Inflammation in ischemia and reperfusion: From mice to men

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GENERAL INTRODUCTION AND OUTLINE OF THE THESIS
SCOPE OF THIS THESIS

The gut is the largest immune organ in the body and may be considered as one unit, functional, anatomical, and metabolical, together with the liver 1. This thesis addresses the cross-talk between basic science and clinical practice in presenting preclinical and clinical studies on inflammatory mediators in intestinal and liver surgery. Molecular mechanisms of ischemia and reperfusion of the gut and liver are investigated in animal models and examples of translation into the human clinical setting are presented. These studies were undertaken with the aim to identify much-needed novel therapeutic options to prevent or ameliorate organ damage due to ischemia and reperfusion in the clinical setting.

Ischemia and reperfusion

Ischemia and reperfusion is a pathological condition which occurs when an initial deprivation of blood flow to tissues or an organ is followed by subsequent restoration of perfusion and concomitant reoxygenation. Perhaps surprisingly, restoration of blood flow and reoxygenation is frequently associated with an exacerbation of tissue injury and a profound inflammatory response (called ‘ischemia-reperfusion (IR) injury’). Ischemia and reperfusion injury contributes to pathology in a wide range of conditions, such as shock, tissue transplantation, myocardial infarction, stroke, certain infections, and arterial disease and trauma. Furthermore, exposure of a single organ to ischemia and reperfusion (for example, the liver) may subsequently cause inflammatory activation in other organs (for example, the intestine), eventually leading to multiorgan failure 1. This multiple organ dysfunction syndrome is the leading cause of death in critically ill patients 2, in particular those with gastrointestinal, liver and skeletal muscle I/R injuries, aortic occlusion-reperfusion, and circulatory shock 2-4. The inflammatory response and multiorgan failure in surgical and critically ill patients is hypothesized to be exaggerated further by the passage of enteric bacteria and endotoxins across the intestinal mucosal barrier to local mesenteric lymph nodes and ultimately to other organs (gut origin of sepsis) 5-7. Despite the fact that ischemia and reperfusion typically occur in a sterile environment, activation of innate and adaptive immune responses occurs and contributes to injury, sharing many features with activation of host immune responses towards invading microorganisms, including activation of the complement system and the attraction of inflammatory cells into the diseased organ 8. This resemblance of response patterns to tissue injury and infecting micro-organisms is explained by involvement of pattern recognition receptors such as toll-like receptors in both conditions. These receptors recognize molecules released during tissue injury, which are called damage-associated molecular patterns or DAMPs, as well as molecular structures present on many pathogens, which are known as pathogen-associated molecular patterns or PAMPs 9,10.

Ischemia and reperfusion: inflammatory mediators

The inflammatory cascade triggered by reperfusion of ischemic tissue has been a focus of elaborate research throughout the previous decades, as enhanced insight into this chain of events can target the selection of future therapies of tissue ischemia. Research into this field has identified key molecular and signaling players that mediate, modulate, or augment cellular, tissue, and organ injury during this disease process, as depicted in Figure 1. Ischemia and reperfusion activates cell death programs, including apoptosis (cell shrinkage, cell membrane blebbing, nuclear fragmentation, and loss of mitochondrial membrane potential and integrity), autophagy-associated cell death (cytoplasmic vacuolization, loss of organelles and accumulation of vacuoles with membrane whorls) and necrosis (progressive cell and organelle swelling, plasma membrane rupture and leakage of proteases and lysosomes into the extracellular compartment) 13,14.

Moreover, limited oxygen availability (hypoxia) as occurs during the ischemic period is associated with impaired endothelial cell barrier function 15 due to decreases in adenylate cyclase activity and intracellular cAMP levels and a concomitant increase in vascular permeability and leakage 16. During ischemia and reperfusion, innate recognition proteins can be self reactive and initiate inflammation against self tissue in a manner similar to the response triggered by pathogens (known as ‘innate autoimmunity’) 17. Studies have demonstrated a role of so-called ‘natural’ antibodies in reperfusion injury 18-20. Natural antibodies consist of pre-existing antibodies that arise spontaneously without apparent antigen exposure early in life. Therefore, natural antibodies exhibit a stable and restricted repertoire that is thought to be a product of natural selection and which provides them with broad specificity for diverse structures, such as phospholipids, carbohydrates, glycoproteins, and nucleic acids.

