Interaction of complement and leukocytes in severe acute pancreatitis: potential for therapeutic intervention

Werner Hartwig,1 Martina Klaß,2 Michael Kirschfink,3 Thilo Hackert, Lutz Schneider, Martha-Maria Gebhard, Markus W. Büchler, and Jens Werner1

1Department of General and Visceral Surgery, 2Department of Experimental Surgery, and 3Institute of Immunology, University of Heidelberg, Germany

Submitted 13 January 2006; accepted in final form 26 May 2006

Hartwig, Werner, Martina Klaß, Michael Kirschfink, Thilo Hackert, Lutz Schneider, Martha-Maria Gebhard, Markus W. Büchler, and Jens Werner. Interaction of complement and leukocytes in severe acute pancreatitis: potential for therapeutic intervention. Am J Physiol Gastrointest Liver Physiol 291: G844–G850, 2006; doi:10.1152/ajpgi.00016.2006.—In acute pancreatitis, local as well as systemic organ complications are mediated by the activation of various inflammatory cascades. The role of complement in this setting is unclear. The aim of the present study was to determine the level of complement activation in experimental pancreatitis, to evaluate the interaction of complement and leukocyte-endothelium activation, and to assess the effects of complement inhibition by soluble complement receptor 1 (sCR1) in this setting. Necrotizing pancreatitis was induced in Wistar rats by the combination of intravenous cerulein and retrograde infusion of glycodeoxycholic acid into the biliopancreatic duct; edematous pancreatitis was induced by intravenous cerulein only. In control animals, a sham operation (midline laparotomy) was performed. Complement activation, leukocyte sequestration, and pancreatic as well as pulmonary injury were assessed in the presence/absence of sCR1. Increased levels of C3a were found in necrotizing but not in edematous pancreatitis. When complement activation in necrotizing pancreatitis was blocked by sCR1, levels of C3a and total hemolytic activity (CH50) were decreased. Leukocyte-endothelial interaction, as assessed by intravital microscopy, and pancreatic as well as pulmonary organ injury (wet-to-dry weight ratio, MPO activity, and histology) were ameliorated by sCR1. As a result of the present study, necrotizing but not edematous pancreatitis is characterized by significant and early complement activation. Based on the interaction of complement and leukocytes, complement inhibition by sCR1 may be a valuable option in the treatment of leukocyte-associated organ injury in severe pancreatitis.

necrotizing pancreatitis; complement activation; soluble complement receptor 1; leukocyte endothelial interaction; lung injury

ACTIVATED COMPLEMENT PROTEINS have long been regarded as possible mediators of organ injury in acute pancreatitis. Evidence of complement activation has been reported by several investigators in the setting of both experimental (13, 25) and human acute pancreatitis (7). Once activated, the complement cascade can initiate organ injury in a variety of ways. For example, the components C5 through C9 assemble to form the membrane attack complexes that destroy plasma cell membranes (29, 30). C5a is a potent chemoattractant for neutrophils and monocytes and stimulates the release of leukocyte lysosomal enzymes, cytokines, and reactive oxygen species. The anaphylatoxins C3a and C5a induce mast cell degranulation and are thought to be involved in airway hyperresponsiveness.

Complement inhibition by the C1 esterase inhibitor has been proven effective in the treatment of experimental severe pancreatitis (23, 34). Supporting these results, complement inhibition by the recombinant soluble complement receptor 1 (sCR1) attenuated leukocyte-endothelium interaction and preserved endothelial barrier function in ischemia-reperfusion injury of the pancreas (28). In a first clinical trial, C1 inhibitor prevented hyperamylasemia in patients undergoing endoscopic sphincterotomy, suggesting a reduced risk of inducing pancreatitis (26). However, conflicting data about the effects of complement inhibition in acute pancreatitis has also been reported; in genetically altered mice that either lack C5a receptor or do not express C5, the complement factor C5a exerted an anti-inflammatory effect (2). Weiser et al. (31) have shown that complement inhibition by sCR1 failed to moderate cerulein-induced edematous pancreatitis in rats. Furthermore, no protective effect of complement inhibition by injection of cobra venom factor has been detected in the choline-deficient and ethionine-supplemented (CDE) diet model of severe pancreatitis (17).

