

PROINFLAMMATORY ACTIVATION OF ENDOTHELIAL CELLS

Ph.D. Thesis

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1. INTRODUCTION

The inflammatory response of endothelial cells is of critical importance in various diseases, such as (septic or anaphylactic) shock, thrombosis, atherosclerosis and chronic venous insufficiency. Controlling inflammation at the level of endothelial cells may efficiently retard or reverse the pathogenetic process; therefore, understanding the differences among the signaling pathways of inflammatory factors is indispensable. Inflammatory signals from exogenous and endogenous sources may stimulate endothelial cells to escalate inflammation through the production of cytokines/chemokines (IL-1 β , TNF α , IL-6, IL-8, MCP-1, etc.) and adhesion molecules (ICAM-1, VCAM-1, E-Selectin).

The three most cited factors that provide inflammatory signals to endothelial cells are lipopolysaccharide (LPS), tumor-necrosis factor- α (TNF α), and interleukin-1- β (IL-1 β).

All these three factors use the nuclear-factor- κ -B (NF κ B) signaling pathway to induce or shut off several genes with NF κ B-binding sites; activation of p38 MAPK, JNK and PI-3-kinase/Akt pathways has also been described in context with LPS, TNF α , and IL-1 β in endothelial cells. Although it is highly important to comprehend inflammatory signaling in endothelial cells, a number of questions are still unanswered or controversial.

Response of endothelial cells to different exogenous factors is a highly complex reaction. Thus, the effects of the stimuli can be

comparable only if they are examined in exactly the same circumstances using multiparametric, pattern-based approach. Although there are numerous publications studying the effects of IL-1 β , TNF α and LPS, none of them have used such an approach.

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, but it also bridges innate and adaptive immunity. The pattern-recognition molecules of the lectin pathway are mannose-binding lectin (MBL) and ficolins (H-, L- and M-ficolin), which form supramolecular complexes with the MASPs (MBL-associated serine proteases). Three MASPs have been identified: MASP-1, MASP-2, and MASP-3. MASP-2 has an unambiguous physiological role: it can autoactivate and cleave C4 and C2 subcomponents, which form the C3-convertase complex (C4b2a)

MASP-1 is the most abundant protease of the lectin pathway of the complement system. Its serum concentration (about 70 nM) considerably exceeds that of MASP-2 (about 5 nM), the enzyme responsible for initiating the proteolytic cascade. Although MASP-1 was the first enzyme identified as a protease member of the lectin pathway, its exact physiological role has not been fully clarified yet. An interesting observation was that MASP-1 is responsible for limited coagulation, because it has several thrombin-like properties, including the cleavage of major thrombin substrates such as fibrinogen.

2. AIMS

The first aim of my dissertation was to compare the LPS-, TNF α -, and IL-1 β -dependent proinflammatory responses of endothelial cells using activation pattern, based on multifactorial cytometric analysis. Changes in gene expression induced by IL-1 β , TNF α and LPS are similar, but not identical. Dissimilarity in gene expression can result from signaling differences; however, no comprehensive study has been conducted, therefore our questions were:

- Are there any differences between LPS, IL-1 β and TNF α signaling at the level of NF κ B nuclear translocation and activation of p38, JNK and Akt pathways? Our aims were to compare the kinetics and maximum response of the three factors in terms of signaling pathways.
- What causes can stand behind of these differences?
- Are there any differences among the LPS-, TNF α -, and IL-1 β -induced ICAM-1, E-Selectin, IL-6, IL-8 and MCP-1 expression?

Responding to these questions stand out a comparative activation pattern of 10 inflammation-related molecules.

In first part of my work I worked with three well-known inflammatory factors, in the second part I was wondering the effect of a poorly studied serine protease, MASP-1. Since MASP-1 is a thrombin-like protease and thrombin can activate several

intracellular pathways in endothelial cells, so we observed the following questions:

- Can MASP-1 cause intracellular Ca^{2+} -mobilization like thrombin?
- Can MASP-1 contribute to the initialization of proinflammatory pathways? Can MASP-1 initiates $\text{NF}\kappa\text{B}$ and p38 MAPK signaling in HUVECs?
- Can MASP-1 induce adhesion molecules or cytokines expression?
- Is MASP-1 a proinflammatory factor in term of endothelial cells?

3. METHODS

Human umbilical vein endothelial cells (HUVEC) were harvested from fresh umbilical cords of normally delivered healthy neonates by collagenase digestion.

NF κ B nuclear translocation and intracellular Ca²⁺-signaling was measured by fluorescence microscopy. Samples were observed and images were recorded using an Olympus IX-81 inverted fluorescence microscope mounted with an Olympus DP70 digital camera. Images were analyzed with analySIS v3.2 software.

p38, JNK, and Akt activation was analyzed by Western blotting. Densitometric analysis was performed using Syngene GeneTools software.

