Molecular mechanisms of complement activation by damaged cells
Ciurana, C.L.F.

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MOLECULAR MECHANISMS OF COMPLEMENT ACTIVATION DURING ISCHEMIA AND REPERFUSION

Caroline L.F. Ciurana\textsuperscript{1} & C. Erik Hack\textsuperscript{1,2}

\textsuperscript{1}Sanquin Research, Department of Immunopathology, Amsterdam
\textsuperscript{2} VU Medical Center, Department of Clinical Chemistry, Amsterdam,
The Netherlands

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Summary

Reperfusion of ischemic organs may induce an inflammatory reaction that damages the reperfused tissues. This negative effect of reperfusion is also known as ischemia/reperfusion (I/R) injury. Among the inflammatory processes involved in the pathogenesis of I/R injury, is activation of complement. Studies in animal models have indicated that inhibition of complement activation potentially may limit I/R injury. However, the molecular mechanisms of I/R-induced complement activation are not clear. In this article we will shortly review the evidence that complement activation contributes to I/R-injury. In addition, we will discuss the molecular mechanisms of this activation, described in the literature. It is concluded that complement is an important mediator of I/R-injury, probably also in humans. The molecular mechanisms causing this activation, likely are multiple and include binding of several proteins such as natural IgM antibodies, mannan binding lectin (MBL) and C-reactive protein (CRP), to the ischemic endothelium. Future studies should reveal to what extent these mechanisms may explain I/R-induced complement activation in humans.

Introduction

Ischemia (I) is a frequent clinical problem occurring amongst others in myocardial infarction, transplantation, vascular surgery and thrombo-embolic disease. The obvious treatment of ischemia is reperfusion of the jeopardized organ or tissue. However, in case ischemia is too severe, reperfusion may exacerbate rather than limit tissue damage, a phenomenon known as I/R-injury. A number of studies have been done to unravel the pathophysiology of I/R-injury, and it is now clear that this condition is mainly due to an inflammation elicited by reperfusion. Hence, limitation of I/R-induced inflammatory reactions is expected to improve the efficacy of reperfusion. Indeed, inhibition of inflammatory mediators improves the beneficial effects of reperfusion of ischemic tissues in animal models. In this article we will summarize the role of inflammation in the pathogenesis of I/R-injury, and discuss in more detail that of the complement system, as this plasma cascade system seems to be a key mediator in this condition.
The inflammatory cascade

Inflammation is an extremely complex interaction of an array of so-called inflammatory mediators resulting in activation of various cells such as leukocytes, endothelial cells and platelets, and in the generation of aggressive molecules including multispecific proteases, oxygen and nitrogen radicals, and various lipid mediators, which degrade proteins and other biomolecules, and cause dysfunction and death of cells. Clinical manifestations of inflammation may range from the classical signs of rubor, calor, tumor, dolor and functio laesa, to organ dysfunction and severe shock. A simplified scheme describing the inflammatory cascade is given in figure 1.

According to the scheme outlined in figure 1, cytokines should be considered as primary inflammatory mediators orchestrating a number of down-stream inflammatory effector mechanisms. The pro-inflammatory effects of cytokines are numerous and include activation of various cells such as endothelial cells, leukocytes and platelets. Part of their effects is exerted by activating nuclear factor kappa B (NF-kB), which induces the synthesis of other cytokines and inflammatory proteins. For example, the expression of adhesion molecules such as Intracellular adhesion molecule (ICAM-1), which increases upon I/R [1], is under control of NF-kB. As depicted in figure 1, plasma cascade systems such as the coagulation and the complement systems, endothelial cells, polymorphonuclear neutrophils (PMN) and platelets cells may be considered as secondary inflammatory mediators. Coagulation is activated when endothelial cells or blood mononuclear cells are stimulated by the cytokines tumor necrosis factor (TNF) or interleukin (IL)-1: the expression of tissue factor, which interacts with coagulation factor VII, is induced, whereas anti-coagulant molecules such as thrombomodulin are down-regulated. Finally, tertiary inflammatory mediators such as multispecific proteases, oxygen and nitrogen radicals are generated, which degrade proteins and phospholipid membranes and induce cell damage and death.

