Initiation of complement activation following oxidative stress.
In vitro and in vivo observations

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Abstract
Ischemia and reperfusion of organs/tissues induce a state of inflammation that can lead to tissue injury. Focus on development of effective therapeutics based on sound pre-clinical work and the role of leukocytes in models of human disease has not lead to a successful clinical trial for anti-leukocyte technologies. For the past >30 years, it has been known that complement activation plays a role in the inflammation and tissue injury associated with ischemia/reperfusion (I/R) injury. In the last 10 years, several complement inhibitors have made their way from the bench to bedside. Will a complement inhibitor eventually be approved for clinical treatment of I/R type diseases? What pathway(s) are involved in I/R injury, and what role do they play? What specific complement components are needed for resolution of inflammation and what components need to be inhibited to decrease tissue injury? This short review will focus on the current state of the art knowledge about complement, complement pathways, complement components and several promising clinical biologics that inhibit complement activation. This review is not a complete review of complement in ischemia/reperfusion injury, but it raises important questions about the role of complement, its pathways and the current knowledge in the area of ischemia/reperfusion injury.

Keywords: MBL, Lectin, Myocardium, Gastrointestinal, Ischemia, Reperfusion

1. Introduction
The endothelium plays an integral role in the maintenance of vascular homeostasis. One of the hallmarks of reperfusion to ischemic tissues is the severe oxidative stress that occurs at the level of the endothelium. Oxidative stress due to ischemia/reperfusion (I/R) has been recognized as a trigger in the initiation of a variety of pathophysiologic conditions. Injury induced by reperfusion (tissue subjected to ischemia followed by restoration of blood flow) is also associated with surgical events including cardiopulmonary bypass, organ transplantation and many surgical procedures.

At least three major components contribute to or initiate I/R injury: oxygen, neutrophils and complement activation. While neutrophil activation and accompanying oxygen radical production may play an important role in the pathogenesis of I/R injury in animal models, translation of these findings to the clinical setting has thus far been disappointing. In this regard, recent clinical trials aimed at taming PMN activation/adherence have failed to reach defined endpoints. Harlan and Winn (2002) have speculated and demonstrated that the reason for these failures may be that other factors, including oxidants, complement and apoptosis, may be more dominant in the clinical setting (Harlan and Winn, 2002; Iwata et al., 2002).

Complement activation and deposition on the endothelium can result in a loss of vascular homeostasis and a pro-inflammatory state. Complement activation following ischemia/reperfusion is associated with myocardial infarction (Hill and Ward, 1971; Weisman et al., 1990; Buerke et al., 1995; 1996; Vakeva et al., 1998; Amsterdam et al., 1995; Jordan et al., 2001), ischemia of the intestine (Wada et al., 2001; Fruchterman et al., 1998; Stahl et al., 2003; Williams et al., 1999; Karpe-Massler et al., 2003; Zhao et al., 2002), hindlimb (Weiser et al., 1996; Kyriakides et al., 1999; Wong et al., 1999; Brock et al., 2001; Toomayan et al., 2003) and kidney (Zhou et al., 2000; Thurman et al., 2003), hemorrhagic shock (Spahn et al., 1999), sepsis (Czermak et al., 1999) and pulmonary injury (Mulligan et al., 1996). While the exact molecular mechanisms of complement activation following oxidative stress have not been fully elucidated, evidence from in vitro and in vivo models suggest that complement activation is an early event and that inhibition of complement activation or its components may offer tissue protection following reperfusion.
2. The complement system

Three separate pathways can activate the complement system: the classical, alternative and lectin pathways (Fig. 1). In order to identify the specific contribution of individual complement pathway(s) or components, distinct depletion or inactivation of these targets needs to be accomplished. For example, elimination of either MBL or C1q specifically inhibits each respective pathway, whereas inhibition of C2 or C4 is non-specific and inhibits both pathways. Novel reagents (knockout mice and monoclonal antibodies) have been, or are currently being created to dissect out the executor of complement activation and complement-dependent injury following I/R.

3. Models examining complement-dependent I/R injury

Work over the past 15 years has established that oxidative stress results in complement activation and deposition on the vascular endothelium (Weisman et al., 1990; Collard et al., 1997, 1998, 1999, 2001). General depletion of complement (Hill and Ward, 1971; Pinckard et al., 1980), specific-complement blockade (Weisman et al., 1990; Buerke et al., 1995, 1998; Vakeva et al., 1998; Amsterdam et al., 1995; Wada et al., 2001; Jordan et al., 2001) or animals deficient of key complement components (Weiser et al., 1996; Zhou et al., 2000; Thurman et al., 2003; Ito et al., 1996) show greatly reduced tissue injury following I/R. Blockade of complement following oxidative stress decreases endothelial adhesion molecule expression (Collard et al., 1999; Buerke et al., 1998; Jordan et al., 2001), production of inflammatory cytokines (Kilgore et al., 1996; Jordan et al., 2001), and preserves endothelium-dependent relaxation (Stahl et al., 1995; Lennon et al., 1996). It has become clear that prevention of complement activation following oxidative stress may greatly decrease vascular injury, subsequent inflammation and tissue injury.

