Assessment and preservation of liver function in hepatic ischemia and reperfusion
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Chapter 6

Inhibition of the classical route of complement attenuates liver ischemia and reperfusion injury in a rat model.


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Submitted
Abstract

Activation of the complement system contributes to the pathogenesis of ischemia/reperfusion (I/R) injury. We evaluated inhibition of the classical pathway of complement by C1-inhibitor (C1-Inh) in an experimental model of 70% partial liver I/R injury in male Wistar rats (n=35). C1-inh was administered at 100, 200 or 400 IU per kg bodyweight into rats, 5 min before 60 min ischemia (pre-I) or 5 min before 24 h reperfusion (end-I). Hundred IU per kg bodyweight significantly reduced the increase of plasma levels of activated C4 as compared to albumin-treated control rats, and also attenuated the increase of the liver enzyme alanine aminotransferase (ALT). These effects were hardly better with higher doses of C1-inhibitor. Administration of C1-inh pre-I resulted in lower ALT levels and higher bile secretion after 24 h of reperfusion than administration at end-I. There were no differences in plasma IL-6 levels at any time and histopathology scores in H&E-stained liver sections after 24 hr of reperfusion between C1-inh and albumin-treated rats. Immunohistochemical assessment indicated that activated C3, the membrane attack complex C5b9 and C-reactive protein (CRP) co-localised in hepatocytes within midzonal areas, which are particularly at risk for I/R injury, suggesting CRP is a mediator of I/R-induced, classical complement activation in rats. We conclude that pre-ischemic administration of C1-inh is an effective pharmacological intervention to protect against liver I/R injury.
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Introduction

Normothermic ischemia of the liver leads to hepatocellular injury which, depending on the time of ischemia, may be aggravated by restoration of flow of oxygenated blood (reperfusion). Pathogenic mechanisms involved in I/R injury have been extensively investigated. Studies in models of myocardial, intestinal, renal and hepatic I/R have provided evidence for the role of complement in the pathogenesis of I/R injury.

The effect of complement activation during liver I/R is diverse. Several complement components become localized in ischemic liver, presumably facilitating opsonisation of injured cells for phagocytosis. Furthermore, plasma levels of activated complement components are increased after liver I/R in pigs and humans. Complement activation products as the anaphylatoxins and the terminal complement membrane attack complex (MAC) have deleterious effects on the liver - amongst others by activating neutrophils- and contribute to vasoconstriction, impaired microcirculation, increased vascular permeability and cell lysis. In rats, depletion of complement before ischemia attenuates superoxide generation by Kupffer cells and accumulation of neutrophils in the liver during reperfusion, thereby suppressing liver I/R injury. Inhibition of the complement pathways by administration of soluble complement receptor type 1 (sCR1) just after the onset of liver ischemia, significantly reduces endothelial complement C3 deposition and the amount of liver necrosis. Taken together, these studies demonstrate that reperfusion of the ischemic liver may induce activation of complement. Hence, inhibition of the complement cascade may be a therapeutic option to reduce the inflammatory damage in postischemic, reperfused liver.

Main activators of complement by the classical pathway are IgM or IgG antibody-antigen complexes, whereas bacteria trigger the alternative and lectin pathways. Also, cytokines, the release of intracellular proteins and reactive oxygen species may be involved in triggering the activation of complement. The molecular mechanism of the observed activation of complement during liver I/R is not clear. A previous study from our department in humans suggests a contribution of the acute phase protein C-reactive protein (CRP).