The human CR1 CD35 is a cell surface glycoprotein on erythrocytes and cells of myeloid lineage that inhibits the classic, alternative, and mannose-binding lectin pathway of complement activation. A soluble form of CR1 has been found in body fluids and shown to inhibit the complement pathways in a manner similar to that of a cell-bound protein. The recombinant form of CR1 inhibits the convertases that activate C3 and C5 complement proteins and acts as a cofactor in the proteolysis of activated C3b and C4b by plasma factor I. SCR1 has a half-life of ~70 h in humans. At doses of >1 mg/kg, sCR1 significantly inhibited complement activity at the levels of C3 and C5 in patients with acute lung injury/acute respiratory distress syndrome (37). In various animal models, the application of sCR1 significantly reduced tissue damage, as demonstrated for myocardial ischemia (32), ischemia-reperfusion injury of the intestine (12), or acute lung injury of various origin (20).

Because of the conflicting results on the effects of complement inhibition in acute pancreatitis, the aim of the present study was to investigate the levels of complement activation in mild and severe acute pancreatitis, to characterize the impact of activated complement on leukocytes in necrotizing pancreatitis, and to evaluate the effects of complement inhibition by...
sCR1 in this setting. We applied the well-established and characterized models of cerulein- and GDOC (glycodeoxycholic acid)-induced pancreatitis. Cerulein pancreatitis reflects well the clinical findings of self-limiting acute edematous pancreatitis (18). GDOC pancreatitis reflects the severe course of acute necrotizing pancreatitis in human and is characterized by marked pancreatic inflammation and necrosis, distant organ injury, and substantial mortality (24). As a result of this study, we report that complement is significantly activated in necrotizing but not in edematous pancreatitis, with a significant reduction of pancreatic tissue injury and pulmonary leukocyte sequestration upon inhibition with sCR1. Since leukocyte-endothelial interaction was abrogated by sCR1, early complement inhibition appears to ameliorate organ injury in severe pancreatitis by a leukocyte-related mechanism.

**MATERIALS AND METHODS**

**Animals**

Inbred male Wistar rats (250–300 g) were used for experiments. Care was provided in accordance with the German law for care and use of laboratory animals. The study was approved by the Regierungsspräsidium Karlsruhe, Germany, committee on animal care. Animals were fasted overnight prior to the experiments but allowed free access to water.

**Anesthesia and Catheter Placement**

Surgical anesthesia was induced by intraperitoneal injection of pentobarbital (10 mg/kg; Nembutal, Sanofi-Ceva, Genova, Germany) and intramuscular injection of ketamine (40 mg/kg; Ketanest 50, Parke, Davis and Company, Berlin, Germany). Two polyethylene catheters (inner diameter 0.5 mm) were inserted into the left carotid artery and the right internal jugular vein, tunneled subcutaneously to the suprascapular area, and exited through a steel tether that allowed the animals’ free movement. The venous catheter was used for infusion regimens, and the arterial line was used for blood sampling and hemodynamic monitoring.

**Induction of Acute Pancreatitis and Experimental Protocol**

Animals were randomly allocated to a control group (sham laparotomy plus saline iv) or two groups of pancreatitis, mild edematous or severe necrotizing disease. Mild pancreatitis was induced by intravenous infusion of the synthetic CCK analog cerulein (5 μg·kg⁻¹·h⁻¹; Takus, Farmitalia, Carlo Erba, Freiburg, Germany) for over 6 h (18). Cerulein was reconstituted in normal saline and infused at 3 ml·kg⁻¹·h⁻¹. Nocrotizing pancreatitis was induced as follows and as described in detail elsewhere (24). The biliopancreatic duct was cannulated with a 24-gauge Teflon catheter (Critikon, Tampa, FL), and bile as well as pancreatic juice were drained by gravity for 5 min with the common hepatic duct clamped at the porta hepatitis. GDOC (Sigma, St. Louis, MO) in glycylglycine-NaOH-buffered solution (pH 8.0, room temperature) was infused retrograde into the biliopancreatic duct at a concentration of 10 mmol/l in a volume (1.2 ml/kg), time (10 min), and pressure (30 mmHg)-controlled fashion, followed by an intravenous infusion of cerulein over 6 h.

