Unravelling molecular and biochemical dysfunction by Shiga toxin: implication for thrombotic microangiopathy in Hemolytic Uremic Syndrome

SUMMARY

Shiga toxins (Stx) has been strongly indicated as the causative agent for typical childhood D+HUS.The kidney is the privileged target of the toxin, with glomerular ischemic changes including tuft retraction and capillary wall thickening, both preceding microvascular thrombosis. Endothelial dysfunction is crucial to the development of microvascular lesions and increasing evidence suggests that Stx/VT by favoring interaction of endothelial cells with leukocytes and platelets, serve to amplify and extend the injury at renal level. Local thrombosis amplifies the inflammatory injury by promoting complement activation -in particular the alternative pathway- and deposition within capillary vessels (**Chapter 2**).

In this thesis further investigations where performed to address the renal toxicity of Stx with the aims of providing insights on the molecular events involved in microvascular lesions in HUS.

In **Chapter 3** we investigated *in vitro* the effect of VT-1 on leukocyte adhesion to vascular endothelium under physiologic flow condition using a parallell plate flow chamber. Incubation of human umbilical vein endothelial cells (HUVEC) with increasing concentrations of VT-1 resulted in a dose-dependent increase in the number of adhering leukocytes to HUVEC. The adhesive response induced by the toxin was quite comparable to the effect of IL-1β, one of the most potent inducer of endothelial cell adhesiveness. Rolling phenomenon was not affected by VT-1. We then explored the role of adhesive molecules in VT-induced leukocyte adhesion. Functional blocking of E-selectin, ICAM-1 and VCAM-1 on endothelial cells with respective antibodies, significantly reduced VT-induced increase in leukocyte adhesion. Pre-exposure of endothelium with TNFα before challenge with VT-1, significantly increased the number of adherent leukocytes under flow.

This observation is consistent with the finding that this cytokine does promote upregulation of endothelial Gb3 receptor and supports toxin-binding. These data identify a novel mechanism by which VT directly modulates leukocyte-endothelium interaction, thus increasing leukocyte adhesion and upregulating adhesive proteins on endothelial surface membrane.

Kidney specimens from HUS children with evidence of Stx-producing *E coli* infection revealed a massive infiltration of polymorphonuclear and mononuclear cells within the glomeruli, along with microvascular injury. Urinary levels of IL-8 and monocyte chemoattractant protein–1 (MCP-1), potent attractants of neutrophils, monocytes/macrophages and T lymphocytes, were elevated during the acute phase of the disease in these patients, suggesting the involvement of these chemokines in the recruitment of inflammatory cells at glomerular level. In **Chapter 4**, we tested the hypothesis that Stx-2 could modulate the endothelial expression of MCP-1 and IL-8 and their functional role on leukocyte adhesion and transmigration. We found that subtoxic concentrations of Stx-2 induced a significant increase in the number of leukocytes adhering to HUVEC, followed by a massive transmigration through the endothelium. MCP-1 and IL-8 mRNA expression was increased after exposure to Stx-2. Blocking of endothelial MCP-1 and IL-8 with the corresponding antibodies significantly reduced Stx-induced leukocyte adhesion and migration either on HUVEC or glomerular endothelial cells. Adenovirus-mediated gene transfer of IkBα down-regulated chemokine expression and also inhibited the adhesion and transmigration of leukocytes in Stx-treated HUVEC. These data suggest that Stx-2 via a transcriptional activation mechanism mediated by NF-kB upregulates MCP-1 and IL-8, key mediators of leukocyte adhesion and transmigration.