Although some studies show that the activation of complement in ischemic liver occurs via the alternative pathway, the involvement of C4, and hence the classical pathway has been demonstrated in human liver. Cl-inhibitor (Cl-Inh) is a member of the serine protease inhibitor (serpin) family and is a major inhibitor of the classical complement pathway. Because of its anti-inflammatory properties, Cl-inh has been evaluated in various animal models for
diseases such as sepsis and myocardial infarction. These studies have yielded promising results, and initial studies with this compound in patients with these diseases have been started. A recent, concise study reports that reperfusion-related microcirculatory disorders were minimised by C1-inh in rat liver. In the present study, the role of the classical pathway in liver I/R and possible therapeutic use of C1-inh for reduction of liver I/R injury was investigated. Purified human C1-inh was administered at different dosages and at different time points in an in vivo rat model of partial liver ischemia. A possible mechanistic role of CRP in the initiation of complement activation was investigated by measurement of specific CRP-complement complexes in plasma.

Materials and methods

Animals

This study was approved by the Animal Experiment Committee of the Academic Medical Center, University of Amsterdam, The Netherlands. Male Wistar rats (n=35; 325-375 g) were purchased from Broekman (Someren, The Netherlands). All rats were allowed to adapt to the laboratory environment for 7 days with free access to water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Rats were housed under standard environmental conditions with a 12-h light/dark cycle. Before use in experiments, rats were fasted overnight with free access to water.

Anesthesia

All animals were anesthetized via inhalation of a mixture of O₂:N₂O (1:1 L/min) and isoflurane 2-3% (Florene, Abbott Laboratories Ltd., Queensborough, Kent, UK). After endotracheal intubation, rats were ventilated (Zoovent ventilator, Instruvet, Amerongen, The Netherlands) and anesthesia was maintained with the same mixture. Adequate ventilation was verified by continuous monitoring of end-tidal CO₂, assuring physiological pH during the entire procedure. A silicone catheter (Ø 0.9 mm) was introduced into the left carotid artery and tunneled subcutaneously to the back of the rats for the assessment of hemodynamic parameters during operation as well as for withdrawal of blood samples and injection of C1-inh, albumin or saline. Arterial blood pressure was maintained at approximately preoperative levels by adjustment of the isoflurane levels. The animals were kept in supine position on a heating pad and rectal temperature was controlled at 37°C with the use of a heating lamp.
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**Operation techniques**

A midline laparotomy was performed. After dissection of the falciform ligament, the afferent vessels to the median and left lateral lobes were exposed by turning the hepatic lobes upwards. An a-traumatic vascular clip was applied to these vessels to induce partial hepatic ischemia (70%) for 60 min, after which the clip was removed and subsequent reperfusion initiated. After surgical closure of the abdomen, the rat was allowed to regain consciousness and was provided with water and food.

After 24 h of reperfusion the rat was anesthetized again for re-laparotomy. A cannula ($\varnothing$ 0.4 mm) was inserted into the distal bile duct and bile was collected during 15 min as a parameter of hepatocyte function. Afterwards the rat was sacrificed under anesthesia by haemorrhage, liver biopsies were taken, frozen in liquid nitrogen and stored at $-80^\circ$C or fixed in 4% (W/V) formaldehyde for future analyses.

**Intervention**

Five minutes before ischemia (pre-I) or 5 min before reperfusion (at the end of ischemia, end-I), either C1-inh (Sanquin, Amsterdam, The Netherlands) at 100 IU/kg, 200 IU/kg or 400 IU/kg or an equivalent amount of human albumin (Cealb®, Sanquin) was administered intravenously.

**Blood sampling**

Blood samples of 500 µl were collected prior to induction of ischemia, after 90 min, 6 h and 24 h of reperfusion in tubes containing lithium heparin or K$_2$EDTA. Blood was centrifuged (10 min at 2,000xg at 4°C). Aliquots of plasma were stored at $-80^\circ$C for further analysis.

**Assessment of hepatocellular injury**

ALT activity in heparin plasma was determined by routine spectrophotometry using alpha-ketoglutaric acid and pyridoxal phosphate (General Clinical Chemistry Laboratory, AMC, Amsterdam. The Netherlands).