Figure 1. Biological processes of ischemia and reperfusion
Natural antibodies are mostly antibodies of the IgM isotype with germline encoded variable regions that provide them with specificity for both microbial and altered self antigens. Thus, natural IgM possess an important function in the first line defense against invading pathogens and in tissue homeostasis by regulating the clearance of cellular debris. Neoepitopes expressed on ischemic tissues may be targets for natural antibody binding during the reperfusion phase with subsequent activation of the complement system, neutrophil recruitment and tissue injury. Hence, ischemia and reperfusion is characterized by autoimmune responses, including natural antibody recognition of neoantigens and subsequent activation of the complement system.

To complicate matters further, an ischemic organ may not immediately regain its perfusion after successful reopening of the vascular supply system due to structural disruption or obstruction of the microvasculature, the so-called no-reflow phenomenon. Tissue edema, endothelial disruption, plugging of capillaries by activated neutrophils and microthrombi, inflammation due to the generation of oxygen-free radicals and activation of complement components, and contraction of neighboring cells are all promoted by reperfusion. Thus, the no-reflow phenomenon results partly from reperfusion injury.

Ischemia and reperfusion: the role of complement

One of the key inflammatory mediators of ischemia and reperfusion is the complement system, which consists of more than 30 fluid-phase and membrane-bound proteins. Complement proteins in plasma amount to more than 3 grams per litre and constitute approximately 15 percent of the globulin fraction. The complement system acts as an immune surveillance system to discriminate among healthy host tissue, cellular debris, apoptotic cells and foreign intruders, varying its response accordingly. Precursorzymogens of the complement proteins circulate in inactive form to be activated locally at sites of infection. During activation, a sequence of enzymatic steps involving cleavage products results in a huge amplification of sterile inflammation during ischemia and reperfusion through complement-mediated recognition of damaged cells and anaphylatoxin release, thereby fueling inflammation and the recruitment of immune cells. The complement system comprises three pathways: the classical pathway, the alternative pathway and the lectin pathway. Each pathway leads to complement activation on pathogen surfaces and is triggered by different molecules on these surfaces.

In the inflammatory phase of ischemia and reperfusion, membrane phospholipids and mitochondrial proteins are redistributed (“flip flopped”) and exposed, enabling direct binding of C1q or mannosebinding lectin (MBL) or indirect binding of complement proteins via molecules such as natural IgM antibodies or C-reactive protein (CRP). Bound C1q and MBL subsequently activate the classical and lectin pathways of complement. Furthermore, complement activation appears to contribute to tissue necrosis following ischemia, amongst others because necrotic cells and tissues lack the regulatory molecules that prevent the activation and binding of complement to normal tissues.

Ischemia and reperfusion: the role of complement in animal models

A multitude of studies have provided compelling evidence for a role of complement activation in ischemia and reperfusion injury using inhibitors of complement components. Furthermore, knock-out mice deficient in specific complement proteins are protected from local and remote injury in I/R models including intestinal I/R, hepatic I/R, myocardial I/R, skeletal muscle I/R, kidney I/R, lung I/R, brain I/R and systemic shock. Consequently, inhibition of the complement system is considered to be a potential target for the treatment of ischemia and reperfusion injury.

Whereas the role of complement in animal I/R models is well established, the contribution of each specific pathway to the initiation of I/R injury remains controversial. The classical pathway of complement has traditionally been regarded to be the main pathway leading to complement-mediated I/R damage. A single type of natural antibody prepared from a panel of B1 cell hybridomas (IgM) has been demonstrated to restore reperfusion injury in antibody-deficient mice, suggesting that reperfusion injury can be considered to be an autoimmune type phenomenon. In mouse models of skeletal muscle and intestinal reperfusion injury, a highly conserved region within non-muscle myosin heavy chain type II A and C was identified as a self target for natural IgM in the initiation of reperfusion injury. More recently, additional neo-epitopes have been identified, for example, the soluble cytosolic protein annexin IV. Together, these studies indicate that neo-epitopes expressed on ischemic tissues are targets for natural antibody binding during the reperfusion phase with subsequent complement activation, neutrophil recruitment and tissue injury. Another potent activator of the classical pathway of complement, C-reactive protein (CRP), has been suggested to play a role in complement activation in a rat model of intestinal ischemia and reperfusion, as depositions of CRP and IgM correlated well with local complement activation. Furthermore, supplementation with human CRP has been shown to increase experimental myocardial infarct size by 40% in a rat model.