Role of inflammation in I/R-injury

Involvement of a variety of inflammatory mediators in I/R-injury has been demonstrated by a number of studies. In general, these studies have shown a) the activation or release of inflammatory mediators following I/R-injury; b) reduction of I/R-injury upon treatment with anti-inflammatory drugs; and c) reduced I/R-injury in animals deficient in inflammatory mediators.
Increased plasma levels of cytokines including TNF, IL-6, IL-8 and others, and increased mRNA levels of pro-inflammatory cytokines support the notion that these mediators play a role in the pathogenesis of I/R-injury. Notably, this increase occurs within hours after reperfusion of ischemic tissues. For example, mRNA of pro-inflammatory cytokines is upregulated in parallel with a decreased expression of superoxide dismutase (SOD) and a rise in oxygen radicals in rat gastro-intestinal I/R model [2]. Definite proof for a role in the pathogenesis of I/R-injury was provided in studies showing that inhibition of cytokines such as TNF, with neutralizing antibodies reduces I/R-injury, as was found in a hind limb ischemia model in rats [3]. Also in human I/R-injury cytokines likely play a role. Plasma levels of IL-6 increase in patients undergoing cardio-pulmonary bypass [4]. Notably, not only pro-inflammatory cytokines increase upon I/R but also anti-inflammatory cytokines such as IL-10 [5], presumably to off-set inflammation in the later stages of I/R.

Among the multiple effects of pro-inflammatory cytokines is stimulation of endothelial cells. Stimulated endothelium actively contributes to inflammatory processes by promoting coagulation, secreting mediators such as cytokines, vasoactive compounds and chemoattracting agents, and expressing adhesion molecules. These processes orchestrate the infiltration of leukocytes in the inflamed tissues. Adhesion molecules, for example selectins, are upregulated during I/R [6], which allows the transmigration of PMN into the ischemic tissue. These cells enhance tissue damage by producing oxygen radicals and other cytotoxic components. Many animal models have shown that the activation of PMN (as well as of complement, see below) during I/R may provoke injury in remote organs, particularly the lungs [7,8]. Both endothelial cells and PMN secrete platelet activating factor (PAF), which activates platelets and PMNs. As a result of the interaction of the endothelium with activated PMN, the endothelium may become dysfunctional. Indeed, impaired functions of ionic canals with influx of Ca2+, formation of reactive oxygen species (ROS), generation of lipid derived mediators and deregulation of nitric oxide (NO) metabolism are among the features observed with endothelial cells exposed to I/R. Damage to the endothelium may result in enhanced vascular leakage, thrombus formation and loss of regulation of microcirculatory flow [9-12]. These phenomena may increase hypoxigenation of the tissues upon reperfusion. This phenomenon is also enhanced by another mechanism involving PMN. During I/R these inflammatory cells become activated within the blood vessels. As a consequence of activation PMN become stiff, and due to their relatively large diameter plug capillaries, which have a smaller diameter. This mechanism is thought to explain the so-called "no reflow" phenomenon, which is often observed when severely ischemic tissues are reperfused. Indeed,
evidence for a role of neutrophils in I/R-injury was provided by studies showing that neutropenia is associated with less I/R-related injury [13]. Furthermore, blockade of ICAM-1 also attenuates I/R-injury [14], suggesting that the interaction of activated PMN with the ischemic endothelium, is an important event in the pathogenesis of I/R-injury.

**INFLAMMATORY CASCADE**

![Diagram of the inflammatory cascade with various mediators, activators and effectors.](Image)

Figure 1: Inflammatory cascade with various mediators, activators and effectors.
The complement system

In addition to the mediators briefly discussed above, the complement system constitutes an important mediator of I/R-injury. Complement has a major function in innate immunity but it also contributes to adaptive immune reactions in particular at low antigen concentrations [15]. The complement system is composed of more than 30 proteins, most of which circulate in peripheral blood as an inactive zymogen. During activation, the various complement components are activated by limited proteolysis in a process, in which one component activates the next one in the cascade and so on. Ultimately, the membrane attack complex (MAC) consisting of the components C5, C6, C7, C8 and polymeric C9 is formed, which can induce cell lysis. Upstream to C5 and central in the complement cascade is the third component, C3. C3 can be activated via various pathways, that are the classical, the alternative and the mannan binding lectin (MBL) pathways (see figure 2). Immune complexes containing IgM or IgG are well known activators of the classical pathway.