**ELISA**

Functional human C1-Inh was detected as described before $^{33}$. Briefly, C1-Inh was bound to plates coated with mAb RII against human C1-Inh $^{34}$ and detected with biotinylated C1s $^{35}$. Notably, this end-stage assay measures the number of functional C1-Inh molecules and not the
kinetics of the interaction between C1s and C1-Inh. Results were compared to those of normal human plasma pool (NHP).

Activated rat C4 was detected with a novel ELISA $^{36}$. Briefly, an IgG fraction of sheep polyclonal Ab against human C4 (SHC4; Department of Immune Reagents; CLB, Amsterdam, The Netherlands), which cross-reacts with activated rat C4 $^{36}$, was incubated at 2 μg/ml, final volume 100 microliter, in 0.1 M carbonate/bicarbonate, pH 9.6, overnight at room temperature in Maxisorb plates (Nunc). Plates were washed with PBS 0.02% (w/v) Tween 20 (PBS-Tween). Rat plasma samples were appropriately diluted in PBS-Tween containing 0.2% (w/v) gelatin and 10 mM EDTA. Hundred μl of each dilution was incubated for 1 h at 4°C, the plates being gently shaken. The plates were washed 5 times in PBS-Tween and incubated with biotinylated IgG fraction of SHC4, diluted in PBS-Tween-0.2% gelatin (PTG), for 1 h at room temperature. Plates were then washed 5 times in PBS-Tween and incubated with streptavidin-polymerized-HRP (Business Unit Reagents, Sanquin) 1:10,000 diluted in PBS containing 2% (v/v) cow milk) for 25 min at room temperature. Finally, the plates were developed with 3, 3', 5, 5'-tetramethylbenzidine (0.1 mg/ml in 0.11 M NaAc, pH 5.5, 0.003% H$_2$O$_2$) and stopped by addition of H$_2$SO$_4$.

Absorption was measured at 450 nm. Levels of activated C4 in the plasma samples tested were compared to those in aged normal rat serum (NRA), which was used as an in house standard. NRA was prepared by incubating normal rat serum for 7 days at 37°C in the presence of sodium azide.

Levels of the cytokine IL-6 were determined with a commercial ELISA according to the manufacturer’s instructions (Pierce Endogen, Rockford, IL, USA). This assay has a sensitivity of < 16 pg/mL.

**Histopathology and histochemistry for C1-inh, C3, C5b9 and CRP**

Formalin-fixed, wax-embedded tissue was stained with haematoxylin and eosin. Liver injury was scored considering the following phenomena: clogging of sinusoids by erythrocyte stasis, hepatocyte vacuolisation, cell death (either nuclear condensation and fragmentation, nuclear fading or eosinophilia), cytoplasmic fading (cytolysis) and granulocyte accumulation. Each of these 5 parameters were scored on a scale from 0 to 5 and added to form a total pathology score.

Immune peroxidase labelling of human C1-inh, rat complement fragments C3 and C5b9 and CRP was performed on acetone-fixed frozen sections (8 μm) using mAbs mouse-anti-human C1-inh (1:40, clone RII IgG1; Sanquin Research), mouse-anti-rat C3 (ED11, Serotec), mouse-anti-rat C5b-9 (1:40, clone 2A1, a kind gift of Dr. W. Couser), rabbit-anti-rat CRP (1:100; affinity
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purified and absorbed with rat plasma depleted for CRP) and mouse-anti-rat IgG1 (1:40, Caltag, Burlingame, CA) as a control antibody for aspecific binding. Primary antibodies were detected with peroxidase-labelled rabbit-anti-mouse IgG (1:200, DAKO, Glostrup, Denmark) or peroxidase-labelled goat-anti-rabbit IgG (1:100, DAKO). Peroxidase activity was visualised by incubating the sections for 12 min with a medium containing 0.5 mg/ml diaminobenzidine (DAB), 10 μM hydrogen peroxide and 50 mM Tris-HCL buffer (pH 7.6). All antibodies were diluted in PTG buffer (PBS, 0.2% gelatin 0.02 % Tween 20).

