Kupffer cell-initiated remote hepatic injury following bilateral hindlimb ischemia is complement dependent

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Brock, Robert W., Robert G. Nie, Kenneth A. Harris, and Richard F. Potter. Kupffer cell-initiated remote hepatic injury following bilateral hindlimb ischemia is complement dependent. Am J Physiol Gastrointest Liver Physiol 280: G279–G284, 2001.—Intravital fluorescence microscopy was applied to the livers of male Wistar rats to test the hypothesis that complement mobilization stimulates Kupffer cells and subsequently initiates hepatic injury after hindlimb ischemia/reperfusion (I/R). Following 3 h of limb reperfusion, hepatocellular viability (serum levels of alanine transaminase and cell death via propidium iodide labeling) decreased significantly from levels in sham-operated animals. Inhibition of complement mobilization with soluble complement receptor type 1 (sCR1) blocked Kupffer cell phagocytosis by 42% following limb reperfusion. These results suggest that the stimulation of Kupffer cells via complement mobilization is necessary but is not the only factor contributing to the early pathogenesis of hepatic injury following hindlimb I/R.

Intravital microscopy; ischemia/reperfusion; liver; complement system; remote injury; microcirculation

RECENTLY, STUDIES FROM OUR laboratory (5, 6) provided the first direct evidence that remote damage to the liver occurs early and progresses following hindlimb ischemia/reperfusion (I/R). In these studies, the initiation of such damage could not be attributed to any deficit within the hepatic microcirculation. However, we did show that Kupffer cells and their subsequent release of tumor necrosis factor-α (TNF-α) played a crucial role in the initiation of this remote hepatic response (6, 22).

Kupffer cells have previously been implicated in the early pathogenesis of both local (18, 35, 40) and remote (6, 15, 16) hepatic damage. Like other phagocytic cells, many antigens and inflammatory signaling molecules stimulate Kupffer cells to a degree of stimulation (“priming”) that is characterized by amplified inflammatory, cytotoxic, and chemotactic states (9). TNF-α, a proinflammatory product liberated by stimulated Kupffer cells, was also implicated as a mediator via promotion of quiescent Kupffer cells to the activated state (43).

Another mediator suggested to be involved, and consequently to be a contributor to the initiation of remote hepatic damage, is mobilization of the complement cascade. This is supported by evidence suggesting that complement contributes to remote lung injury after intestinal I/R (14, 46). In addition, the occurrence of complement mobilization has been confirmed in numerous hindlimb I/R studies (2, 25, 45). In fact, it is generally accepted that complement deposition and activation occurs following skeletal muscle I/R (21, 41, 44). For example, Bless et al. (2) found complement systemically mobilized after hindlimb I/R and suggested that it was likely to be because of the release of a complement-activating product from the ischemic limbs. Further support for a role of complement in Kupffer cell priming lies in the fact that these macrophages possess membrane receptors to several complement components (26). Moreover, recent work shows that complement mobilization indirectly causes damage to the hepatic parenchyma via Kupffer cell stimulation (19, 23).

In this study, we sought to determine whether the initiation of hepatic injury following a distant inflammatory insult occurs as a result of complement-mediated priming of Kupffer cells. To accomplish this, we blocked Kupffer cell function with GdCl₃ and used an in vivo inhibitor of complement mobilization [soluble complement receptor type 1 (sCR1)]. This complement inhibitor was shown to suppress both the classic and the alternative pathways of the complement cascade in the rat (42). By incorporating these interventions with our whole organ-to-cellular approach, we provided direct in vivo evidence in support of a role for a complement-dependent mechanism, the priming of Kupffer cells, in the remote hepatic damage that follows hindlimb I/R.
MATERIALS AND METHODS

Animals. All animals (male Wistar rats, 200–250 g) in this study were randomly assigned, with a minimum of four animals per group. The protocols described herein were approved by the University of Western Ontario Council on Animal Care and undertaken in accordance with the criteria outlined by the Canadian Council on Animal Care.