This pathway is also activated by other agents, including bacterial components and C-reactive protein (CRP) [16]. Activators of the classical pathway bind and activate the first complement protein, C1. C1 consists of three components, C1q, which binds to the activator, and the pro-enzymes C1r and C1s, which both are present as dimers in the C1 complex, and become active enzymes upon activation. Activated C1s activates C4 and C2, which together forms the bimolecular C4b2b complex. This complex is a C3-convertase being able to activate C3.

Bacterial products can activate complement in an antibody-independent fashion via at least two different mechanisms. One of these involves alternative pathway activation, which leads to the formation of the C3bBb complex. This bimolecular complex, another C3-convertase, is stabilized by properdin (P). The other mechanism starts with the binding of MBL, a protein resembling C1q, to sugar residues, particularly mannose, in the bacterial wall. Bound MBL then will activate so-called MBL-associated serine proteinase-1 and/or -2 (MASP-1 and MASP-2, respectively), which are homologous to C1r and C1s. Activated MASP can either directly activate C3, or indirectly via activation of C4 and C2. Many molecular aspects of this MBL-pathway are not clear yet. There is evidence for the involvement of other collectins (MBL and C1q both belong to this superfamily of proteins) such as ficolin. Furthermore, another MASP, MASP-3, has been identified recently, but its role is far from clear yet.
Inflammatory effects of complement activation products

All three pathways of complement ultimately lead to the activation of C3 and the formation of MAC, which can penetrate into the cell membrane to form a pore. Insertion of various MAC complexes in the membrane induces lysis of the cells. Smaller amounts of MAC are not necessarily lethal to cells, but rather may signal the cells to produce inflammatory mediators, which in part results from NF-κB activation. Among the effects of sublytic amounts of MAC are adhesion molecule expression and the synthesis of pro-inflammatory cytokines [17,18]. Also C5b-7 and C5b-8, incomplete forms of MAC, can activate intracellular mechanisms [19]. MAC is not the only inflammatory complement product. During activation of C4, C3 and C5 small peptides, C4a, C3a and C5a, respectively, are cleaved off, which because of their biological effects are known as the anaphylatoxins. In particular C5a is a potent peptide being able to attract, activate and degranulate PMN, and to induce bronchoconstriction and vasodilation due to its effects on smooth muscle cells (contraction in the airways, relaxation in the arterioles, respectively). Furthermore, the anaphylatoxins are able to activate and degranulate mast cells and platelets, and have many more inflammatory effects [20-22].

Regulation of complement activation

In order to control excessive activation and to overcome unwanted detrimental effects, complement activation is regulated at various levels. Two main classes of regulators exist: fluid phase and membrane-bound regulators. Some regulators act as enzyme inhibitors (Cl-inhibitor, C1-Inh), some are protease (factor I) that serve to degrade active complement components into inactive species, whereas others, such as factor H, compete with cofactors of the complement enzymes [15,23]. Factors H and I are fluid phase inhibitors that regulate amplification and the alternative pathway, whereas C4-binding protein (C4bp), C1-Inh and factor I regulate both the classical and the MBL pathways. Membrane cofactor protein (MCP or CD46), decay accelerating factor (DAF or CD55), and complement receptor 1 (CR1 or CD35) are membrane-bound proteins that inhibit all pathways at the level of the C3 and C5 convertases [24].

