.1. Publications related to the dissertation

1) Jani PK*, Schwaner E*, Kajdácsi E, Debreczeni ML, Ungai-Salánki R, Dobó J, Doleschall Z, Rigó J Jr, Geiszt M, Szabó B, Gál P, Cervenak L, (2016) Complement MASP-1 enhances adhesion between endothelial cells and neutrophils by up-regulating E-selectin expression. MOLECULAR IMMUNOLOGY 2016 Jul;75:38-47.
   * Shared lead authorship

   IF: 3.236

2) Schwaner E, Németh Z, Jani PK, Kajdácsi E, Debreczeni ML, Doleschall Z, Dobó J, Gál P, Rigó J, András K, Hegedűs T, Cervenak L, (2017) Transcriptome analysis of inflammation-related gene expression in endothelial cells activated by complement MASP-1. SCIENTIFIC REPORTS 2017 Sep 5;7(1):10462.

   IF: 4.122

Cumulated impact factors of the publications, related to the dissertation: 7.358

6.2. Other publications

1) Megyeri M, Jani PK, Kajdácsi E, Dobó J, Schwaner E, Major B, Rigó J Jr, Závodszky P, Thiel S, Cervenak L, Gál P, (2014) Serum MASP-1 in complex with MBL activates endothelial cells. MOLECULAR IMMUNOLOGY 2014 May;59(1):39-45.

IF: 2.973

2) Debreczeni ML, Németh Z, Kajdácsi E, Schwaner E, Makó V, Masszi A, Doleschall Z, Rigó J, Walter FR, Deli MA, Pál G, Dobó J, Gál P, Cervenak L, (2019) MASP-1 Increases Endothelial Permeability. FRONTIERS IN IMMUNOLOGY 2019 May 3;10:991.

IF: 4.716

Cumulated impact factors of the publications, unrelated to the dissertation: 7.689