Surgical procedure. Details of the preparation and model (hindlimb I/R) have been described previously (5). Briefly, animals were anesthetized with inhalational isoflurane (5% induction, 2% surgery, and 1% maintenance; Abbott Laboratories, Saint-Laurent, PQ, Canada), and the left carotid artery was cannulated for sampling and monitoring of systemic arterial pressures. The left jugular vein was also cannulated for drug administration. All animals were chronically prepared with a swivel (Stoelting, Wood Dale, IL) and fluid resuscitated with normal saline (2–4 ml/h). Fentanyl citrate (2 μg·100 g body wt −1·h −1) was used for analgesia. Tourniquets applied with pressure sufficient to block arterial blood flow were applied above the greater trochanter of each leg. After 4 h of ischemia, the tourniquets were removed just before recovery from anesthesia. Sham-operated animals underwent the same protocol but with no ischemia. Hepatocyte death and sinusoidal perfusion were measured following 3 h of hindlimb reperfusion.

Experimental groups. Animals were randomly assigned to either a 3-h sham-operated group (no hindlimb ischemia), a 3-h hindlimb reperfusion group (I/R), a 3-h hindlimb reperfusion group with complement inhibition (sCr1; generously donated by Avant Immunotherapeutics, Needham, MA), a 3-h hindlimb reperfusion group with a blockade of Kupffer cell function (GdCl₃, Sigma, St. Louis, MO), or a 3-h hindlimb reperfusion group with a combination of complement inhibition and Kupffer cell blockade (sCr1 + GdCl₃). The novel complement inhibitor sCr1 (Lot no. 92M027) was administered intravenously at a dosage previously determined to be effective (20 mg/kg body wt, just before limb reperfusion) (19, 46). Furthermore, the in vivo application of the sCr1 acts with great efficacy to suppress serum complement mobilization (10, 11, 42). Therefore, any effect caused by the administration of sCr1 is assumed complement dependent. GdCl₃ (1 mg/100 g body wt, 24 h before hindlimb ischemia) was administered via tail vein injection to suppress Kupffer cell function and deplete Kupffer cell number (12). The dosage of GdCl₃ used had previously been confirmed to be specific for liver macrophages (1, 12). Therefore, we believe the effect of GdCl₃ pretreatment can be attributed to Kupffer cells.

Intravital videomicroscopy. Details of this technique have been described previously (5). Briefly, animals were reanesthetized with isoflurane near the third hour of hindlimb reperfusion. The left lobe of the liver was then exposed and reflected into a saline bath on the warmed stage (37°C) of an inverted microscope (Nikon, Tokyo, Japan). Once in the bath, the liver was covered with plastic film (Dow Chemical, Paris, ON, Canada) to maintain a stable environment and field of view. Ten random views of the liver microcirculation, near the organ surface, were observed on a video monitor at a magnification of ×600 using a ×20 objective lens (CF Achromat 20/0.4; Nikon). Subsequent images were then projected onto a charge-coupled device video camera (Dage-MTI, Michigan City, IN) and recorded for later offline analysis.

Analysis of hepatocellular death and sinusoidal perfusion. The nuclei of dead hepatocytes were labeled with an intravascular bolus of the fluorescent vital dye propidium iodide (PI; 0.05 mg/100 g body wt; Sigma) before the third hour of hindlimb reperfusion (13). These fluorescently labeled nuclei were observed with exposure to excitation wavelengths of 510–560 nm and an emission barrier filter of 590 nm. This index of hepatocellular death was expressed as the number of PI-labeled nuclei per unit volume of liver (1 × 10⁻⁵ mm³).

Sinusoidal perfusion was determined using previously accepted stereological techniques (8, 34) described in detail elsewhere (5). In brief, a point-counting grid was used to provide a >95% confidence that the density of points counted was proportional to the density of sinusoids within an area of interest. A sinusoid was then counted only if a point from the predetermined grid landed within the dimensions of the vessel. The state of sinusoidal perfusion during the 1-min observation period was determined according to the following guidelines: 1) continuously perfused sinusoids were limited to those that retained red blood cell perfusion; 2) intermittently perfused sinusoids were those in which red blood cell perfusion terminated for any period of time; and 3) nonperfused sinusoids were those that were devoid of red blood cell flow. Each state of perfusion was expressed as a percentage of the total number of sinusoids counted. Both hepatocyte death and sinusoidal perfusion were evaluated for the periportal and pericentral regions of the hepatic lobule.

Hepatic phagocytosis. The assessment of hepatic phagocytosis was determined in a separate set of animals (n = 11) using methods adapted from Post et al. (32). Briefly, carboxylate-modified, fluorescently labeled microspheres (Molecular Probes, Eugene, OR; diameter = 1 μm; excitation 505/515) were injected as an intra-arterial bolus (3 × 10⁸ microspheres/kg body wt in 1 ml of isotonic saline) 3 h after limb reperfusion. Within 10–15 randomly selected microscopic fields of view (7.6 × 10⁴ μm²), the number of adherent microspheres was evaluated within the first 10 min after injection and quantified by the number of adherent microspheres per field of view during 10 s of observation. Microspheres in postsinusoidal venules were not included because of the lack of Kupffer cell localization.