TLR4 localization and LPS internalization was measured by fluorescence microscopy. In case of colocalization experiments Immunofluorescence was analyzed with a Olympus Fluoview 500 confocal microscope, equipped with 40x and 60x objectives. Pearson's Colocalization Index (CI) was calculated with the Image Correlator Plus plugin of ImageJ 1.34i software.

Expression of adhesion molecules was detected by cellular ELISA. E-selectin and ICAM-1 expression was measured after 6 and 24 hours treatment, respectively.

Cytokine/chemokine production was analyzed by ELISA from supernatants of treated cells for ICAM-1 cellular ELISA.

Statistical analyses were done with GraphPad Prism v4.02. Significant differences were assumed if $p < 0.05$.

4. RESULTS

4.1. Comparing the effect of LPS, TNF α and IL-1 β proinflammatory factors

Kinetics of NF κ B nuclear translocation

First, we determined the dose of LPS, TNF α , and IL-1 β capable of inducing maximal NF κ B nuclear translocation, then we used these concentrations in the following experiments. We treated the cells with 1 μ g/ml LPS, 10 ng/ml TNF α or 1 ng/ml IL-1 β for 3.75, 7.5, 15, 30, 60, 120 or 240 minutes. LPS induced significant nuclear translocation of NF κ B only after 30 minutes, and translocation reached maximum at 120 minutes. Both TNF α and IL-1 β elicited NF κ B nuclear translocation with an onset at 7.5 minutes, and a peak at 30 minutes.

Kinetics of p38, JNK and Akt phosphorylation

HUVECs were treated with LPS, TNF α , and IL-1 β for 10, 30 or 60 minutes in the same concentrations used in NF κ B activation measurements, to ensure comparability. Then, we analyzed the phosphorylation of p38, JNK and Akt by Western blot. In case of p38 and JNK LPS reached its maximum effect only after 60 minutes, and its effect decreased after 120 minutes, whereas TNF α and IL-1 β reached this maximum after 10 and 30 minutes, respectively. Similar to the induction of NF κ B translocation, there were differences – in addition to kinetics – also in the maximum effect of the three factors.

MAPK phosphorylation level was the highest after IL-1 β treatment, whereas the effect of TNF α and LPS was smaller. In contrast to the MAPKs and NF κ B pathways, only slight phosphorylation of Akt by the three factors was observed, with the same kinetics.

Ca²⁺-signaling

Intracellular Ca²⁺-mobilization was studied by fluorescence microscopy, using Fluo-4-AM labeled HUVECs. Compared to vehicle, thrombin and bradykinin (used as positive controls) induced an intense Ca²⁺-response. By contrast, none of the three inflammatory factors elicited any elevation of intracellular Ca²⁺-concentration.

Localization of TLR4 in HUVECs

It was described earlier in human coronary endothelial and intestinal epithelial cells, TLR4 is not a cell surface receptor of LPS and its intracellular localization might influence the signaling pathways mediated by TLR4. Thus, we examined the localization of TLR4 in HUVECs by fluorescence microscopy, which clearly revealed that TLR4 is predominantly located in the perinuclear region and not in the cell membrane. Using BODIPY-FL C5 Ceramide as a Golgi marker to identify the cytoplasmic compartment in which TLR4 is localized, we found that TLR4 is strongly co-localized with the Golgi complex.

Kinetics of LPS internalization

We treated the cells with biotinylated LPS for 2, 10, 30 or 60 minutes and after incubation with streptavidin-conjugated Alexa488, investigated the localization of LPS and its co-localization with the plasma membrane. After 2 minutes, LPS showed a diffuse cell membrane staining and co-localization with PECAM-1 membrane protein. At 10 minutes, the co-localization decreased significantly, after 30 and 60 minutes, LPS was almost completely internalized and could be found mainly around the nucleus.

Expression of adhesion molecules and cytokines

We asked whether the dissimilar kinetics and/or efficiency of signaling pathways induced by LPS, TNF α and IL-1 β could result in a different pattern of adhesion molecules and cytokines. E-Selectin expression of HUVECs could not be stimulated by LPS as efficient as by TNF α or IL-1 β . In contrast, ICAM-1 expression did not differ significantly at 24 hours after stimulation by LPS, TNF α or IL-1 β .

IL-6 production was minimally induced by TNF α , LPS provoked stronger IL-6 secretion, whereas IL-1 β was the superior. IL-8 was induced most efficiently by IL-1 β , while LPS and TNF α had less effect. Production of MCP-1 was highly and similarly enhanced by all the three inflammatory molecules.