The formation of MAC is inhibited by several plasma proteins such as vitronectin S protein and Clusterin SP40, apolipoprotein J, AI and All, but also by the membrane-bound complement regulators, C8 binding protein (C8bp), and protectin (CD59). The membrane-
bound regulatory proteins are differently expressed depending on the cell type [25]. Endothelial cells in general are well endowed with membrane-bound complement regulatory proteins, presumably to protect these cells from so-called innocent bystander lysis by activation processes induced by complement activators in the neighborhood. This protection may be lost upon I/R: glycosyl phosphatidyl inositol (GPI)-anchored regulators CD35 and CD59 are released during inflammation, which renders the cells more sensitive to complement attack. This was shown both in vitro [26], in a rat model for acute myocardial infarction (AMI), as well as in human AMI [27,28]. Notably, membrane-bound complement regulators may be upregulated upon I/R as well, though this phenomenon needs more than 24h, and hence is probably not important for the protection of cells during I/R injury, as this damage occurs within a few hours after reperfusion [29,30].

Complement activation during I/R

Complement involvement in the inflammatory process induced by I/R has been proposed more than 3 decades ago by Hill et al. [31]. Since then, a number of studies have shown that complement is activated during I/R-injury [24,32-36]. In general, this activation occurs within minutes after reperfusion to diminish within 30 to 60 minutes [35]. To assess whether complement activation contributes to the pathophysiology of I/R-injury or constitutes an epiphenomenon, initially the effect of cobra venom factor (CVF) was evaluated in animal models. CVF binds to factor B, which subsequently becomes activated by factor D, and activates native C3 into C3a and C3b, and C5 into C5a and C5b. As it is not regulated by factors I and H, the complex of CVF with factor B constitutes an unregulated C3-convertase which within 1-2 hours depletes circulating complement, an effect that may last up to 24-48 hours. Hence, pretreatment with CVF renders an animal virtually complement-deficient during this period. CVF pretreatment reduced I/R-injury in a number of animal models [37,38]. As CVF extensively depletes the complement system by activation within the vascular compartment, its application is associated with serious side effects such as dyspnoea, hypotension and others. Hence, this agent cannot be used in clinical practice. Last decade a number of complement inhibitors, which do not have this disadvantage of intravascular activation of the system, has been developed and evaluated for their effects in animal models for I/R-injury. All these inhibitors, which include C1-Inh [32,33], sCD35 or sCR1[34-36] as well as DAF/MCP hybrid molecules [39] showed beneficial effects in these models. Antibodies against C5 or the MAC complex also showed beneficial effects [40-42].
Final proof for the involvement of complement in I/R-injury came from experiments in complement-deficient mice [43], which showed significantly less I/R-injury than their wild-type littermates. The deficient animals developed normal I/R-injury when supplemented with the lacking protein. The studies discussed above clearly demonstrate that complement is activated during I/R and contributes to the injury induced by reperfusion.

Likely the most toxic complement activation products are generated at the level of C5, since inhibition of C5 activation by monoclonal antibodies, which allow C3 activation, reduces I/R-injury. These activation products likely are C5a and/or MAC, as is discussed in a previous paragraph.

Mechanisms of complement activation during I/R

A number of studies have shown that the classical, the alternative as well as MBL pathways could be involved in the development of I/R-injury depending on the model, the type of tissue and the time course of inflammation [44].

Involvement of IgM

IgG or IgM antibodies bound to antigens are strong activators of the classical complement pathways. Observations in mouse models have revealed that in particular IgM may be involved in the pathogenesis of I/R-injury. Initial experiments with mice deficient in rag (RAG1-/- (C57BL/6J-RAG1™/Mom) and RAG2-/-), and hence unable to form immunoglobulins, had about half the I/R-injury in a hind-limb model as compared to wild-type mice. Deficient mice substituted with polyclonal IgM had I/R-injury comparable with wild-type mice. Finally, it was established that upon reperfusion IgM bound to the endothelium in the ischemic tissues, although the epitopes for this IgM are not known at present [45]. Similar results were obtained in a mouse model for intestinal I/R [46].