In D+HUS, thrombotic microangiopathy, defines a lesion of vessel wall thickening and intraluminal platelet thrombosis that occlude the microcirculation of the kidney and other organs. The reason why thrombi form only on arterioles and capillaries is not known. **In Chapter 5** we studied whether Stx-1 directly affected endothelial antithrombogenic properties promoting thrombus formation on human microvascular endothelial cells (HMEC-1) under high shear stress. HUVEC were used for comparison, as large vessel endothelium. VT-1 directly induced platelet adhesion on cultured endothelial cells perfused with whole blood in a flow chamber system under shear stress levels high enough to mimic the ones encountered in the microcirculation. This effect was more pronounced on VT-treated endothelial cells of microvascular (HMEC-1) in respect to large vessel (HUVEC) origin, since basal expression of Gb3 receptor in HMEC-1 was 50-fold higher than in HUVEC. In the attempt to identify the adhesive proteins involved in platelet-endothelium interactions elicited by VT-1, we first focused on von Willebrand factor (vWf), which is indispensable substrate to promote thrombus formation. Blocking the binding of vWf to platelet glycoprotein 1b by aurintricarboxylic acid and the glycoprotein $\alpha_{IIb}\beta_3$ integrin by chimeric 7E3 Fab resulted in a significant reduction of VT-induced platelet deposition, indicating a role of vWf-platelet interaction at high shear stress in this phenomenon.

Inhibition of endothelial vitronectin receptor, P-selectin and PECAM-1 with specific antibodies markedly reduced the endothelial surface area covered by thrombi. These data along with the observation of a strong expression of these adhesive proteins due to upregulation/redistribution, on the endothelial surface of HMEC-1 after VT challenge, provide insights on the determinants possibly involved in the process of microvascular thrombosis associated with D+HUS.

Podocytes are sensitive to the toxic effects of Stx-1 and 2 isoforms, as documented either in cultured cells or in human renal biopsies. Podocytes, a crucial component of the glomerular filter, are highly specialized epithelial cells endowed with foot processes. They possess a contractile structure that respond to vasoactive hormones to control glomerular capillary surface area and in turn ultrafiltration coefficient. Effacement of foot processes occurs in many proteinuric nephropathies and is accompanied by rearrangement of the actin cytoskeleton. Instrumental in studying the effect of Stx on podocytes, in **Chapter 6** we first set up the technique to maintain in culture immortalized mouse podocytes and to induce differentiation of podocytes which in this conditions stop to proliferate and express high levels of synaptopodin. Then, we assessed whether protein overload -reproducing the condition of exaggerated plasma protein traffic through the glomerular capillary barrier- affects intracellular pathways, leading to cytoskeletal architecture changes and ultimately to podocyte dysfunction. We have found that in podocytes the abnormal uptake of plasma proteins induces Rho kinase-dependent F-actin cytoskeletal rearrangement leading to cell dedifferentiation. Such structural changes translate into the activation of FAK in turn responsible for NF-kB- and Ap-1 dependent ET-1 gene upregulation. ET-1 overproduction may act on the podocyte contractile apparatus altering the glomerular capillary surface area thus leading to protein permeability dysfunction. These results indicate podocyte as a novel cellular target for the toxic effect of excess plasma ultrafiltered protein.

Since podocytes express Stx specific receptor, are susceptible to Stx citotoxicity and represent an important source of vasoactive molecules, we investigated in **Chapter 7** whether Stx-2 modulates the expression and production of the vasoconstrictor peptide, Endothelin-1 (ET-1), taken as candidate mediator of podocyte dysfunction. Stx-2 enhanced ET-1 mRNA and protein through the activation of NF-kB and Ap-1 to the extent that

transfection with dominant negative mutant of IKB kinase2 or with Ap-1 decoy oligodeoxynucleotide reduced ET-1 mRNA. We further investigated the intracellular signals activated by Stx-2 possibly involved in the upregulation of ET-1 gene. A role for p38 and p42/44 MAPK in mediating NF-kB-dependent gene transcription induced by Stx-2 has been proposed based on data that Stx-2 phosphorylated p38 and p42/44 MAPK and that inhibitors of both MAPK reduced transcription of NF-kB promoter luciferase reporter gene construct induced by Stx-2. Additionally, Stx-2 induced F-actin redistribution and intercellular gap formation via ET-1 induction since cytoskeletal changes were prevented by ET_A receptor blockade. Findings that podocyte challenge with ET-1 induced podocyte F-actin redistribution and in parallel increased protein permeability, unravel ET-1 as a major mediator of the toxin-induced effect. In summary, our data are the first to document that podocyte is a functionally relevant target of Stx, a novel stimulus for the synthesis of ET-1 synthesis that controls in autocrine and paracrine fashion glomerular remodeling and hemodynamic derangement in HUS.