4.2. Effect of MASP-1 on endothelial cells

MASP-1 elicits Ca²⁺-response

Application of MASP-1 caused a significant increase in cytosolic [Ca²⁺], in a concentration-dependent manner. Comparing the Ca²⁺-signal of MASP-1 to that of thrombin as positive control showed similar kinetics of the Ca²⁺-response; however, MASP-1 consistently elicited a lower signal, compared to thrombin. In contrast to MASP-1, its cognate molecule, MASP-2 (the physiological function of which has largely been elucidated), was unable to trigger any Ca²⁺-signal in the cells – even at a very high concentration.

Activation of NFκB and p38 MAPK pathways

We tested whether MASP-1 can contribute to the initialization of proinflammatory pathways. HUVECs treated with MASP-1 for 1 h induced NFκB nuclear translocation in a dose-dependent manner, although their overall effects were smaller, than that of LPS. HUVECs treated with MASP-1 for 30 min induced strong, dose-dependent phosphorylation of p38 MAPK.

Expression of adhesion molecules and cytokines

E-selectin, IL-6 and IL-8 expression could not be stimulated by MASP-1 (860 nM) as efficient as by LPS or IL-1β, but MASP-1 provoked stronger IL-6 and IL-8 secretion than TNFα.

5. DISCUSSION

We describe for the first time the differences and similarities of LPS, TNF α , and IL-1 β in the induction of endothelial inflammatory response by using a pattern of 10 inflammation-related molecules (Figure 1.).

- We demonstrated that signaling of LPS was less effective than that of IL-1 β , and was significantly slower than that of TNF α and IL-1 β , which can be partially explained by the special localization of Toll-like receptor 4 (TLR4). We showed that TLR4 is mainly localized in Golgi apparatus in HUVEC.
- The proinflammatory capacity of TNF α was similar to that of IL-1 β in inducing NF κ B nuclear translocation, while IL-1 β was the strongest activator of MAPK pathways
- We showed that the delay in the LPS-induced NF κ B and MAPK signaling pathways does not seem to result from Ca²⁺- or Akt-signaling.
- Expression of E-Selectin, IL-6 and IL-8 induced by LPS, TNF α and IL-1 β were also dissimilar, whereas we did not find such a difference in ICAM-1 and MCP-1 expression. Due to the higher induction of E-selectin and IL-8, IL-1 β might have more important role in neutrophil recruitment than LPS and TNF α .

	IL-1 β	TNF α	LPS
NF κ B	Red	Red	Yellow
P38 MAPK	Orange	Red	Yellow
JNK	Orange	Red	Yellow
Akt	Orange	Orange	Orange

	IL-1 β	TNF α	LPS	MASP-1
NF κ B	Red	Red	Orange	Yellow
p38 MAPK	Red	Orange	Orange	Yellow
JNK	Red	Orange	Orange	N/A
Akt	Yellow	Yellow	Yellow	N/A
Ca-signal	White	White	White	Red
E-selectin	Red	Red	Orange	Yellow
ICAM-1	Red	Red	Red	N/A
IL-6	Red	White	Orange	Yellow
IL-8	Red	Yellow	Orange	Orange
MCP-1	Red	Red	Red	White

Figure 1. Pattern of the kinetics (upper panel) and maximal (lower panel) responses induced by LPS, TNF α , IL-1 β and MASP-1.

Upper: We compare the kinetics of signaling pathways stimulated by the three factors. Red: the factor reached its maximal effect within 15 minutes; orange: the maximal effect was around at 30 minutes; yellow: maximal effect was at 60 minutes or later.

Lower: We compared the relative maximal effect of the three factors for each parameter applying two-way ANOVA. Red: the most efficient activators in each line. Orange: significantly weaker activation than that of the strongest factor. Yellow: significantly weaker activation than that of the second strongest factor. Grey, N/A: Not available.

- By above mentioned parameters we identified a signaling and expression pattern for the three proinflammatory molecules. This pattern illustrates how complex a proinflammatory process can be, and may enable us to predict and compare the pathomechanism of various inflammatory diseases.

In contrast to the three well-known proinflammatory factors, the effect of MASP-1 is poorly defined. Activation of endothelial cells by MASP-1 leads to proinflammatory phenotype and points to a novel connection between the complement- and coagulation systems during the inflammatory process.

- MASP-1, at its plasma concentration, triggered significant Ca^{2+} -response in HUVECs and the kinetics of the responses elicited by MASP-1 and thrombin were similar. MASP-2, the other important protease of the lectin pathway failed to induce Ca^{2+} -response even at a very high concentration.
- MASP-1 induced both NF κ B nuclear translocation and p38 MAPK phosphorylation in a dose-dependent manner, as well as secretion of IL-6 and IL-8 cytokines. Activation of these pathways leads to a complex change in the phenotype of HUVECs.
- This well-characterized proinflammatory phenotype enhances leukocyte adhesion and transmigration, smooth muscle cell proliferation, and remodeling of the extracellular matrix.

6. LIST OF PUBLICATIONS

Publications related to the thesis

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