In the first set of experiments, EDTA blood was collected from animals at baseline, 30 min, and 1, 2, 3, and 6 h after induction of acute pancreatitis for C3a and total hemolytic activity (CH50) measurements. In animals with necrotizing pancreatitis, human recombinant sCR1 (TP10, provided by AVANT Immunotherapeutics, Needham, MA) was infused at baseline and at 3 h at a dosage of 12 mg/kg per application. Organ injury and leukocyte sequestration in the pancreas and lungs were assessed by histology, MPO activity, and wet-to-dry weight ratio. In a second set of experiments, leukocyte-endothelial interaction and pancreatic microvascular perfusion were determined at 6 h by intravital microscopy.

**C3a and CH50 Measurement**

Blood from rats was collected on ice in syringes containing EDTA at a final concentration of 10 mM. Immediately after collection, blood was centrifuged at 500 g for 10 min. The plasma was retained and then stored at −80°C until assayed. C3a in plasma was quantitated by a commercially available ELISA (BMA Biomedicals, Augst, Switzerland), using a specific antibody against a neoepitope of rat C3αdesArg, a relatively stable degradation product of C3a, that is not exposed on native, noncleaved C3. CH50 of plasma was determined by hemolytic assay (19).

**MPO Activity**

Excised pancreatic and pulmonary tissue samples were rinsed with saline, blotted dry, snap-frozen in liquid nitrogen, and stored at −80°C. MPO activity was measured as previously described (10), and is expressed as units per milligram protein. Total protein content in samples was determined by BCA protein assay, provided by Pierce Biotechnology.

**Histopathological Analysis**

Histopathological evaluation of pancreatic injury was performed in a blinded fashion. In brief, the head of the pancreas was removed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Coronal sections were made in the plane of the flattened pancreas and stained with hematoxylin and eosin. Edema, hemorrhage, inflammation, and acinar necrosis were evaluated using a well-established scoring system (degree of injury, 0–3; 0 = no injury and 3 = worst injury) (24).

**Edema Assessment**

Pancreatic and pulmonary edema was evaluated by measuring the wet-to-dry weight ratio. A segment of the pancreatic tail was removed immediately after death, trimmed of fat, and weighed. The water content was determined by calculating the wet-to-dry weight ratio from the initial weight (wet weight) and its weight after incubating at 160°C for 24 h (dry weight).

**Intravital Microscopy**

Microcirculatory alterations in the pancreas of rats with necrotizing pancreatitis with/without sCR1 administration were assessed by intravital microscopy as described in detail elsewhere (11). Briefly, after a midline laparotomy was performed, the pancreas with the duodenal loop was gently exteriorized and placed in an immersion chamber containing Ringer’s lactate maintained at 37°C. The pancreatic microcirculation was then evaluated in epillumination using an fluorescence microscope (Leitz, Wetzlar, Germany). Therefore, animals were injected with FITC-labeled erythrocytes (0.5 ml/kg, hematocrit 50%) for capillary blood-flow measurements and Rhodamin 6G (bolus of 0.1 ml) for in vivo staining of leukocytes and quantitation of leukocyte-endothelium interaction. Mean capillary red blood cell velocity was analyzed in four different regions of the pancreas in each rat. The mean red blood cell velocity in each area was calculated by averaging the velocity of erythrocytes in 15 ± 2 capillaries. According to their interaction with the endothelial lining, adherent and rolling leukocytes were assessed in postcapillary venules with a diameter of 25–40 μm. Adherent leukocytes (stickers) were defined as cells that did not move or detach from the endothelium within the observation period of 30 s (5). Rolling leukocytes (rollers) were defined as those white cells moving at a velocity ≤5⁄3 of that of erythrocytes in the centerline of the venule (36). Both stickers and rollers were expressed as the number of cells per square millimeter of vessel surface, calculated from the diameter and length (100 μm) of the vessel.
segment studied. Off-line analysis of video recordings was performed in a blinded fashion using a computer-assisted microcirculation analysis system (Cap Image; H. Zeintl, Heidelberg, Germany).

Statistical Analysis

Values are means ± SE. ANOVA was used to show an overall difference between groups, and the Student’s t-test was used to make pairwise comparisons of normal distributed parameters. P values <0.05 were considered to be statistically significant.