Liver enzymes. Blood samples for serum alanine transaminase (ALT) activities were collected at three different time points as an index of hepatocellular damage (mild through severe injury): 1) preischemia, 2) prereperfusion, and 3) 3 h of hindlimb reperfusion. ALT activity (U/l) was tested using standard spectrophotometric techniques.

Statistics. Main effects were determined using standard ANOVA procedures. If any significance was found, values were tested further by using the Student-Newman-Keuls post hoc comparison. The Kruskal-Wallis ANOVA on ranks was used with Dunn’s test if the criteria for parametric tests were not satisfied. A probability of 0.05 was accepted as statistically significant, and the sample sizes for each experimental group provided a statistical power of >85%.

RESULTS

Indirect estimates of hepatocellular damage. Bilateral hindlimb ischemia resulted in a significant increase in ALT activity following 3 h of reperfusion (Fig. 1; P < 0.001). Both the inhibition of complement and suppression of Kupffer cell function proved to be effective in reducing this early increase (P < 0.001). No additional benefit was provided by the combination of these treatments. Measures of ALT activity, before and at the end of 4 h of hindlimb ischemia, showed no significant changes.

Direct index of hepatocyte death. Hepatocyte death was found to be significantly elevated within both the
periportal and pericentral regions of the liver lobule 3 h after hindlimb I/R (Fig. 2; $P_{0.002}$). There were no apparent differences between these lobular regions in any of the experimental groups. The cell death that occurred from this systemic inflammatory insult was significantly reduced by all interventions, although there appeared to be a trend toward a greater reduction by GdCl$_3$ pretreatment or the combination of treatments.

**Direct index of sinusoidal perfusion.** Total sinusoidal perfusion (continuously + intermittently perfused sinusoids) remained unchanged following 3 h of hindlimb I/R (Fig. 3). However, an increase in its heterogeneity was apparent, as characterized by a significant shift from continuously perfused to intermittently perfused sinusoids in both regions of the lobule. Such perfusion heterogeneity was significantly diminished after sCR1 administration but only returned to baseline levels after pretreatment with GdCl$_3$ or a combination of the treatments ($P < 0.0001$). There were no significant changes to the number of nonperfused sinusoids during any of the interventions (sham operation, $1.8 \pm 0.2\%$; I/R, $6.5 \pm 2.1\%$; sCR1, $4.0 \pm 0.8\%$; GdCl$_3$, $2.8 \pm 0.6\%$; GdCl$_3$ + sCR1, $3.1 \pm 1.1\%$).

![Fig. 2. Following 4 h of hindlimb ischemia, pretreatment with GdCl$_3$ ($n = 5$), sCR1 ($n = 5$), and GdCl$_3$ + sCR1 ($n = 5$) resulted in protection from hepatocyte death at 3 h of limb reperfusion ($n = 6$). On pretreatment of these animals with GdCl$_3$ ($n = 6$), soluble complement receptor type 1 (sCR1; $n = 5$), and GdCl$_3$ + sCR1 ($n = 4$), marked protection was provided at 3 h. I/R, ischemia/reperfusion. Values are means $\pm$ SE. *$P < 0.001$ vs. preischemia and prereperfusion; †$P < 0.001$ vs. all other groups at 3 h of limb reperfusion; ‡$P < 0.001$ vs. sham at 3 h of limb reperfusion only.

![Fig. 3. Four hours of hindlimb ischemia did not result in any reductions to total sinusoidal perfusion [continuous (solid bars) + intermittent (open bars)] in either the perportal (A) or pericentral (B) regions after 3 h of limb reperfusion. However, there was an increase in the degree of perfusion heterogeneity, which reverted back to normal levels following pretreatment with sCR1 ($n = 5$), GdCl$_3$ ($n = 4$), and GdCl$_3$ + sCR1 ($n = 4$). The protection from sCR1 was not as beneficial as that of the other interventions. Solid areas represent continuously perfused sinusoids, and open areas designate intermittent perfusion. Values are means $\pm$ SE. *$P < 0.0001$ vs. all other groups; †$P < 0.001$ vs. all other groups.](http://ajpgi.physiology.org/)

