Inflammation in ischemia and reperfusion: From mice to men
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GENERAL DISCUSSION

Recent developments in complement-mediated I/R injury

Tissue injury following ischemia and reperfusion (I/R) may occur in multiple clinical situations such as myocardial infarction, hepatic surgery, intestinal ischemia, transplantation, shock, trauma and sepsis. Tissue ischemia can induce local hypoxic changes that increase tissue susceptibility to injury following reperfusion. Therefore, restoration of blood flow after ischemia is not always beneficial but rather may induce both local and systemic inflammatory responses, which may result in enhanced tissue injury in the ischemic organ and to multiple organ failure, leading to morbidity and mortality1. This detrimental effect of reperfusion of an ischemic tissue or organ is called ischemia-reperfusion (I/R) injury. Identification of the molecular and cellular mechanisms of I/R injury may facilitate development of new therapies which are urgently needed for the treatment of ischemic syndromes.

This thesis explores some players in I/R injury activation including the complement system, associated inflammatory mediators and mechanisms regulating gut commensal bacteria in animal and human models. As described in Chapter 1 and Chapter 2 of this thesis, arising at an integrated view on molecular mechanisms involved remains challenging due to the complexity of these processes and limitations to progress in the field of inflammation and tissue injury. A more detailed understanding of this intricate network established between complement and tissue injury is crucial to bridging the gap between promising therapeutic approaches in preclinical studies and effective treatments in the human setting.

Intestinal I/R injury

The gut is a critical target organ when, in case of hypotension, blood flow is redistributed to preserve central hemodynamic stability. In such conditions, intestinal I/R may occur leading to the loss of gut barrier function2. To complicate the development of therapeutic interventions, human diseases leading to such severe stress state are characterised by a heterogeneity, ranging from intestinal ischemia due to acute occlusive mesenteric ischemia to surgery, shock and sepsis. As illustrated by the data described in Chapter 7, intestinal ischemia can be potentially life-threatening. At the time of diagnosis of intestinal ischemia, therapeutic options are often limited to resection of necrotic intestine due to extensive ischemic tissue injury. The mechanism of intestinal I/R injury appears to be multi-factorial. The complement system3-6 and natural antibodies1,7-9 are considered to play a central role in intestinal I/R injury, though numerous other players have been implicated in intestinal I/R injury as well. These include C-reactive protein10, lipid peroxidation and reactive oxygen species formation11, cytokines and chemokines12, B cells13, T cells14-15, neutrophils16, adhesion molecules17-18, platelets19, IL-17A20, free fatty acids21, and Toll-like receptor22. In addition, a number of self-targets have been identified to trigger inflammation during ischemia including non-muscle myosin heavy chains type II (NMHC-II) subtype A and C23 or aggregated actin cytoskeleton24. Brief periods of ischemia are believed to lead to an alteration in surface epitopes, resulting in binding by natural IgM and activation of the complement system25. In addition to natural antibodies, autoantibodies, which recognize self-antigen, may cause tissue injury after I/R, suggesting that autoimmunity may contribute to pathogenic ischemic conditions in humans25-27. Studies further suggest that the natural IgM-ischemic antigen complex provides a binding site for mannan binding lectin (MBL), which subsequently leads to activation of complement and results in tissue injury11,23,28. Hence the classical and lectin pathways have been put forward as main players in intestinal I/R injury induction. Recent reports demonstrate that also human natural antibodies, like those of mice, are capable of inducing I/R injury in the murine intestinal model29. However, as described in Chapter 6 we were not able to confirm reduced histological damage upon intestinal I/R in two strains of Rag1-/- mice in our experimental set-up as described in literature. Moreover, supplementation with known complement activators IgM and CRP did not enhance tissue damage. Whereas this unexpected disparity between our observations and literature will need to be studied further, these results emphasize that extrapolation of findings in mouse models of intestinal I/R injury to human conditions, should be treated with caution. These findings may imply a role of other complement mediators than human immunoglobulin and may implicate the involvement of multiple recognition molecules in mediating I/R-injury rather than a single specific complement-dependent initiating factor.