RESULTS

Complement Activation in Acute Pancreatitis

C3a in plasma. Strong complement activation was found in necrotizing pancreatitis as reflected by significantly increased levels of C3a compared with controls, whereas C3a levels in edematous pancreatitis were not significantly increased (Fig. 1). C3a levels increased very early in the course of necrotizing pancreatitis right after intraductal infusion of glycodeoxycholic acid into the biliopancreatic duct. The highest levels of C3a were found at 1 h after infusions were started. No significant changes in C3a concentrations were seen in control animals with sham laparotomy (Fig. 1). Animals with necrotizing pancreatitis that were treated with sCR1 showed markedly lower plasma levels of C3a to 30–40% of those in necrotizing pancreatitis without sCR1 treatment (Fig. 2, P < 0.01, ANOVA).

CH50. Hemolytic activity of plasma, as assessed by complement-dependent lysis of sensitized sheep erythrocytes, was decreased in necrotizing pancreatitis compared with controls, reflecting moderate complement consumption (Fig. 3). Successful in vivo inhibition of complement in sCR1-treated rats with necrotizing pancreatitis was reflected by significantly reduced hemolytic activity levels. In animals with cerulein pancreatitis, hemolytic complement activity was comparable to controls.

Pancreatic and Pulmonary Injury

Histopathological alterations. Regular pancreatic morphology was seen in sham-operated animals that were infused with Ringer’s solution for 6 h (Fig. 4A). Histopathological sections of the pancreas of animals with cerulein-induced acute pancreatitis were characterized by marked interstitial edema and moderate leukocyte infiltration (Figs. 4B and 5). Animals with necrotizing GDOC pancreatitis showed severe acinar cell necrosis throughout the pancreas, marked edema, and leukocyte infiltration (Figs. 4C and 5). When animals with necrotizing pancreatitis were treated with sCR1, the severity of pancreatic injury was significantly reduced (Fig. 4D). Histological scoring showed a significant reduction of edema and necrosis in sCR1-treated animals (Fig. 5).

Edema formation/wet-to-dry weight ratio. Reflecting the findings from histopathological evaluation of the pancreas, wet-to-dry weight ratios were significantly increased in animals with edematous and necrotizing pancreatitis compared...
with controls. Treatment of necrotizing pancreatitis with sCR1 led to significantly reduced edema formation compared with untreated animals (Fig. 6).

As known from previous studies, significant pulmonary edema in the model of necrotizing pancreatitis develops late after intraductal infusion of GDOC (10). As in the present study, where animals were killed at 6 h after induction of pancreatitis, wet-to-dry weight ratios of the lungs did not show any differences between animals with edematous, necrotizing, and sCR1-treated pancreatitis (Fig. 6).

Leukocyte sequestration/MPO activity. MPO activity is a well-established marker for the quantitation of leukocyte sequestration in tissue. MPO activity was increased in the pancreas and lungs of animals with necrotizing pancreatitis compared with controls. Whereas sCR1 infusions did not change MPO levels in the pancreas, levels in lung tissue were significantly decreased (Fig. 7).

Pancreatic microvascular perfusion, as assessed by mean erythrocyte velocity in capillaries, showed significantly decreased values in necrotizing pancreatitis compared with controls (control, 0.95 ± 0.07 mm/s vs. GDOC, 0.52 ± 0.04 mm/s; P < 0.001). Application of sCR1 significantly ameliorated these changes (0.77 ± 0.07 mm/s, GDOC vs. GDOC + sCR1, P = 0.015).

