Commentary

Complement in Ischemia Reperfusion Injury

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In the current issue of The American Journal of Pathology Stahl et al describe an important role for the alternative complement pathway in ischemia reperfusion (I/R) of the intestine, using factor D-deficient mice. The requirement for the complement system in I/R-induced tissue injury has been established, but the contribution of the various complement pathways (classical, alternative, and lectin), is largely unknown because, until recently, the tools for such an investigation were not available. The complement split product, C5a, and the terminal membrane attack complex, C5b-9 (MAC), are believed to be responsible for complement-mediated I/R-induced tissue injury, and, therefore, may be targets for therapeutic interventions in clinical settings. More precise understanding of the relevant complement pathway(s) involved in the final generation of C5a and MAC will define the options for therapeutic intervention.

I/R-Mediated Tissue Injury

During ischemia, cells and tissues undergo rapid changes which lead to perturbations in signaling pathways and surface molecule expression. Depending on the time and severity of ischemia, toxic products accumulate intracellularly, leading to apoptosis and necrosis, resulting in loss of organ function. At a certain duration and severity of ischemia, injury may be completely or partially reversible or irreversible. Besides ischemia caused by different types of vascular occlusion, as in most cases of myocardial infarct, ischemia injury also appears to play a major role in organ transplantation. After re-establishment or re-connection of the vasculature to the circulation, oxygen is re-applied and repair mechanisms are set into place. During the time of re-perfusion, accumulated toxic metabolites are flushed into the system, which may affect other organs and may negatively influence the process of regeneration in the ischemic organ.

In injury due to ischemia, two major components involved in events leading to injury are well known, namely, complement activation and neutrophil stimulation with accompanying oxygen radical-mediated injury. Under ischemic conditions, reduced oxygen supply leads to enhanced neutrophil adherence to endothelial cells due to increased surface expression of adhesion molecules on endothelial cells. This ultimately results in diapedesis of neutrophils and their oxidative burst, which results in oxygen radical production. These events are thought to contribute to the tissue damage during ischemia reperfusion in various organs.

In I/R injury, complement activation was described during myocardial infarction over 30 years ago and has led to numerous investigations on the contribution of the complement system to I/R tissue injury.

Pathways of the Complement System

The complement system can be activated through different mechanisms. The classical pathway is activated by antigen-antibody interaction, which then leads to activation of C1q, followed by C2- and C4-dependent cleavage of C3 (by C3 convertase C4b2a) and, ultimately, cleavage of C5 by formation of the C5 convertase (C4b2a3b). In the lectin pathway, serum mannose binding lectin (MBL), which is homologous to C1q, recognizes microbial surface mannose and triggers activation of MBL-associated proteases (MASP1–3). This interaction leads to the same formation of C3 and C5 convertases (as described above). The alternative pathway is activated by presence of lipopolysaccharide (LPS), and, to a certain extent, spontaneously generated C3b. In this pathway, C3 binds to factor B and forms a complex, which is cleaved by factor D to form the alternative C3 convertase, C3b(H2O)Bb. Properdin acts as an amplifying activator and stabilizes this complex, enabling the cleavage product C3b to bind to it, thus forming the alternative C5 convertase (C3b3bBb). All pathways, therefore, use C3 and cleave C5, which results in the powerful pro-inflammatory cleavage products C5a and C5b-9. These two products of complement activation are believed to be mainly responsible for I/R injury. C5a has been shown to exert numerous pro-inflammatory effects such as a chemotactic effect on neutrophils, release from phagocytic...
cells of granular enzymes,11 production in neutrophils of superoxide anion,15 vasodilatation, and increased vascular permeability.15 C5b-9 has been demonstrated to have major contribution to complement-mediated tissue injury after I/R.14,15 and causes relaxation of coronary arteries.16

The complement system is controlled by natural fluid phase inhibitors, namely factor I (for the lectin and classical pathways), factor H (alternative pathway), carboxypeptidase N (CPN, classical, and lectin pathways) and C1 inhibitor (C1INH), (classical and lectin pathways). Factor I facilitates the cleavage of C4b and C3b (together with the cofactor C4BP) and degradation of C3 and C5 convertases (together with factor H in the alternative pathway). CPN inactivates C3a, C5a, and C4a by removing the terminal arginine. C1INH inhibits C1r, C1s, and MASP. There are a few membrane-bound inhibitors such as complement receptor 1 (CR1/CD35), the membrane cofactor protein (MCP/CD46), and the decay accelerating factor (DAF/CD55), which all act as cofactors for factor I, facilitating degradation of the convertases and C3b as well as C4b.