3.1. Myocardial ischemia and reperfusion (M/I/R)

Models examining complement activation following MI/R have suggested a potential novel therapeutic approach to cardiovascular disease. Inhibitors of complement activation have been developed with promising clinical applications for the prevention of patient morbidity and mortality resulting from MI/R complications (Table 1). Two of these inhibitors (e.g., TP10 and pexelizumab) have completed phase II clinical trials. Pexelizumab has additionally completed a Phase III clinical trial (PRIMO-CABG) for improving surgically induced outcomes following coronary artery bypass.

Early in vivo MI/R studies used cobra venom factor (CVF) to non-specifically deplete complement to reduce inflammation and tissue injury. Depletion of C3 by administration of CVF significantly reduced inflammation and myocardial necrosis in dogs, rats and baboons (Hill and Ward, 1971; MacLean et al., 1978; Maroko et al., 1978; Pinckard et al., 1980). However, the soluble form of complement receptor type one (sCR1; TP10) was the first complement-specific inhibitor utilized for the prevention of MI/R injury (Weisman et al., 1990). Distinct regions of sCR1 bind C4b or C3b components of the C3 and C5 convertases and displace these subunits from their respective catalytic complexes (Weisman et al., 1990). In addition, sCR1 assists in the factor I mediated cleavage of both C4b and C3b. Thus, sCR1 is a non-specific complement inhibitor capable of inhibiting the classical, lectin and alternative pathways. sCR1 treatment during MI/R attenuates infarction associated with decreased deposition of C5b-9 complexes along the coronary endothelium and decreased leukocyte infiltration after reperfusion (Weisman et al., 1990). In a similar study, administration of sCR1 prior to MI/R decreased infarct size and the infiltration of PMNs into the area at risk (AAR) (Smith et al., 1990). However, in a rat MI/R model examining an alternatively glycosylated form of sCR1 (TP20), which possesses...
the sialyl Lewis\(^x\) ligand for P-, E- and L-selectin adhesion molecules (Zacharowski et al., 1999; Foxall et al., 1992), there was no significant difference in the cardioprotection mediated by sCR1sLe\(^x\) in comparison to sCR1, as measured by infarct size and production of cardiac troponin T (cTnT marker for cardiac damage) (Zacharowski et al., 1999). Thus, the "anti-PMN" ligand modified TP-10 (i.e., TP20) data suggest that complement activation is proximal to PMN mediated injury.

Of the various forms of sCR1, TP-10 alone has completed a Phase II clinical trial in cardiopulmonary bypass. Although TP10 (sCR1 from Avant Pharmaceuticals) was well tolerated and beneficial to patients in early Phase III trials, results from a Phase II trial ending in February 2002 failed to meet its primary endpoint. However, sub-group analysis of the data from male patients in a high-risk population undergoing open-heart procedures demonstrated significantly decreased mortality and infarct size. Furthermore, this significant decrease in both endpoints was not observed in female subjects (presented at the Late Breaking Clinical Trials Session of the 2003 American Heart Association Scientific Sessions in Orlando, Florida). Thus, additional trials are planned in 2004 to assess the overall potential of TP10 in the prevention of MI/R injury following coronary artery bypass grafting (CABG).

The plasma-derived protein C1 esterase inhibitor (C1-INH) has also been adapted for inhibition of complement activation following MI/R. C1-INH covalently binds the serine proteases C1s and C1r of the classical complement pathway and the MASP-1 and MASP-2 proteases of the lectin pathway (Sahul and Lambris, 2000; Matsushita et al., 2000), and thus is also "non-specific". Further, recent data suggest that C1-INH also inhibits the alternative pathway (Jiang et al., 2001). In a feline MI/R model, administration of C1-INH attenuated infarction, decreased neutrophil infiltration, increased recovery of myocardial contractility and preserved endothelial function (Buerke et al., 1995). Similar observations were also observed in rats and pigs (Buerke et al., 1998; Horstick et al., 1997).