To demonstrate co-localisation, single sections of I/R liver were sequentially stained for CRP and C5b9. Immunostaining for CRP was performed as described above and followed by intensive rinsing with double distilled water and PBS, incubation with mouse-anti rat C5b-9 and peroxidase-labelled rabbit-anti-mouse IgG as described above and visualisation (12 min) with 4-chloro-1-naphthol (2.24 mM, Sigma) dissolved in 0.2% dimethyl formamide and 0.3% ethyl alcohol, 10 μM hydrogen peroxide and 50 mM Tris-HCl buffer (pH 7.6).

Statistical analysis

Results are expressed as mean ± SEM. A paired Student t-test for analysis of matched data and one-way ANOVA followed by a Newman-Keuls post-test for analyses between groups were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA). A p-value <0.05 was considered significant.
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Results

Bile secretion

Bile secretion after 24 h of reperfusion was higher in all rats treated with any dose of C1-inh before induction of ischemia as compared to albumin-treated rats. Rats treated with 200 IU/kg C1-inh before ischemia showed better bile secretion than rats treated with the same dose at the end of ischemia. Only treatment with 400 IU/kg of C1-inh at the end of the ischemic period rendered a significantly improved bile secretion when compared to albumin-treated rats (Fig. 1).

![Figure 1](image)

Bile secretion (means ± SEM) measured during 15 min after 24 h of reperfusion (24 h R). Rats were treated with albumin (white bar), 100 IU/kg C1-inh pre-I (black bar), 100 IU/kg C1-inh end-I (black chequered bar), 200 IU/kg C1-inh pre-I (dark grey bar), 200 IU/kg C1-inh end-I (dark grey chequered bar), 400 IU/kg C1-inh end-I (light grey bar) and 400 IU/kg C1-inh pre-I (light grey chequered bar). All rats treated with C1-inh pre-I showed higher bile secretion than albumin treated rats (* significant versus albumin treated rats, p<0.01). Rats treated with 200 IU/kg C1-inh pre-I showed better bile secretion than rats treated with the same dose at the end of ischemia (# significant versus 200 IU pre-ischemia, p<0.05).

Hepatocellular injury

The increase of plasma ALT levels in rats treated with C1-inh was significantly less than that in the albumin-treated rats. Only after 24 h of reperfusion this reduction in ALT levels reached statistical significance (p<0.001) in all experimental groups. After 24 h of reperfusion, rats treated with either 200 or 400 IU/kg of C1-inh before ischemia showed significantly lower ALT levels when compared to rats treated with 100, 200 or 400 IU/kg of C1-inh at the end of the ischemic period (Fig. 2).
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Functional C1-inh

Administration of either 100, 200 or 400 IU/kg of human C1-inh resulted in an increase of plasma C1-inh levels by 1.9, 3.8 and 6.2 IU per ml, respectively (one IU is the amount present in pooled human plasma). As expected, human C1-inh was not found in the rats treated with albumin. Plasma C1-inh levels between groups treated with either 100, 200 or 400 IU/kg of C1-inh remained significantly different during 24 h of reperfusion (not shown). No differences in C1-inh levels existed between rats treated with equivalent amounts of C1-inh administered either before ischemia or at the end of the ischemic period (Fig. 3). Mean plasma half-life was 4.4 h.

Figure 2
Plasma ALT (means ± SEM) measured pre-ischemia (pre-I), after 90 m of reperfusion (90 m R), 6 h (6 h R) and 24 h of reperfusion (24 h R) in rats receiving albumin or C1-inh (bars are coded as in Fig. 1). After 24 h R, ALT levels in C1-inh treated rats were significantly lower than in albumin-treated rats (* p<0.001) and ALT levels were lower in rats treated with C1-inh before ischemia than in rats treated at the end of ischemia (# and $ significant versus end-ischemia treated rats, p<0.05).