**Blockade of the Complement System in Experimental I/R**

Following establishment of the role of complement activation in tissue injury during I/R, the complement system became a major therapeutical target for experimental I/R. In early studies, C3 depletion was achieved following infusion of cobra venom factor and reported to be beneficial during I/R in kidney and heart.17,18

In other investigations, recombinant soluble CR1 (sCR1) showed significant beneficial effect in models of myocardial I/R.19,20 experimental lung and liver transplantation,21–24 and in a model of intestinal I/R.25 sCR1 has also been suggested to provide neuronal protection in a model of stroke26 and protection in I/R injury in skeletal muscle.27

Another attempt of complement inhibition was the use of C1 esterase inhibitor, which has demonstrated especially protective effects in myocardial I/R28–30 and in models of lung transplantation.31,32 In addition, this therapy suppressed endothelial adhesion molecule expression during myocardial I/R,33 suggesting also direct influence on neutrophil-mediated I/R injury. In a recent study, the classical/lectin pathway activation was blocked using a novel C1s inhibitor in a model of myocardial I/R.34 In xenograft transplantation, a new chimeric inhibitor was developed, derived from human DAF (CD55) and MCP (CD46), two natural membrane-bound inhibitors (as above).35 An attempt was made to specifically inhibit activation of the lectin pathway by using monoclonal antibodies against rat mannose binding lectin (MBL), which resulted in reduction of post-ischemic myocardial reperfusion injury in rats.36 MBL antibodies have also reduced complement deposition on endothelial cells in vitro after oxidative stress.37

As mentioned earlier, C5a is believed to be a major factor for complement-mediated I/R tissue injury. Several studies have targeted C5 or C5a, using blocking antibodies or inhibitors. In I/R models of rat intestine, both C5 as well as C5a blockade resulted in protection from I/R injury.25,38,39 In myocardial infarcts in rats, antibodies to C5 significantly inhibited necrosis and cell apoptosis as well as neutrophil infiltration.40 In pigs, use of antibodies directed against C5a led to reduced myocardial injury and reduced coronary endothelial dysfunction after I/R.41,42

In kidney I/R experiments with C4 knockout mice, there was no significant tissue protection, suggesting a more significant role for the alternative complement pathway, since C3, C5, and C6 knockout mice were protected from injury.14 These findings were somewhat in contrast with earlier studies demonstrating that, in both C3 and C4 knockout mice, animals were equally protected from I/R injury of skeletal muscle and intestine,43,44 suggesting an important role of the classical/lectin pathway of complement activation in I/R injury. The findings have raised the question as to whether the contribution of complement activation pathways in I/R injury may be organ-dependent. Due to lack of specific inhibitors of the alternative complement pathway in rodents, data from experiments with C4 knockout mice have been the only indirect way of assessing the contribution of the alternative complement pathway for I/R injury so far.

Most attempts to inhibit complement activation during I/R injury have targeted the classical and lectin pathway (eg, C1 esterase inhibitor, C1s antagonist), the lectin pathway (MBL antibodies) or all three pathways (sCR1, anti-C5/C5a). Recent studies suggest that C1 inhibitor also regulates the alternative complement pathway by down-regulating the convertases of the alternative pathway, specifically by inhibiting C3b.45 Such data suggest that the pathways may be not as independent as originally proposed, and that “cross-talk” and interplay exist.

**Intestinal I/R and the Role of the Alternative Pathway: How Do the Findings of Stahl et al Relate to Existing Literature?**

Recently, recombinant human factor D and antibodies have become available,46 as well as factor D knockout mice.47 The study of Stahl et al reflects the first use of these animals for defining the role of the alternative pathway in complement-mediated I/R injury in the intestine.1 Knockout mice (factor D−/−) had significantly less tissue damage in the intestine and lung when compared to heterozygote control animals and also showed significantly less C3 deposition in tissues. This was accompanied by less myeloperoxidase activity, a sign for reduced neutrophil accumulation, in the intestine and lung of factor D−/− mice. Infusion of recombinant human factor D restored the injury in factor D−/− mice as demonstrated by LDH measurements, while infusion of antibodies to human factor D protected mice from intestinal I/R injury again. In addition, factor D−/− mice showed only minimal tissue staining for C3. These results demonstrate a predominant role for the alternative pathway in I/R injury in the intestine of mice. In contrast, the findings of Wil-
liams et al suggested a predominant role of the classical pathway for I/R injury in the intestine of mice by showing reduced organ staining for C3 in C4 knockout mice and protection from injury. Obviously, such findings speak against each other. It is possible that knockout mice deficient of C4 or factor D are not directly comparable, because it is well known that significant differences exist between strains. It is also possible that knockout mice are not only specifically deficient of one pathway. For instance, it has been demonstrated that factor B knockout mice also lack C2, due to close gene proximity, implicating deficiencies in all three pathways. Another possible explanation for the observed differences is that classical and lectin pathway (C4-dependent) may be required for initial complement activation, while the alternative pathway may play a crucial role for amplifying complement activation. In such a scenario, C4-deficient mice would not necessarily show complement deposition because the required initiation of complement activation would not take place and the alternative pathway could not exert its amplifying effect. From this point of view, the specific role of the alternative complement pathway should only be investigated by direct blocking of alternative pathway specific mediators such as factor B or factor D. Thus, since Stahl and colleagues used recombinant factor D and antibodies to factor D to reverse effects observed in factor D-deficient mice, their data appear to be well controlled and convincing.