Human clinical trials have substantiated the cardioprotective effects of C1-INH administration during cardiopulmonary bypass (CPB) or myocardial infarction accompanied with thrombolytic therapy (Horstick et al., 2001; Horstick, 2002; de Zwaan et al., 2002). However, the protective effects of C1-INH during MI/R are dose-dependent and excess administration of C1-INH may induce severe adverse effects during a cardiac event. In an attempt to prevent capillary leakage, administration of C1-INH during CPB in a group of thirteen newborn babies induced great vein thrombosis in all patients and resulted in nine deaths (Horstick et al., 2001). An additional study analyzing the dose-response of C1-INH administered intravenously 5 to 10 min before coronary reperfusion demonstrated that when C1-INH was administered at an optimal dose, it significantly protected ischemic tissue from reperfusion injury (Horstick et al., 2001), whereas, high doses of C1-INH provoked detrimental side effects (Horstick et al., 2001). Therefore, C1-INH is a promising candidate for protection against MI/R injury, however continued dose-response analysis profiles are required to demonstrate appropriate safety and continued efficacy in the clinical setting.

The above treatment scenarios are not specific for identification of specific pathways or components responsible for tissue injury following MI/R. There has been significant success with monoclonal antibodies (mAb) directed at specific complement components for prevention of MI/R injury. In a porcine model of MI/R, animals treated with mAb to the anaphylatoxin C5a prior to reperfusion showed attenuated infarction, although this protection was not accompanied by a decrease in PMN infiltration (Amsterdam et al., 1995). Rats treated with C5 mAb (which blocks both C5a and C5b-9) demonstrated attenuated infarct size, neutrophil infiltration and apoptosis in the myocardium (Vakeva et al., 1998). This pre-clinical work led to the initiation of the recently completed phase II clinical trials (COMMA and COMPLY) discussed below.

Administration of a humanized anti-C5 scFv (pexelizumab) decreased overall patient mortality associated with acute myocardial infarction in the COMMA and COMPLY trials, but failed to meet the primary endpoint (Mahaffey et al., 2003; Granger et al., 2003). In addition to these studies, patients requiring concomitant CABG plus CPB, and treated with pexelizumab showed reduced myocardial injury and accompanying disorders during a phase IIa clinical trial (Shernan and Collard, 2001; Fitch et al., 1999). Furthermore, the findings of a phase III anti-C5 mAb clinical trials (PRIMO-CABG) were recently presented at the Late Breaking Clinical Trials Session of the 2003 American Heart Association Scientific Sessions in Orlando, Florida. Although the primary endpoint for this study was not reached, the study demonstrated an overall reduction in post-operative patient morbidity and mortality. Thus, while additional Phase III clinical trials for MI and CPB may be necessary, it is likely that pexelizumab may be approved for clinical use in the near future. The pexelizumab and TP-10 clinical findings suggest that complement is involved in cardiovascular disorders and may be involved in enhancing cardiac related deaths.

It is apparent that activated complement mediates tissue injury following MI/R; although it remains unclear which complement pathway is responsible for the initiation of complement activation. Complement inhibitors used in published data to date could not fully discriminate between the classical, lectin and alternative pathways, and therefore would not directly address this issue. However, examination of early events in complement activation following oxidative stress in vitro and in vivo have shed new light on our understanding of the initiation of complement-dependent MI/R injury. Collard and colleagues showed that C2 is required for deposition of C3 on the endothelium, indicating that the classical or lectin pathway is responsible for initiation of inflammatory events following oxidative stress (Collard et al., 1997). Further,
Reoxygenation of stressed endothelium increases complement activation and cellular C3b deposition independent of antibody (Fig. 2) (Vakeva and Meri, 1998). Depleting or inhibiting mannose-binding lectin (MBL) decreases complement activation on endothelium following oxidative stress (Collard et al., 2000, 2001; Montalto et al., 2001; Lekowski et al., 2001). Further, MBL and C3 co-localize on stressed endothelium and anti-MBL mAbs inhibit MBL deposition and complement activation (Collard et al., 1997). Additionally we have shown that MBL and C3 co-localize in vivo on rat myocardium following MI/R (Collard et al., 2000). Moreover, specifically blocking the lectin complement pathway with anti-MBL inhibitory mAbs protects rat hearts from I/R injury, reduces PMN infiltration and attenuates pro-inflammatory gene expression (Jordan et al., 2001). In support of our anti-MBL treatment in rats undergoing MI/R, we have extended these observations to complement knockout mice. At the 2004 ACC meeting, we demonstrated that C2/factor B KO mice have significantly smaller infarcts than WT mice (Walsh et al., 2004). Reconstitution of the lectin and classical pathways by addition of human C2 restored infarct size to that observed in WT mice. C1qKO mice have larger infarcts than WT mice, yet anti-C5 treatment protected the myocardium, demonstrating that while complement (i.e. activated C5) plays a role in mouse MI/R, the pathways involved do not include C1q. These data in C1qKO mice also suggest a potential mechanism for the adverse events observed in studies using large doses of C1-INH (Horstick et al., 2001). Thus, it appears that some C1q may be needed to "protect" the myocardium during oxidative stress.