DISCUSSION

In acute pancreatitis, increasing knowledge about the pathogenesis of local and systemic complications has resulted in the establishment of widely accepted treatment algorithms (27).
Complement activation in acute pancreatitis correlates with the severity of the disease, as reflected in two rat models of different severity used in our study, demonstrating significantly increased levels of C3a in necrotizing pancreatitis. In contrast, C3a levels remained unchanged in edematous disease and sham-operated control rats. Additionally, measurements of CH50 blood support this finding. Complement consumption could only be seen in necrotizing but not in mild pancreatitis. As a result of these findings, complement inhibition by sCR1 was omitted in the setting of edematous pancreatitis in our study. Previously, an inverse correlation has been demonstrated between CH50 titers and trypsin (1), whose serum concentration is elevated from an early stage and in correlation to the severity of acute pancreatitis (10). Since the complement components C3 and C5 are susceptible to direct cleavage by the pancreatic enzyme trypsin (16, 22, 33), trypsin may play a key role in the activation of complement in this disease. Clinical findings are in agreement with our results. It has been reported that the central complement components of both pathways, C3 and C4, are significantly decreased in patients with pancreatic necrosis compared with edematous pancreatitis (3). Furthermore, in patients with acute pancreatitis, the plasma levels of C3a and sC5b-9 measured during the first week of the disease have been proposed to represent a sensitive marker for the prediction of severe acute pancreatitis (6).

As demonstrated in our study, sCR1 proved effective in the inhibition of complement activation. In animals injected with sCR1, the plasma levels of C3a were reduced to about 30–40% of those of untreated animals with necrotizing pancreatitis. The effective inhibition of the complement cascade reaction in vivo is also reflected by decreased levels of CH50 in sCR1-treated animals. In our model of necrotizing disease, sCR1 attenuated local and systemic organ injury. Pancreatic edema and necrosis were significantly decreased compared with untreated animals. Likewise, leukocyte sequestration in the lungs, as assessed by MPO activity, was decreased by sCR1. These findings are in agreement with other studies (15, 23, 34) indicating a possible therapeutic role of complement inhibition in the setting of acute pancreatitis. However, two recent studies (2, 31) that could not confirm these beneficial effects applied the model of mild cerulein-induced pancreatitis. Importantly, and in agreement with these results, we have not been able to detect any complement activation or consumption in this model in the present study.

In acute pancreatitis, pancreatic and, in particular, pulmonary injury is associated with the effects of proteases, cytokines, and reactive oxygen species released from activated leukocytes. Polymorphonuclear neutrophils have been shown to accumulate in these organs, with increased metabolic activity in severe necrotizing pancreatitis compared with mild disease (8). Applying intravital microscopy, the present study provides clear evidence that complement contributes to leukocyte activation in acute pancreatitis. When sCR1 was administered in animals with necrotizing pancreatitis, the amount of

However, those are only symptomatic interventions, e.g., adequate fluid management to improve pancreatic microcirculation or prophylactic administration of antibiotics to prevent infection of pancreatic necrosis. Therapeutic interventions that are aimed to specifically alter the course of the disease have not been established yet or have been proven ineffective in large randomized controlled trials (4, 14). In the present study, we provide evidence that inhibition of complement activation by the prophylactic administration of sCR1 results in the attenuation of pancreatic as well as pulmonary injury in necrotizing pancreatitis. The beneficial effects of sCR1 are at least partly based on its inhibition of complement-triggered leukocyte and endothelium activation. However, complement activation occurs very early in the disease and may, therefore, demand therapeutic intervention just shortly after the onset of symptoms in the clinical setting.

In general, there are three pathways of complement activation: the classical, the alternative, and the mannose-binding lectin pathway (29, 30). The classical pathway is activated by antigen-antibody complexes or antibody-coated targets, the mannose-binding lectin pathway is activated by microbes with terminal mannose groups. The alternative pathway, probably more important in the setting of acute pancreatitis, can be activated by suitable targets in the absence of an antibody. All pathways proceed by means of sequential activation and assembly of a series of proteins, leading to the formation of a complex enzyme capable of binding and cleaving a key protein, C3, which is common to all pathways. Thereafter, the three pathways proceed together to form a membrane-attack complex, which ultimately causes cell lysis (29, 30). The activated complement components C3a, C3b, and C5a are all strong chemoattractants and are capable of activating leukocytes and endothelial cells, thereby triggering the inflammatory response in various human diseases.