Gut microbiota stimulate the normal development of the humoral and cellular mucosal immune system during neonatal life and maintain homeostasis of intestinal immune system thereafter.30,31. Whereas gut commensal bacteria are important in maintaining the gut mucosal barrier, recent findings suggest that they also play essential roles in driving pathogenic inflammatory responses32. Disturbances in microbial colonization of the gut brought about by major abdominal surgery and/or intestinal I/R syndromes are therefore considered to cause loss of gut barrier function. This disturbed barrier function is assumed to be the first step leading to bacterial translocation and has been implied to be a major contributor to the systemic inflammatory response syndrome, resulting in dysfunction of postoperative immune responses33 or multiple-organ dysfunction syndrome34-35 if the magnitude of ischemia is severe or the volume of ischemic mesenteric tissue is large34,35. Indeed, germ-free mice exhibit reduced local (intestinal) and remote (lung) injury following mesenteric I/R relative to conventional mice36. Furthermore, depletion of gut commensal bacteria using broad-spectrum antibiotics have been shown to reduce intestinal I/R injury37. However, the role of gut microbiota in acute intestinal inflammation and injury is still underdetermined and controversial, as emphasized by findings in Chapter 9, in which we propose that bacterial translocation to some extent occurs in the normal gut. The phenomenon of bacterial translocation appears insufficient to explain the development of multi organ dysfunction syndrome in the critically ill. Instead, gut injury and the systemic spread of non-microbial, tissue injurious factors that reach the systemic circulation via the intestinal lymphatics may play a major role in the development of a septic state38.

Hepatic I/R injury

Liver dysfunction has been strongly linked to the extent of hepatic I/R injury, and is an unavoidable consequence of the surgical procedures. Although the precise mechanisms responsible for liver dysfunction are not completely understood, complement appears to play an important role in both I/R and liver regeneration. Studies using rat models indicate
a central role for complement in hepatic I/R. Complement inhibition with soluble complement receptor 1 (SCR1), C1-inhibitor and C5a receptor antagonist protect against excessive tissue injury in rat models of hepatic I/R 37-40, and a role for the terminal cytolitic membrane attack complex (MAC) has been demonstrated 41. In addition to its role in hepatic I/R, recent evidence indicates that complement activation, in particular the classical pathway, is required for normal liver regeneration, following either liver resection or toxic injury 42-47. The classical pathway of complement has been suggested to play a role in complement activation in human livers after partial hepatectomy 48. We observed a similar time course of IgM and CRP deposition as C3 in Chapter 3 and hence suggest a role for both molecules in the activation of complement in the setting of hepatic I/R. However, the exact role of complement and its different pathways remains to be elucidated, moreover since a plethora of cellular and humoral players have been proposed to contribute to hepatic I/R injury 49-52. Hence, experimental models have shown hepatic I/R, as intestinal I/R, to be a multifactorial and intriguing phenomenon that probably cannot be attributed to a single pathway. Responses to the strategies aimed at reducing hepatic I/R injury, such as described in Chapter 4, might depend on the surgical procedure and on aspects of liver tissue 10. Systemic lidocaine has been reported to reduce I/R injury in the heart 53,54, lung 55,56 and brain 57,58. In our rat hepatic I/R models, systemic lidocaine in therapeutic concentrations neither attenuated hepatocellular damage nor improved postoperative liver function. Hence the beneficial effects of lidocaine in I/R injury may be organ specific.

Myocardial I/R injury

Myocardial I/R injury is typical for myocardial infarction. After myocardial infarction, an inflammatory response takes place that causes further damage to viable tissue around the infarct, most likely through accelerated apoptosis. A major clinical goal is to determine optimal strategies for minimizing myocardial necrosis and optimizing cardiac repair by modulation of this inflammatory response following acute myocardial infarction (AMI). This is of major therapeutic significance, as infarct size is known to be a strong independent predictor of post infarction mortality 59. During myocardial reperfusion, the acute ischemic myocardium is subjected to several abrupt biochemical and metabolic changes, such as mitochondrial reenergization, the generation of reactive oxygen species (ROS), intracellular Ca2+ overload, the rapid restoration of physiologic pH, and inflammation, all of which interact with each other and may mediate cardiomyocyte death 60,61. Also complement, probably triggered by CRP and natural IgM antibodies may contribute to cell death following myocardial ischemia 62,64. In Chapter 8 we describe a possible role of a subset of natural antibodies, anti-phosphorylcholine IgM antibodies, in amplification of inflammation after AMI. More recently, studies have proposed a responsibility of the lectin pathway in mediation of myocardial I/R injury 65-67. Despite these substantial research efforts and the large number of studies dedicated to identify agents modulating the inflammatory response after AMI, until now this has not resulted in new clinical interventions 68,69. The large number studies on myocardial I/R injury probably reflects the many different mechanisms contributing to the necrosis that follows I/R injury 68. Patient outcome after myocardial infarction almost certainly depends upon the combined activation of several distinct but interrelated signaling pathways, suggesting that a combination of treatments targeted to different pathways at the end may be the therapy of choice, and modulation of complement may be one of them 70.