Alongside described effects mediated by the classical pathway of complement, the lectin pathway has recently gained increasing attention. Functional inhibition of MBL binding to stressed endothelium inhibited complement activation and subsequent C3b deposition. Preservation of rat myocardium from myocardial I/R injury, inflammation and complement activation was observed upon anti-MBL-A monoclonal antibody treatment. Hence, pinpointing the molecular events leading to complement activation in I/R-injury to the different pathways remains a subject of debate.

Ischemia and reperfusion: inflammatory mediators and complement in human diseases

The fact that the relative role of each pathway appears to differ among the organ involved in ischemia complicates matters. Furthermore, ischemic syndromes probably represent a heterogeneous group of conditions, despite several similarities in the biological responses. Essential differences include a systemic reduction in perfusion, such as occurs during shock, versus local ischemia and reperfusion of a single organ. Similarly, differences in inflammatory
responses to ischemia may result from the temperature of the jeopardized organ. Warm ischemia - as occurs, for example, during myocardial ischemia and reperfusion - and cold ischemia - such as those that occur during organ transplantation after cooling of the organ with a cold perfusion solution - seem to have a different impact on organs10.

Hence, extrapolation of the results of animal studies on complement-mediated I/R injury into a unifying theory applicable in clinical I/R syndromes remains a major challenge. However, in order to identify new targets for future interventions, better insight into the chain of inflammatory events leading to tissue damage remains essential. One way to achieve this is to investigate involvement of complement in ischemic syndromes in the clinical setting.

Myocardial infarction is a clinical situation in which regional ischemia and reperfusion of a single organ occurs. Hence, this condition provides a good human model of ischemia and reperfusion. Many studies on complement-mediated tissue injury in humans have actually been focussing on myocardial infarction. Activation of complement has been demonstrated in areas of tissue infarction. CRP was found to be co-localized with activated complement components in infarcted tissue and co-localization of IgM with complement and CRP was demonstrated in infarcted myocardium65-67. Furthermore, the inhibition of complement has been shown to reduce the extent of tissue destruction in experimental models of myocardial ischemia and reperfusion68,69. Furthermore, C3 and C4 have been shown to correlate with the incidence of myocardial infarction in a population-based study70.

Another clinical situation with IR is patients undergoing surgery for intestinal ischemia or other types of major abdominal surgery. Patients with this condition have a high risk for developing the systemic inflammatory response syndrome (SIRS), sepsis and organ dysfunction following surgery1. Alongside the systemic inflammatory response, disturbances in microbial colonization of the gut, the largest immune organ in the body, are proposed to cause further dysfunction of immune responses51.

Ischemia and reperfusion: setting the scene for therapeutic intervention

Studies in animal models have indicated that inhibition of the complement system is an effective approach to prevent ischemia and reperfusion injury. However, clinical application of complement inhibitors to reduce tissue damage during I/R has been somewhat disappointing. Nowadays, a few anti-complement therapeutics have been approved for clinical use, such as human C1-inhibitor for hereditary angioedema72 and Eculizumab, a humanized anti-C5 monoclonal antibody for the treatment of paroxysmal nocturnal hemoglobinuria73. As yet, no complement inhibitor has been approved for use in the setting of IR injury, e.g. for the treatment of myocardial infarction or in organ transplantation. However, several complement inhibitors have been used either as rescue therapies or in clinical studies. In a phase II trial in patients with ST-elevation myocardial infarction undergoing percutaneous coronary intervention, the C5 complement inhibitor Pexelizumab did not measurably influence infarct size, but significantly reduced 90-day mortality74. C1-inhibitor has been used as a rescue therapy in patients undergoing emergency surgical revascularisation after failed percutaneous transluminal coronary angioplasty75. A gender-specific positive effect on the incidence of death and myocardial infarction was demonstrated in two trials using sCR1 in patients undergoing cardiac surgery76,77.

The complexity of the complement system and incomplete mechanistic insight into the functional consequences of manipulating individual components of the cascade may contribute to difficulties in therapeutic targeting of complement pathways10. Important questions remain about the role of specific inflammatory mediators of tissue damage and the translation of effects shown in animal models into the human setting. Species, tissue, and organ differences represent key factors in unraveling the mechanism of tissue injury and in developing novel diagnostic approaches and therapies.
REFERENCES


OUTLINE OF THE THESIS

In this thesis, the role of the complement system and other inflammatory mediators of innate immune defence in ischemia and reperfusion (I/R) injury in animal models and human clinical conditions is investigated.