### 3.2. Other important models of I/R injury

In addition to MI/R injury, the involvement of complement has become evident in models of gastrointestinal (Zhao et al., 2002; Wada et al., 2001; Stahl et al., 2003; Williams et al., 1999; Karpel-Massler et al., 2003; Frachterman et al., 1998), skeletal (Weiser et al., 1996; Toomayan et al., 2003; Brock et al., 2001; Wong et al., 1999; Kyriakides et al., 1999), and renal (Zhou et al., 2000; Thurman et al., 2003) I/R and organ transplantation. Williams et al. suggested a predominant role of the classical pathway for initiation of I/R injury in the intestine of mice by showing reduced organ staining for C3 and protection from injury in C4KO and IgM (RAG1−/−) deficient mice (Williams et al., 1999). However, loss of C4 inhibits both lectin and classical pathways. We recently found that factor D KO mice are protected from G/I/R injury, and reconstitution with human factor D restored the injury (Stahl et al., 2003). Further, we demonstrated attenuated (but not complete) C3 deposition in the lung and gut following gastrointestinal I/R compared to heterozygotes, suggesting an initiating role for either the lectin or classical pathway (Stahl et al., 2003). Recent data from our lab demonstrates that C2/factor B KO mice are protected from intestinal and pulmonary injury following G/I/R (Hart et al., 2003). Addition of C2 restored the injury to both organs. Further, C1qKO mice display C3 deposition, and have enhanced inflammation and injury compared to WT mice following G/I/R. These data, similar to our MI/R studies in complement KO mice, suggest that the lectin pathway initiates complement activation following G/I/R. Interestingly, a recent clinical manuscript suggests that initial complement activation during thoracoabdominal aortic aneurysm repair is also MBL mediated and amplified through the alternative pathway (Fiane et al., 2003). Collectively, these data delineate a prominent role for the lectin pathway in the initiation of G/I/R injury and perhaps in other I/R instances.

Complement activation and deposition also occurs following skeletal muscle I/R (Kyriakides et al., 1999; Weiser et al., 1996; Wong et al., 1999; Brock et al., 2001; Toomayan et al., 2003).
et al., 2003). Weiser et al. demonstrated an important role of the lectin and/or classical pathway during skeletal I/R by showing that C3- or C4-KO mice were protected against I/R injury based on a significant reduction in vascular permeability (Weiser et al., 1996). The data suggest that activation is partly mediated by antibody and thus classical complement activation by demonstrating that mice deficient in serum immunoglobulin (RAG2−/−) were protected from injury and that this injury could be restored by reconstitution with serum from normal mice. However, the role of the lectin pathway cannot be ruled out, as MBL would have been present in the serum. Recently Toomayan et al. demonstrated that treatment of rats with C1-INH reduced complement activation and improved contractile function in reperfused skeletal muscle (Toomayan et al., 2003), also suggesting a role for the lectin and/or classical pathway in skeletal I/R injury. The specific role of the lectin and classical pathway will need to be further evaluated by using C2/factor B, Clq and MBL A/C KO mice, as well as using specific mAbs.

In contrast to intestinal and skeletal muscle I/R, renal I/R experiments with C4KO mice demonstrate no significant tissue protection, while C3-, C5- and C6-knockout mice were protected from injury (Zhou et al., 2000), suggesting that the membrane attack complex is responsible for complement-mediated tubular damage. Recently, another study confirmed the importance of the alternative pathway in renal I/R injury by demonstrating that factor B KO mice were protected from injury (Thurman et al., 2003). However, a supporting role for the lectin or classical pathways was not ruled out.

4. Summary

Thus, it is still unclear which specific complement pathways are involved in the primary mechanism of initial complement activation in I/R injury. However, collectively the data suggest that the lectin pathway plays an important role in the myocardium and the gastrointestinal system. This then raises the question of what is the role of Clq? These findings also raise the question as to whether the contribution of complement pathways may be organ dependent (e.g. is the kidney really alternative pathway dependent?). To dissect the specific contribution of each complement pathway and to determine the overall picture for I/R in each organ system, further studies using specific inhibitors of the classical, alternative or lectin pathway, in addition to using genetically modified mice will be needed. Our lab is actively making such mAbs to rat complement components. What components should we block? and (3) What pathways remain include: (1) What pathways should we inhibit? (2) What components should we block? and (3) What pathways or components are needed for resolution? Until recently it has been difficult to identify the contribution of each individual pathway given the complex association of the different complement pathways and the limited resources to target selectively a specific pathway of the complement system. As specific and genetically modified mice become readily available, the specific contribution of each complement pathway and the overall picture for each organ system will become more apparent.

References


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