Towards complement-mediated therapies

Various complement inhibitors are currently in preclinical or clinical development 71,72. Despite the ubiquitous presence of complement, relatively few side effects have been reported for complement-directed therapy 73. Since the approval of the first complement-specific drug eculizumab, an antibody against complement component C5, a multifaceted armory of therapeutic inhibitors has been proposed for the diverse array of complement-mediated pathologies. Importantly, translational research has emphasized that the diverse array of complement-mediated pathologies, with distinct underlying mechanisms, demands a multifaceted arsenal of therapeutic strategies. Complement may not be the main driving force in some of these disorders, but it may still be a critical factor that can tip the balance between induction and resolution of inflammation 71. The development of complement inhibitors in prevention of I/R injuries will have to focus on whether to inhibit all or specific components of complement activation. Whereas certain disease processes may require varying specific inhibition as others may not, it is not yet clear what the overall ramifications will be in either component inhibition or complete cascade inhibition. Some components may surely need to remain active as in the recognition of pathogens to prevent bacterial sequela or for low level expression for which certain protection during I/R may be required 72. Whereas complement inhibition is expected to prove successful in cases in which complement activation participates in the initial events of muscle cell destruction, as in autoimmune myocarditis or autoimmune muscle disorders, in pathologic conditions in which complement is recruited by degenerating or dying muscle cells, as in ischemia, the ideal approach is probably to modulate rather than abruptly blunt complement activation. Beneficial effects of complement action with regard to waste disposal, recruitment of stem cells, regeneration, angiogenesis, and better utilization of energy sources under hypoxic conditions will probably need to be preserved in successful disease treatment 73.

Challenges and future directions in the translation of animal models to human disease

One of the challenges and limitations to progress in the field of complement-mediated tissue injury is the multitude of species, tissue, and organ differences encountered as well as the heterogeneity of diseases involving human tissue damage. Furthermore, in working towards therapeutic strategies, translational problems between mouse and human systems are expected to be encountered. Most of the current knowledge on ischemia and reperfusion is derived from studies derived in mice. In large part due to the high degree of genetic homology with humans, rodents have been considered prime experimental subjects. Reports on the sequencing of human and mouse genomes reveal only approximately 300 genes are specific to one species or the other. Thus the majority of over 20,000 human genes described to date 73 are shared with mice. Nevertheless, significant differences between mouse and human immune systems exist 74,75. Hence we should keep in mind that some aspects of the human immune system cannot be studied in mice, and vice versa. Disparity between findings in animals and in patients and inconclusive results of clinical studies have been seen in many inflammatory diseases and I/R injury, including myocardial
infarction\textsuperscript{60,68,76} and in our intestinal I/R model in Rag\textsuperscript{1/-} mice in Chapter 6. Apart from technical issues such as hypothermia, differences in the anatomy of the organs of various species and subspecies, differences in the experimental models used, and differences in the modes of administration, dosage, and metabolic breakdown of the drugs all may contribute to the observed differences. Thus, it is very important to choose the animal species and the experimental model and to standardize the protocol according to the clinical question under study\textsuperscript{50}. On the other hand, many paradigms translate well between both species, and, in addition, mice can be genetically manipulated relatively easily. Hence, mouse models will most probably continue to provide important information for human disease. However, in developing future therapeutic options for the human setting, we should be cautious and treat mouse data more as a lead to expected findings in the human setting.

The \textit{in vitro} and \textit{in vivo} molecular techniques that have been the mainstay of complement I/R research are now being aided by a new era of -omics based research. Traditional bioinformatics and chemoinformatics have already been used widely in drug-target elucidation. It is however clear that a better understanding of the genetic expression and transcripts involved in complement induced I/R injury is needed in order to develop new classes of therapeutics to either alter the expression levels of complement genes or to inhibit specific transcripts. It is also of vital importance to understand the genetic polymorphisms that are involved or influenced by I/R\textsuperscript{72}. Because virtually every drug and drug candidate functions at the molecular level, one practical approach forward is to raise the bar by requiring molecular detail in the animal model studies indicating whether the model mimics or fails to mimic the molecular behavior of key genes, key pathways, or the genome-wide level thought to be important for the relevant human disease\textsuperscript{75}. 
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