Figure 3
Percentage of functional C1-inh compared to normal plasma pool (NMP) measured pre-ischemia (pre-I), after 90 m of reperfusion (90 m R), 6 h (6 h R) and 24 h of reperfusion (24 h R) in rats receiving albumin or C1-inh (bars are coded as in Fig. 1). Plasma C1-inh levels between groups treated with either 100, 200 or 400 IU/kg of C1-inh remained significantly different during 24 h R (significance not shown). Bars represent means ± SEM.
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Complement activation

All rats treated with C1-inh, irrespective of dosage and either before or at the end of ischemia, had lower plasma levels of activated C4 after 90 min and 6 h of reperfusion than the albumin-treated rats. After 24 h of reperfusion, no significant differences in activated C4 levels between C1-inh- and albumin-treated rats were observed (Fig. 4). Although activated C4 levels after 90 min of reperfusion were dissimilar in rats treated with equivalent amounts of C1-inh either before or at the end of ischemia, significance was not reached (C1-inh pre-I versus C1-inh end-I, 100 IU/kg p=0.18, 200 IU/kg p=0.09, 400 IU/kg p=0.06). Complete inhibition of the classical pathway of complement could not be achieved with any dosage of C1-inh, since all rats still had a significant increase of activated C4 levels after 90 min of reperfusion when compared to pre-ischemic levels. However, after 6 h of reperfusion, activated C4 levels were not different when compared with pre-ischemic levels. After 24 h of reperfusion activated C4 levels in several C1-inh-treated experimental groups were higher than pre-ischemic levels. In albumin-treated rats activated C4 levels were consistently higher during reperfusion as compared to pre-ischemic levels (Fig. 4).

IL-6

As expected, IL-6 levels rose after 90 min and 6 h of reperfusion (Fig. 5). However, no differences between peak levels of IL-6 were observed between C1-inh-treated and albumin-treated rats.

Figure 4
Percentage of activated C4 compared to normal rat plasma aged pre-ischemia (pre-I), after 90 min of reperfusion (90 m R), 6 h of reperfusion (6 h R) and 24 h of reperfusion (24 h R). Albumin showed the highest increase in plasma activated C4 levels after 90 m R and 6 h R. Bars represent means ± SEM.

* significantly different from C1-inh treated rats (p<0.001); $ significantly different from pre-I levels (p<0.05).
Figure 5
IL-6 levels in plasma (mean ±SEM) before ischemia (pre-I), after 90 min of reperfusion (90 min R), 6 h of reperfusion (6 h R) and 24 h of reperfusion (24 h R) in rats receiving albumin (white bar), 100 IU/kg C1-inh pre-I (black bar), 100 IU/kg C1-inh end-I (black chequered bar), 200 IU/kg C1-inh pre-I (dark grey bar), 200 IU/kg C1-inh end-I (dark grey chequered bar), 400 IU/kg C1-inh pre-I (light grey bar) and 400 IU/kg C1-inh end-I (light grey chequered bar). IL-6 levels peaked in all animals after liver I/R at 90 min and 6 hr of reperfusion. There was no significant alteration of IL-6 levels in C1-inh-treated animal compared to albumin-treated rats.

Histopathology and histochemistry for C1-inh, C3, C5b9 and CRP
Histopathological analysis of liver injury in H&E-stained paraffin sections according to a semi-quantitative score (see Material & Methods) revealed no differences between I/R livers at 24 hr after treatment with either albumin or C1-inh (Fig. 6).

