Signaling in leukocyte transendothelial migration: a roadmap for homing of progenitor cells
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Summary

This thesis describes the process of transendothelial migration of leukocytes, in particular of hematopoietic progenitor cells. Transendothelial migration can be divided into distinct stages. In brief, the leukocytes first roll over the endothelium, then adhere to the endothelium and finally migrate through the endothelial cleft towards the underlying tissues such as, in the case of progenitor cells, the bone marrow stroma. The leukocytes are guided in this complex process by chemokines, small molecules that are able to attract subsets of leukocytes. For hematopoietic progenitor cells, the chemokine SDF-1 (or CXCL12) as well as its receptor CXCR4 are very important; blocking antibodies to CXCR4 prevent homing of progenitor cells to the bone marrow, and homing is impaired in SDF-1- and CXCR4-deficient mice. Our current knowledge about transendothelial migration of leukocytes and the accompanying signalling events in leukocytes as well as in endothelial cells are reviewed in chapter 1.

SDF-1 is presented by the endothelial cells in vivo, presumably through heparin sulphates on the apical side. Rolling leukocytes or progenitor cells will bind immobilized SDF-1 through CXCR4 and subsequently adhere more strongly to the endothelium. We have found that CXCR4 polarizes upon binding to SDF-1 immobilized to endothelial cells. Moreover, detailed analysis revealed that CXCR4 was recruited to lipid rafts and that these rafts were necessary for proper SDF-1-induced CXCR4 signalling and migration. These findings are described in chapter 2.

Leukocytes adhere to the endothelium through leukocyte integrins and endothelial adhesion molecules such as ICAM-1 and VCAM-1. Subsequently, the leukocytes migrate through the endothelial cell-cell junctions. The junctional function depends on the homophilic binding protein vascular-endothelial (VE)-cadherin. Inhibition of VE-cadherin function not only increases the permeability of the endothelial monolayer but also promotes migration of hematopoietic progenitor cells across the endothelial monolayer. However, blockade of ICAM-1 and VCAM-1 still inhibits efficient migration across endothelial cells that lack VE-cadherin function, indicating that the progenitor cells effectively use the endothelial cells to migrate across them. To mimic progenitor cell adhesion to the endothelium, we clustered VCAM-1, an important molecule for the homing of progenitor cells, with specific antibodies. Clustering of VCAM-1 induces intracellular signaling in the endothelial cells, resulting in a loss of VE-cadherin-mediated endothelial cell-cell contacts and a drop of the electrical resistance across the endothelial monolayer. VCAM-1-induced loss of cell-cell contacts.
appears to depend on the generation of reactive oxygen species (ROS) and requires activation of the small GTPase Rac1. Moreover, we showed that p38 MAP kinase is involved in this pathway, downstream of ROS production. We suggest that the VCAM-1-Rac-ROS-p38 signalling pathway in endothelial cells is activated by the adhesion of hematopoietic progenitor cells to the endothelium and that this pathway is required for efficient migration of leukocytes in general and hematopoietic progenitor cells in particular across endothelial monolayers. These findings are described in chapters 3 and 4.

To study in more detail the ROS production in endothelial cells, we made use of a cell-permeable active mutant of Rac1, Tat-RacV12 and describe in chapter 5 that this mutant increases ROS production in endothelial cells. Moreover, active Rac1 induced loss of cell-cell contacts in a ROS-dependent fashion, underscoring the idea that ROS is involved in the regulation of endothelial cell-cell contacts. Detailed analysis with confocal imaging microscopy revealed increased levels of phosphotyrosine at sites of cell-cell contacts. Moreover, biochemical analysis showed increased phosphorylation levels of the VE-cadherin complex, in particular of α-catenin. In line with the suggestion that ROS is involved in the regulation of cell-cell contacts, scavenging of ROS prevented increased phosphotyrosine levels at sites of cell-cell contacts.

To further explore the regulation of endothelial cell-cell contacts, chapter 6 focuses on the endothelial cell-cell contacts and in particular on VE-cadherin. Loss of endothelial cell-cell contacts by disruption of VE-cadherin-specific cell-cell interactions resulted in a rapid increase in β-catenin tyrosine phosphorylation. Detailed observation of endothelial cell-cell contacts showed that local loss of contact is immediately followed by the induction of local membrane ruffles. This observation is underscored by the finding that loss of cell-cell contact leads to transient and rapid RhoA activation, required for cell contraction, and a prolonged Rac1 activation, required for contraction (chapter 5) and cell-spreading. It appeared that Rac1 localizes with VE-cadherin to cell-cell borders and that inhibition of active Rac1 prevents the restoration of endothelial cell-cell junctions. The initial loss of cell-cell contact is preceded by increased production of ROS. Moreover, induced loss of VE-cadherin-specific cell-cell contacts is inhibited by scavenging of ROS. These studies suggest that ROS play a key role in the regulation of VE-cadherin-mediated endothelial cell-cell contacts and thus in transendothelial migration of leukocytes. Moreover, it suggests that VE-cadherin functions as a signaling molecule.

Chapter 7 highlights the effects of ICAM-3 on the regulation of bone-marrow endothelial cell-cell junctions. ICAM-3 is generally thought to be expressed exclusively on
human leukocytes, although ICAM-3 expression on endothelial cells in tumour vasculature has also been reported. We found ICAM-3 expression on primary human umbilical vein endothelial cells as well as on human bone-marrow endothelial cells. The level of ICAM-3 expression was not affected by inflammatory stimuli such as TNF-α or IL-1β, and endothelial ICAM-3 appeared not to be involved in transendothelial migration. Surprisingly however, clustering of ICAM-3 reduced the electrical resistance of monolayers of bone-marrow endothelial cells but had no effect on the resistance of monolayers of endothelial cells derived from umbilical veins. The drop in resistance depended on ROS production, in line with the previous observations that ROS regulates endothelial cell-cell contacts. Although we describe here the effects of ICAM-3 clustering on bone-marrow endothelium, it is as yet unclear what the role of ICAM-3 on these endothelial cells is.

To study the source of endothelial ROS, we focused in chapter 8 on the expression and localization of components of the oxidase-generating complex, as previously described in phagocytic cells. It appeared that the regulatory components for the phagocytic NADPH-oxidase complex are also expressed in endothelial cells. Moreover, endothelial cells express mRNA for NOX2 (gp91phox) as well as for a homologue, NOX4. Importantly, NOX4 was found to be expressed at approximately 100-fold higher mRNA levels compared to NOX2. Confocal imaging of GFP-fusion proteins revealed that NOX4, together with the other NADPH-oxidase components p47phox and p67phox, localizes at the endoplasmic reticulum. We further demonstrated that endothelial ROS are required for full restoration of stimulus-induced loss of intercellular adhesion. Future, detailed studies are required to reveal the precise regulation of ROS generation in endothelial cells.

Finally, the findings that are described in this thesis are summarized in chapter 9 and further discussed to incorporate them into our current model of transendothelial migration of leukocytes.