![Fig. 1. Levels of serum alanine transaminase (ALT) measured at preischemia (open bars), prereperfusion (hatched bars), and 3 h (solid bars) of reperfusion. After 4 h of hindlimb ischemia, these levels were only significantly increased after 3 h of limb reperfusion ($n = 6$). On pretreatment of these animals with GdCl$_3$ ($n = 6$), soluble complement receptor type 1 (sCR1; $n = 5$), and GdCl$_3$ + sCR1 ($n = 4$), marked protection was provided at 3 h. I/R, ischemia/reperfusion. Values are means $\pm$ SE. *$P < 0.001$ vs. preischemia and prereperfusion; †$P < 0.001$ vs. all other groups at 3 h of limb reperfusion; ‡$P < 0.001$ vs. sham at 3 h of limb reperfusion only.](http://ajpgi.physiology.org/)
Direct index of Kupffer cell phagocytosis. Following 3 h of hindlimb I/R, hepatic microsphere adherence was enhanced significantly (289%) compared with sham-operated animals (Fig. 4; \( P < 0.03 \)). Treatment with sCR1 was found to depress microsphere adherence by 42% following I/R \( (P < 0.03) \). Thus the phagocytosis of microspheres was not completely blocked by sCR1.

DISCUSSION

Evidence suggests that injury to the liver following a remote inflammatory insult occurs in a biphasic manner, much like that of local liver injury (17–19). More specifically, the early phase of injury may be neutrophil independent, whereas the latter was neutrophil dependent (24). Support for this comes from studies of both remote and local hepatic injury that suggest the initiation of hepatocyte damage was mediated primarily by Kupffer cells (6, 17, 18). Despite the similarity of cellular mechanisms in these disparate injuries, the signaling cascades that lead to the stimulation of Kupffer cells in a remote injury process remain elusive.

The results of the present investigation demonstrate that complement inhibition with sCR1 caused a significant reduction in early hepatic injury and microvascular heterogeneity, with a reduced Kupffer cell phagocytic activity following hindlimb I/R. Also, the combination of complement inhibition with interruption of Kupffer cell function did not provide any additional protection over their individual effects. Such evidence suggests that systemic complement mobilization contributes to the priming of Kupffer cells with enhanced phagocytic activity. Also, the data from this study not only extend but confirm our previous work (5, 6) suggesting that Kupffer cell-initiated damage to the liver occurs following hindlimb I/R.

Complement receptor type 1 (CR1), a membrane-bound regulator of complement mobilization, enzymatically displaces the catalytic subunits from C3 and C5 convertases of both the classic and alternative complement pathways, as well as possessing cofactor function for the degradation of C3b and C4b by factor I (42). As a result, CR1 prevents the generation of anaphylatoxins (i.e., C3a and C5a), as well as assembly of the membrane attack complex (29). A truncated form, sCR1, that lacks both the cytoplasmic and transmembrane domains but retains the full regulatory activity of the membrane-associated CR1, was first introduced by Weisman et al. (42). The in vivo potency of this inhibitor has been demonstrated in numerous animal models of I/R injury (7, 19, 46), illustrating its protection against organ damage.

Since ALT principally resides within the cytoplasm of hepatocytes, its measure in the serum may occur as a result of cell necrosis and/or an increase in membrane permeability (33). With that, differences in the levels of ALT suggest that a large fraction of hepatocytes experienced some degree of membrane compromise. These estimates of hepatocellular damage were confirmed by directly evaluating hepatocyte death with intravital microscopy and PI, a vital dye that only labels the nuclei of cells suffering lethal damage (i.e., hepatocyte death) (13). Thus the relatively small proportion of PI-positive hepatocytes that occur in our model of systemic inflammation represent only a minor segment of total organ injury. This corroborates previous work suggesting that although hepatocytes experience changes in viability the magnitude of cell death accounted for <5% of the total population of hepatocytes (5). Although the treatments with sCR1 and GdCl\(_3\) resulted in reduced hepatocellular death, there appeared to be a trend toward greater protection afforded by GdCl\(_3\) compared with sCR1. Together, these data support the hypothesis that complement mobilization contributes to Kupffer cell stimulation and that the Kupffer cells in turn generate a cytotoxic response compromising hepatocyte viability during the initial limb reperfusion phase. However, complement is not likely the only mechanism involved with priming Kupffer cell activation (6, 9).