Figure 6
Liver pathology was scored in H&E-stained sections considering the following phenomena: clogging of sinusoids by erythrocyte stasis, hepatocyte vacuolisation, cell death (either nuclear condensation and fragmentation, nuclear fading or eosinophilia), cytoplasmic fading (cytolysis) granulocyte accumulation. Each of these 5 parameters were scored on a scale from 0 to 5 and added to form a total pathology score. No difference in pathology score was observed after treatment with either albumin or C1-inhibitor.
In frozen sections of ischemic-reperfused liver, after control immune histochemistry without primary antibody, focal light brown staining was found on pericentral and midzonal hepatocytes, likely due to non-specific adhesion of secondary antibodies and staining with the chromogen DAB. Upon incubation with rat IgG protein, a control for non-specific adhesion of plasma proteins to injured cells, a similar light brown staining in ischemic foci was observed. In all ischemic livers, intense dark brown staining was observed in granulocytes, showing high levels of endogenous peroxidase and non-specific conversion of DAB colour product (Fig. 7 A, B).

Human C1-inh was never demonstrated in livers at 24 after administration of the compound, neither on sinusoidal endothelium nor on hepatocytes or any other constituent of the liver. Rat complement C3 was found on the hepatocyte plasma membrane both in normal and ischemic-reperfused livers. Occasionally, C3 staining was more intense in the cytoplasm of hepatocytes in pericentral and midzonal areas of I/R liver (Fig. 7 C, D).

Complement protein C5b9 as well as CRP were never observed in non-ischemic livers and appeared very intense in midzonal and pericentral hepatocytes in I/R livers. Incubation of serial sections and double staining of single sections showed that these proteins co-localised (Fig. 7, E-H and Fig. 8).
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Figure 7
Immune histochemistry for IgG (A, B), complement C3 (C, D) and C5b9 (E, F) and CRP (G, H) on a series of frozen sections of normal liver (A, C, E, G) and liver after 60 min of ischemia and 24 hr of reperfusion (B, D, F, H). In pericentral and midzonal areas, hepatocytes show clear staining for C5b9 and CRP (arrowheads). After ischemia/reperfusion, complement C3 was mainly increased at the plasma membrane of hepatocytes (insert in D, arrowheads). No cytoplasmic staining of native C3 is observed in normal (C) or ischemic-reperfused livers (D). Arrows point at granulocytes showing non-specific, endogenous peroxidase staining. (cv, central vein; pv, portal vein; original magnification with 10x objective and for inserts 40x objective)
Figure 8
In a single, frozen section of ischemic-reperfused liver, sequential staining for CRP and complement C5b9 was performed. Arrows show co-localisation of CRP and C5b9 in hepatocytes. (Original magnification with 100x objective)
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Discussion

Inappropriate complement activation is an important mediator of I/R injury after major surgery. Activation of the classical complement pathway in this type of tissue damage can occur via Ab-dependent as well as Ab-independent mechanisms, which in the latter case may involve the direct binding of C1q to damaged cells and in situ deposited acute phase proteins. To prevent undesired effects of complement activation, the therapeutic application of complement inhibitor C1-inh, a physiological inhibitor of the serine proteases C1r and C1s of the classical pathway, and of the manna binding lectin associated serine proteases (MASPs) of the manna binding lectin pathway, has been preliminary tested in man. Herein, C1-inh was tested in a rat model of liver I/R injury at different doses and different time points. In our study exogenous administration of C1-inh significantly attenuated liver I/R injury after 24 h of reperfusion, which is in agreement with previous studies. Furthermore, we show that pre-ischemic administration of C1-inh was more effective in attenuating liver I/R injury than administration of C1-inh at the end of the ischemic period. With respect to the recommended dose of C1-inh, our study suggests that a dose of 100 IU/kg is sufficient.

We observed increased bile secretion, reduced ALT leakage and lower levels of activated C4 when C1-inh was administered before the start of ischemia. This suggests that either C1-inh should be distributed thoroughly within the liver microcirculation or perhaps should be bound to the sinusoidal endothelium to exert its effect. The observations also suggest that at least part of the complement activation is initiated during the period of ischemia and is inhibited by C1-inh. A state of minimal flow hypoxia during ischemia, due to collateral circulation, cannot be excluded.