Similar studies in mice suggested beneficial effects of blockade of C5 with antibodies during I/R. A recombinant soluble form of complement inhibitor (Crry) has been used in an intestinal I/R model in mice. In mice, even a delayed infusion (30 minutes after start of reperfusion) of Crry resulted in significantly reduced organ injury, which was not neutrophil dependent. An earlier study had already suggested that intestinal I/R injury in mice was unaffected by neutrophil depletion. The latter findings stand in contrast to the results of Stahl et al and to the results in the intestinal I/R models in the rat in which a clear neutrophil dependency has been described. In 1992, Hill et al showed that intestinal I/R injury in the rat was significantly decreased when animals were treated with sCR1. Parallel to these findings in the intestine, the authors described reduced MPO levels in the lung, similar to the finding in the current study of Stahl et al. The findings of Hill et al were then confirmed in a similar model, using sCR1 in rats. Since sCR1 is believed to inhibit activation of all three pathways, no pathway distinction could be made. Besides these studies, treatment with anti-C5 antibodies has been demonstrated to result in significantly reduced intestinal injury in the rat after I/R. A recent study also reconfirms these findings in the rat and points out the role of C5a by using a novel receptor antagonist, demonstrating that pretreatment with a single intravenous dose (1 mg/kg body weight), or a single oral dose (10 mg/kg), significantly inhibited intestinal edema and mucosal damage. These findings were associated with decreased TNFα and haptoglobin serum levels. This study represents the first experimental oral therapeutic success in complement blockade during I/R.

So far, intestinal I/R injury has mainly been investigated in mice and rats. Even though there seems to be a dispute about the contribution of neutrophils, all results reported suggest beneficial effects of various attempts of inhibition of complement activation. To solve the discrepancy between the findings of Stahl et al and Williams et al and to answer the question of the contribution of classical or lectin pathways to intestinal I/R injury, it will be necessary to conduct experiments that specifically block the classical pathway (eg, inhibitors of C1q) and the alternative pathway (eg, inhibitors of MBL). Such inhibitors are currently not available, but are subjects of investigation.

Complement Inhibition in Clinical Settings of I/R

Even though numerous experimental studies have demonstrated beneficial effects of complement inhibition for I/R-induced injury, there have been only a few clinical trials so far. The heart is, by far, the most studied subject in this context. Experimental data revealed early that complement products of the classical pathway were associated with acute ischemic heart injury in rats, in rabbits, and in humans. In 1990, Weismann and colleagues demonstrated that sCR1 significantly inhibited post-ischemic myocardial inflammation and necrosis. These findings were extended in the rat by the use of sCR1 containing sialyl Lewisx, enhancing the function of sCR1. Similar findings were made in cats, pigs and rats using C1 esterase inhibitors. This led to the first successful use of C1-inhibitors in patients receiving emergency coronary surgery for failed percutaneous transluminal coronary angioplasty in 1998.

In 1998, Vakeva et al demonstrated in rats that blockade of C5 with a monoclonal antibody significantly reduced the extent of myocardial injury after I/R suggesting an important role for the complement products, C5a and C5b-9. Earlier, studies had shown that blockade of C5a resulted in reduced myocardial endothelial dysfunction and reduced myocardial injury after I/R in pigs. Such studies led to the first clinical use of anti-C5 treatment for patients undergoing cardiopulmonary bypass operation. In these studies, patients treated with anti-C5 showed reduced post-operative myocardial injury, cognitive defects, and blood loss, suggesting a broad range of benefits for the patient.

In rats, treatment with antibodies to mannose-binding lectin significantly reduced myocardial I/R injury, indicating a role for the lectin pathway but until now in clinical trials, MBL has not yet been targeted.

Conclusions

Data from numerous experimental studies of I/R injury in different organ systems as well as first clinical trials support the thesis that inhibition of the complement system offers a therapeutic target for reducing harmful tissue injury in various clinical settings of I/R. So far, clinical trials in humans have focused on ischemic conditions of the heart. Given the complex "cross-talk" of the different complement pathways and the limited amount of tools to
selectively target a single pathway of complement activation, the involvement or contribution, especially of the alternative pathway, has not yet been investigated in detail. The current study of Stahl and colleagues presents the first use of alternative-complement-pathway-deficient mice in experimental I/R. The authors conclude from their findings that inhibitors of the alternative complement pathway represent potential therapeutical targets. This is certainly possible, but it would require a situation in which the alternative pathway plays a predominant role.

However, as pointed out in the information provided above, any complement activation results in production of C5a and the membrane attack complex (C5b-9), which, in concert with neutrophils, are believed to be mainly responsible for tissue injury during I/R. From this point of view, targeting C5/C5a may represent the most potent strategy to inhibit complement-induced organ injury during I/R.

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