Involvement of C-reactive protein (CRP)

CRP is an acute phase protein that upon binding to a ligand can activate the classical complement pathway. It also inhibits the activation of the alternative pathway by increasing the binding of regulator factor H to C3b [47]. A ligand for CRP may be lyso-phospholipids generated in the cell membrane [16,48]. These lyso-phospholipids may be generated in a membrane by interaction with phospholipase A2 (PLA2) with a flip-flopped membrane [49], as occurs in damaged cells.
In a post-mortem study of AMI patients, CRP was found to be co-localized with activated C3 and C4 on cardiomyocytes [50], suggesting this mechanism may be relevant for ischemia of tissues in humans. Human CRP indeed was found to enhance reperfusion-induced cell death in a rat model for myocardial infarction [51]. Pasceri et al. [52] have shown in vitro that endothelial cells exposed to I/R, had an increased expression of adhesion molecules (ICAM-1, VCAM-1 and E-Selectin) upon binding of CRP in the presence of serum, an effect that likely is complement-mediated [50].

**Mitochondrial disturbance**

Many in vitro data emphasized a role for mitochondria in the onset of complement activation by I/R injury. In these studies mitochondria were shown to be able to activate complement when added to normal human serum. Sera deficient in immunoglobulins allowed complement activation to a similar extent as normal sera, ruling out involvement of immunoglobulins in this activation process [53]. A few candidate proteins being able to activate C1 have been purified from mitochondria. Also cardiolipin was described to bind C1q and was detected in vivo in cardiac lymph upon reperfusion [54]. In another study from Giclas et al. [55], both classical and alternative pathways were activated in serum incubated with human heart mitochondrial membrane constituents. Though the molecular mechanism causing complement activation by mitochondria is not completely resolved yet, these studies clearly demonstrated an intriguing mechanism of complement activation. However, whether this mechanism really contributes to complement-mediated damage during I/R is not clear since it requires exposure of mitochondria to extracellular proteins, i.e. complement components. This implies that the cells providing mitochondria for this activation process are already permeable, and probably dead.

**Other mechanisms of activation**

Many studies have been undertaken to elucidate the mechanism of activation of the complement during I/R. Various activation mechanisms other than those described above, were suggested, and included activation of the classical, MBL but also of the alternative activation pathways. Since activation of C4 has been observed without apparent involvement of immunoglobulins, the role of the MBL pathway in I/R was studied. Indeed, cultured endothelial cells exposed in vitro to hypoxia followed by reoxygenation bound MBL [56]. Cytokeratin 1, which is upregulated upon I/R, was identified as a ligand for MBL [57]. Although a role for MBL was also pointed out in I/R in vivo as well [58], it remains to be
Figure 2: Complement activation cascades. The 3 different pathways are depicted here and inhibitors are also indicated in bold. Inflammatory mediators are indicated in bold, italic, underlined symbols.
established to what extent this mechanism contributes to I/R-injury in man.

Erythrocytes, of which the membranes are disturbed and flip-flopped upon oxidative stress, were shown to activate complement in vivo via the alternative pathway. This induced recruitment of neutrophils and amplified the inflammatory process [59-61]. A flip-flop of the cell-membrane likely occurs on endothelial cells upon I/R. Thus, it is conceivable that a similar activation process may take place on these cells, at least during I/R. Subendothelial extracellular matrix, exposed to blood upon endothelial disruption, also has been suggested to activate complement [62]. In addition, oxidized molecules generated by I/R such as oxidized LDL [17], were found to activate proteases that in their turn activate the alternative pathway of complement. Apoptotic endothelial cells can also activate the classical complement pathway in vitro. Among the mechanisms causing this activation, IgM deposition may be involved [63]. On the other hand, evidence is accumulating that apoptotic cells directly or indirectly via adaptor molecules such as CRP or serum amyloid P component [64] may activate complement.

Conclusions

It is now well accepted that complement plays a role in the initiation of I/R injury in animals. However, whether this occurs in humans as well remains to be established. Inhibition of complement reduces I/R-injury significantly, though not completely. Hence, complement inhibitors constitute an attractive option for therapy in clinical conditions resulting from I/R-injury. Complement is likely activated during I/R via multiple mechanisms. Unraveling the relative contribution of these mechanisms to I/R-injury in man, probably allows for the best therapeutic approach to reduce this injury.

Literature


Molecular mechanisms of complement activation during ischemia reperfusion