Inhibition of an early step in the classical pathway is of relevance in view of the pro-inflammatory effects of early products of the complement activation cascade, such as C4a. Although in this study the classical pathway of complement (i.e. level of activated C4) was only partially blocked by C1-inh, hepatic I/R injury (i.e. ALT leakage from hepatocytes) was significantly attenuated. This suggests that C1-inh exerts its effects not only via reduction of classical complement activation. C1-Inh is also a major inhibitor of the lectin pathway of complement activation, the contact activation system and the intrinsic pathway of coagulation. It is, therefore, endowed with anti-inflammatory properties. These additional effects of C1-inhibitor probably explain the therapeutic benefit of C1-Inh independent of an effect on the classical complement activation.
The differences in liver I/R injury between albumin and C1-inh treated rats were most apparent after 24 h of reperfusion. This late beneficial effect of complement inhibition can be explained by the modulating effects of C1-inh on the inflammatory response, resulting in a blunted late phase of I/R injury, which is mainly propagated by activated neutrophils.

The finding that preischemic C1-inh administration has a more beneficial effect on liver I/R injury after 24 h of reperfusion versus administration of C1-inh at the end of ischemia is intriguing. Although this may be explained in various ways, we favour the explanation that the better effect of C1-inh given before ischemia, is due to inhibition of an activation process that already starts in the ischemic tissue before reperfusion. Indeed, we found a tendency towards lower activated C4 levels after 90 min of reperfusion when rats were treated with C1-inh-treated pre-ischemia. Support for this reperfusion-independent activation of complement is provided by experiments in dogs undergoing experimental myocardial infarction. In these animals it was observed that C1-inh produced a significant cardioprotective effect when coronary vessels were permanently occluded (WTh Hermens, CE Hack et al., manuscript in preparation).

Hepatic ischemia is associated with the release of a variety of acute reactant cytokines such as IL-6, a cytokine with pleiotrophic biological effects including induction of the acute phase reaction. Interleukins and oxygen free radicals, formed as a result of complement activation itself, may give positive feed-back to complement activation. IL-6 also appears to have unique protective properties in enhancing hepatocyte proliferation and preventing ischemic cell death. We observed no differences between peak levels of IL-6 between C1-inh-treated and albumin-treated rats at 6 hr after liver ischemia. Hence, complement inhibition likely does not interfere with hepatoprotection by IL-6 after liver ischemia.

Histopathological examination revealed no differences between I/R livers at 24 hr after treatment with either albumin or C1-inh. It should be noted that when it comes to assessing liver injury, tissue morphology is seldom conclusive. Bile secretion however, as parameter of hepatocyte function, did show differences between groups, analogous to the ALT levels.

C1-inh has been found to bind to sinusoidal endothelium or the sinusoidal pole of the liver trabeculae, linked to sinusoidal endothelium, after 8 hr of cold storage in UW solution containing C1-inh and 2 hr of reperfusion. We could not demonstrate that human C1-inh was retained by the livers at 24 hr after administration of the compound. We did find C3 deposition on plasma membranes of hepatocytes both in normal and postischemic liver. The mouse-anti-rat antibodies did not discriminate between native C3 and activation fragments of C3. In normal livers, the low background staining in the cytoplasm of hepatocytes as well as the more intense
staining of the hepatocyte membranes results from the synthesis of native C3 by these cells. In I/R livers, more cytoplasmic C3 was found in pericentral and midzonal hepatocytes. Whether this is native or activated C3 cannot be distinguished. Deposition of the anti-C3 antibodies on liver cells shows marked heterogeneity, which is likely related to the mainly midzonal expression of I/R injury, but perhaps also to variability in sensitivity to complement activation. We made no attempt to quantify the amount of C3 in hepatocytes after administration of C1-inh or albumin, considering the large variation that results from assessment of liver sections.