Sinusoidal blood flow in normal rat liver has been shown to be heterogeneous in nature and has been proposed to become more so during pathological conditions (5, 28, 39). Although the total number of perfused (continuous + intermittent) sinusoids remained unchanged in the present study, the heterogeneity of sinusoidal perfusion was increased following hindlimb reperfusion. For example, increases in intermittently perfused sinusoids occurred secondary to decreases in the number of continuously perfused sinusoids. However, it is unlikely that such perfusion heterogeneity would have significantly influenced hepatocellular viability, since the ratio of continuously-to-interruptently perfused sinusoids was high and the liver parenchyma is highly tolerant to transient red cell stasis (27).

Kupffer cells likely play an important role in the increased perfusion heterogeneity, especially since their stimulation is believed to contribute to changes in sinusoidal blood flow (27). Following sCR1 and GdCl\(_3\) pretreatment, the increased perfusion heterogeneity returned to normal levels. Notwithstanding, the effect observed with sCR1 was not as great as that of GdCl\(_3\).
Such differences may have been the result of more than one mechanism being involved in the stimulation of Kupffer cells (9). In addition to this Kupffer cell mechanism, endothelin-1, carbon monoxide, and/or nitric oxide have been shown to be important modulators of microvascular perfusion (30, 31). It is also commonly believed that leukocyte recruitment into the liver may induce perfusion abnormalities (20). However, we have been unable to show evidence of significant leukocyte accumulation over the time periods studied (unpublished data).

Although circulating microspheres are subject to phagocytosis by Kupffer cells, uptake by sinusoidal endothelial cells may also take place, usually when Kupffer cells are unable to keep up with the phagocytic challenge (36–38). Since the dose used (3 × 10⁸ microspheres/kg body wt) was well below the total number of Kupffer cells found in rat liver (≈17 × 10⁸/g tissue (4)), the phagocytic challenge should not have overcome Kupffer cell activity. Additionally, adherence of any microsphere to the sinusoidal wall has been shown to be internalized by Kupffer cells in <1 min (3). As a result, the differences observed in this study likely only represent alterations to Kupffer cell phagocytic activity. Following the systemic inflammatory response imposed by our model, Kupffer cell phagocytic activity was enhanced. Such stimulation is likely a result of the washout of the ischemic limb into the systemic circulation on reperfusion. Complement is believed to be one component of this Kupffer cell-priming sequelae. Confirmation of this is provided by the reduction in microsphere adherence following inhibition of complement mobilization, which demonstrates the importance of complement to Kupffer cell priming.

The results of this study are unique in that they not only provide direct evidence of remote hepatic injury but help to further elucidate the roles of Kupffer cells and complement mobilization in the initiation of such injury. Our model of systemic inflammation caused, within 3 h of its induction, a significant compromise to hepatocellular viability and augmented perfusion heterogeneity with enhanced Kupffer cell phagocytosis. Following treatment with sCR1, these parameters saw moderate protection and diminished Kupffer cell phagocytic activity. Together, these results support a role for Kupffer cells in the induction of remote hepatic injury and implicate the mobilization of the complement cascade as an initial activator of these macrophages in the early stages of such injury. Thus, on the basis of this and our previous work, we propose the following events for the contribution of complement mobilization to the early pathogenesis of remote hepatic injury following normotensive hindlimb I/R. Hindlimb I/R results in a systemic washout of posts ischemic sequelae, including proinflammatory mediators (i.e., TNF-α) and cellular debris (i.e., myoglobin), causing an initially minor injury induced either directly or indirectly by Kupffer cells (22). This sequelae also involves the liberation of complement and other factors, which, in turn, further stimulate Kupffer cells to induce a transient release of TNF-α (6). The increased levels of TNF-α likely lead to continued stimulation of previously quiescent Kupffer cells, thereby propagating Kupffer cell cell activation. The combination of cytokine and complement priming of Kupffer cells results in enhanced phagocytosis, leading to the initiation and early progression of hepatic parenchymal death.

In conclusion, the evidence from this study confirms that hepatic injury following a remote inflammatory insult occurs via Kupffer cell stimulation with enhanced phagocytosis and suggests that such injury is complement dependent. However, a certain degree of injury can still occur with little or no complement mobilization. In this way, the data suggest that a number of mediators and signaling cascades are involved in the pathogenesis of remote hepatic injury. With such a built-in redundancy, the lack of one of these mediators is easily compensated for, suggesting that the development of therapeutic strategies should be multifactorial.

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