Complement activation in acute pancreatitis correlates with the severity of the disease, as reflected in two rat models of different severity used in our study, demonstrating significantly increased levels of C3a in necrotizing pancreatitis. In contrast, C3a levels remained unchanged in edematous disease and sham-operated control rats. Additionally, measurements of CH50 blood support this finding. Complement consumption could only be seen in necrotizing but not in mild pancreatitis. As a result of these findings, complement inhibition by sCR1 was omitted in the setting of edematous pancreatitis in our study. Previously, an inverse correlation has been demonstrated between CH50 titers and trypsin (1), whose serum concentration is elevated from an early stage and in correlation to the severity of acute pancreatitis (10). Since the complement components C3 and C5 are susceptible to direct cleavage by the pancreatic enzyme trypsin (16, 22, 33), trypsin may play a key role in the activation of complement in this disease. Clinical findings are in agreement with our results. It has been reported that the central complement components of both pathways, C3 and C4, are significantly decreased in patients with pancreatic necrosis compared with edematous pancreatitis (3). Furthermore, in patients with acute pancreatitis, the plasma levels of C3a and sC5b-9 measured during the first week of the disease have been proposed to represent a sensitive marker for the prediction of severe acute pancreatitis (6).

As demonstrated in our study, sCR1 proved effective in the inhibition of complement activation. In animals injected with sCR1, the plasma levels of C3a were reduced to about 30–40% of those of untreated animals with necrotizing pancreatitis. The effective inhibition of the complement cascade reaction in vivo is also reflected by decreased levels of CH50 in sCR1-treated animals. In our model of necrotizing disease, sCR1 attenuated local and systemic organ injury. Pancreatic edema and necrosis were significantly decreased compared with untreated animals. Likewise, leukocyte sequestration in the lungs, as assessed by MPO activity, was decreased by sCR1. These findings are in agreement with other studies (15, 23, 34) indicating a possible therapeutic role of complement inhibition in the setting of acute pancreatitis. However, two recent studies (2, 31) that could not confirm these beneficial effects applied the model of mild cerulein-induced pancreatitis. Importantly, and in agreement with these results, we have not been able to detect any complement activation or consumption in this model in the present study.

In acute pancreatitis, pancreatic and, in particular, pulmonary injury is associated with the effects of proteases, cytokines, and reactive oxygen species released from activated leukocytes. Polymorphonuclear neutrophils have been shown to accumulate in these organs, with increased metabolic activity in severe necrotizing pancreatitis compared with mild disease (8). Applying intravital microscopy, the present study provides clear evidence that complement contributes to leukocyte activation in acute pancreatitis. When sCR1 was administered in animals with necrotizing pancreatitis, the amount of
leukocyte stickers in the pancreas was significantly decreased, reflecting attenuated leukocyte-endothelial interaction. Previously, we have demonstrated that Mac-1, an adhesion molecule from the integrin family that mediates firm adhesion of leukocytes to endothelium, was expressed on both neutrophils in acute pancreatitis and neutrophils incubated with trypticized serum (9, 15). Complement inhibition by sCR1 decreased Mac-1 upregulation in this setting, which, in turn, may attenuate leukocyte-endothelial interaction, as demonstrated by the present study.

Recent studies on the use of sCR1 in ischemia-reperfusion injury of the pancreas showed results comparable to our study. Prophylactic complement inhibition improved pancreatic microcirculation. The amount of adherent leukocytes in postcapillary venules was significantly reduced by sCR1, and capillary microperfusion was improved (28). Also, lung injury after intestinal ischemia-reperfusion has been ameliorated by complement inhibition (12). Complement activation has been documented in patients with adult respiratory distress syndrome (ARDS) and those at risk for ARDS (21), with substantial local complement activation in the lungs as demonstrated by bronchoalveolar lavage studies (35). Our data indicate that complement inhibition by sCR1 may also be a promising tool in the treatment of pancreatitis-associated pulmonary complications. Whether sCR1-associated complement inhibition may be beneficial in the clinical setting of severe pancreatitis, where patients are admitted to the hospital with a delay of hours to days after onset of symptoms and frequently present with established organ failure (14), remains to be investigated.

ACKNOWLEDGMENTS

We thank H. Marsh, AVANT Immunotherapeutics, Needham, MA, for kindly providing sCR1.

This study has previously been presented at the Annual Meeting of the American Pancreatic Association, Chicago, Illinois, November 4–5, 2004 and at the 122nd Annual Meeting of the German Society of Surgeons, Munich, April 5–8, 2005.

REFERENCES

28. Von Dobschuetz E, Bleiziffer O, Paherons S, Dellian M, Hoffmann T, and Messmer K. Soluble complement receptor 1 preserves endothelial...