The MAC or C5b9 complex was found in the same areas (and by serial sectioning suggesting the same hepatocytes) as C3. This co-localisation indicates that the increased binding of anti-C3 antibodies to the cytoplasm of hepatocytes represents deposition of activated C3. The co-existence of activated C3 and C5b9 on hepatocytes in areas renowned for ischemic/reperfusion injury suggests that these hepatocytes may die through MAC-mediated cell lysis.

CRP co-localised with both activated C3 and C5b9 in hepatocytes in ischamically injured midzonal areas whereas periportal areas (notably most resistant to ischemic injury) were negative for CRP and complement. These results suggest CRP directly participates in local inflammatory processes, possibly via complement activation, after binding of a suitable ligand. Lagrand et al. first hypothesised that CRP fixed to injured plasma membranes in infarcted myocardium could promote local activation of the complement system via the classical route in humans. Although a previous attempt failed to demonstrate this phenomenon in rats, recent findings have proven that rat CRP activates the endogenous complement system in rats. Our own study in ischemic human livers also suggested a contribution of the acute phase protein CRP, but candidates like reactive oxygen species and immunoglobulin M can not be excluded. Whether mitochondrial constituents play a role as ligand in the molecular mechanism of complement during I/R in the liver, as is suggested from studies in ischemic myocardium, remains to be evaluated.

Hepatocytes and sinusoidal endothelial cells, which have a defective expression of complement-regulatory proteins in normal liver, might therefore be at risk for complement-mediated injury. This may explain in part the high susceptibility of the liver to complement-mediated injury as shown in experimental models.

Complement activation leads to the formation and release of complement-derived peptides (C3a and C5a), potent pro-inflammatory mediators, which contribute to neutrophil accumulation, contraction of smooth muscle cells, increased vascular permeability and activation of Kupffer and endothelial cells. These mechanisms all contribute to impaired hepatic
microcirculation upon reperfusion, which is an important determinant in hepatic I/R injury. Although there is no doubt that complement is activated by ischemic liver, it remains to be established that inhibition of this activation is at the basis of the beneficial effects of C1-inh. Thus, if inhibition of the classical activation of complement is the main inhibitor of I/R injury, the question remains which effect of the complement cascade is attenuated: (1) release of C3a and C5a for attraction of leukocytes, (2) opsonisation of injured cells for macrophage recognition and phagocytosis, and/or (3) lysis of cells by formation of the MAC? It has been suggested that part of the cardioprotective effect of C1-inh is related to a diminished infiltration of neutrophils because of lower myeloperoxidase (MPO) activity in the ischemic myocardium of treated animals. It was also shown that increases of circulating C3a, and to a lesser extent C5a, were attenuated by intracoronary application of C1-inh in an experimental pig model of myocardial ischemia and reperfusion. Also, the inhibitory effect of C1-inh on leukocyte rolling and adhesion has been shown in rat mesentery and liver. Lysis of hepatocytes by formation of the MAC leads to a rise of ALT in plasma, which was largely reduced by administration of C1-inh in our study.

Although activation of the classical complement pathway is causally involved in harmful complement activation during liver I/R, other pathways are likely to be involved as well. The alternative pathway amplifies the complement activation cascade induced via any pathway at the level of C3. The lectin pathway can be involved in complement activation after endothelial oxidative stress. Next to inhibiting the C1 complex, C1 inhibitor also affects the lectin pathway of complement activation and the contact system. Therefore, further definition of the contribution of the various complement pathways in liver I/R is of major importance for the development of an effective, specific, and safe treatment, e.g. in transplantation medicine.

We conclude that our results provide further evidence for the pivotal role of classical complement activation in mediating liver I/R injury. Our study also demonstrates that pre-ischemic administration of C1-inh is more effective in reducing liver I/R injury than administration just prior to reperfusion, representing an effective pharmacological intervention to protect against liver I/R. Furthermore, our data support the hypothesis for CRP-mediated complement activation in liver I/R.

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