Chapter 2 presents an extensive review of literature on the complement system and the pathogenesis of I/R injury. Complement activation has been demonstrated to play an important role in I/R of a number of organs, such as the liver, intestine, heart, kidney and lung. The chapter critically reviews the role of the different pathways of complement in animal I/R models studies as well as in human disease conditions and describes therapeutic interventions targeting complement-mediated tissue injury. Hence an integrated view on molecular mechanisms involved in I/R-induced complement activation is presented.

In Chapter 3, a rat model of hepatic I/R injury is used to investigate hepatocellular injury and inflammation. I/R injury is an important cause of liver damage occurring during surgical procedures including hepatic resections. The acute phase protein C-reactive protein (CRP) and immunoglobulin M (IgM) are known activators of the classical pathway of complement and have been demonstrated to aggravate I/R injury in various organs. However, the number of studies exploring the role of CRP, IgM and the complement system in the pathogenesis of hepatic I/R injury is limited. In this chapter the time course of complement activation, IgM and CRP are related to tissue damage and inflammation in order to gain insight into the mechanism of hepatic I/R injury.

In Chapter 4 the setting of hepatic I/R injury is further explored. Remnant liver function after partial liver resection is influenced by a number of factors, including the extent of resection, hepatocellular damage induced by I/R injury as well as regenerative capacity. Hence, reduction of I/R injury potentially reduces the risk of postoperative liver failure. The local anaesthetic and anti-arrhythmic agent lidocaine has been reported to reduce I/R injury in the heart, lung and brain. This chapter investigates the effect of systemic lidocaine on hepatocellular damage and liver function using two distinct rat models of hepatic I/R injury.

In Chapter 5 a murine intestinal model of I/R injury is used to explore tissue damage. As only trace amounts of CRP occur in the circulation of wild-type mice, this system is employed as a natural knock-down system for evaluation of the role of CRP. We first evaluated binding of human CRP to murine apoptotic cells in vitro and thereafter assessed the effects of this protein on tissue injury in the I/R model.

In Chapter 6 intestinal I/R injury is further investigated. In several studies mice deficient in immunoglobulin production (Rag1⁻/⁻) were shown to be protected from local and remote intestinal I/R injury. More specifically, in this model the lack of natural IgM recognizing neo-epitopes in ischemic tissue is claimed to result in deficient complement activation upon I/R. As Rag1⁻/⁻ mice are devoid of endogenous CRP or IgM, we used the intestinal I/R model to address the question whether human IgM or CRP can compensate for the lack of mouse endogenous natural IgM to induce complement activation in I/R. Hence, histological damage upon intestinal I/R was evaluated in the intestinal I/R model in Rag1⁻/⁻ and wild-type (WT) mice. Furthermore, tissue damage after supplementation with known complement activators IgM and CRP was evaluated.

In Chapter 7 a human model of intestinal tissue damage is studied to assess outcomes of surgery. Patients undergoing surgery for acute occlusive mesenteric ischemia represent a heterogeneous patient category with an extensive degree of tissue damage. This retrospective review examines our center’s experience in diagnosis and treatment of this patient category and identifies risk factors of mortality.

In Chapter 8 ischemia and reperfusion is examined in human acute myocardial infarction. A subset of circulating natural antibodies (anti-phosphorylcholine IgM) and binding of IgM to damaged cells may enhance infarct size and the post-infarct inflammatory response. This hypothesis is investigated by evaluation of these parameters, as well as measuring circulating levels of inflammatory mediators such as activated complement, CRP, interleukin-6 (IL6), interleukin-8 (IL8), and secretory phospholipase A2 to assess the post-infarct inflammatory response.

Chapter 9 explores the phenomenon of impaired intestinal permeability as observed after ischemic periods in patients undergoing major abdominal surgery. In a randomized controlled trial, the cascade of events consisting of mucosal damage, intestinal permeability, bacterial translocation and subsequent local inflammatory responses is investigated. The effect of prophylactic administration of probiotics and selective decontamination of the digestive tract on perioperative gut barrier function is studied in comparison with standard treatment to gain insight in the chain of events leading to postoperative inflammatory responses.

In Chapter 10 the outcomes of this thesis are summarized and discussed.

Finally, Chapter 11 describes recent developments in complement-mediated I/R injury and discusses challenges and future directions in the translation of animal models to human disease.