Soluble complement receptor 1 preserves endothelial barrier function and microcirculation in posts ischemic pancreatitis in the rat

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Von Dobschuetz, E., O. Bleiziffer, S. Pahernik, M. Dellian, T. Hoffmann, and K. Messmer. Soluble complement receptor 1 preserves endothelial barrier function and microcirculation in posts ischemic pancreatitis in the rat. Am J Physiol Gastrointest Liver Physiol 286: G791–G796, 2004. First published December 23, 2003; 10.1152/ajpgi.00407.2003.—Components of the activated complement cascade are considered to play a pivotal role in ischemia-reperfusion-induced organ injury. With the use of intravital epifluorescence microscopy, we investigated the effect of complement inhibition by the recombinant soluble complement receptor 1 (sCR1; TP10) on the effect of macromolecular microvascular permeability, functional capillary perfusion, and leukocyte endothelium interaction in posts ischemic pancreatitis. Anaesthetized Sprague-Dawley rats were subjected to 60 min of normothermic pancreatic ischemia induced by micro-clipping of the blood-supplying arteries of the organ. Rats who received sCR1 (15 mg/kg body wt iv; n = 7) during reperfusion showed a significant reduction of permeability (1.77 ± 1.34 × 10^-8 cm/s; n = 7) from 279 ± 15.7 to 330 ± 3.7 cm/s; n = 7) and the number of leukocytes adherent to postcapillary venules was significantly reduced (from 131 ± 87 to 163 ± 71 mm^-2; n = 7) by sCR1 compared with vehicle treatment. Complement inhibition by sCR1 effectively ameliorates pancreatic ischemia-reperfusion-induced microcirculatory disturbances and might be considered for treatment of posts ischemic pancreatitis.

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0 ± 2. During the experiment, arterial Po2 was kept >90 mmHg by adjustment of FiO2 and Pco2 was kept <45 mmHg by alteration of the breathing frequency. Hematocrit in arterial blood was measured by a Coultercounter T540 (Coulter Electronics, Hialeah, FL).

Animal model and experimental protocol. After transverse laparotomy, complete ischemia of the pancreas was induced by clipping the four blood-supplying arteries (left gastric artery, gastroduodenal artery, splenic artery, and caudal pancreateoduodenal artery) to the pancreas by means of microvascular clips (closing force 70 g; Aesculap, Tuttingen, Germany). The complete microsurgical technique was described previously (7). Sham-operated animals were immediately reperfused after the surgical procedure without induction of ischemia. After a stabilization period of 15 min, animals were randomly assigned to three groups: (1) sham-operated group without ischemia (sham control, n = 7); (2) 1 h ischemia group receiving 3 ml/kg body wt iv vehicle (ischemia saline). With the use of water for injection as reconstitution vehicle, sodium phosphate (5.76 mg), and sodium chloride (5.48 mg) as buffer solutions. The experiments were discontinued by an intravenous injection of potassium solution containing 5 mg/ml sCR1 was prepared, aliquoted into sterile test tubes, and stored at −70°C until use. sCR1 was further injected into three sham-operated animals (15 mg/kg body wt iv sCR1 (ischemia sCR1, n = 7). sCR1 was further injected into three sham-operated animals (15 mg/kg body wt iv sCR1; ischemia control, n = 7); and) 1 h ischemia group receiving 15 mg/kg body wt iv sCR1 (ischemia control, n = 7). The solutions were standardized to be equal in volume before the injection time point. A saltwater immersion contrast enhancement of microvessels and 0.1 ml 0.2% rhodamin 6G (excitation 530 nm/410 nm, emission 580 nm) was injected, and the identical capillary field was analyzed in three animals by measuring the length of capillaries and the diameter with an CAMA image analyzing system (developed by Dr. Zeintl, Heidelberg, Germany) (9). The dynamic microhematocrit (HTm) of the pancreatic capillary field was measured in a separate experiment by the method described by Savelius and Duling (22) using the following formula: 

\[ HTm = \left( \frac{V_{BRC} \times LF \times MCV \times 100}{V_{BRC} \times (D/2)^2} \right) \]

Brieﬂy, rat erythrocytes were labeled with ﬂuoresceinsulphocyanate (Sigma, St. Louis, MO). The erythrocytes were injected into the animal, and red blood cell ﬂux (FRBC) of labeled cells, the average cell velocity (vBCR), and diameter of the vessel (D) were measured in the pancreatic capillary ﬁelds. Labeled cell fraction (LF) of the whole capillary length was determined by using a cell sorter analyzer (Becton Dickinson, Heidelberg, Germany). Mean corpuscular volume (MCV) was calculated from a full blood cell count (Coulter Electronics, Hialeah, FL). FCD and leukocyte adherence were measured by the following setup: 0.2 ml of 2.5% BSA labeled with the ﬂuorochrome ﬂuorescein isothiocyanate (Sigma) for contrast enhancement of microvessels and 0.1 ml 0.2% rhodamin 6G (497 mol wt; Sigma) for in vivo staining of cytochrome c-containing cells (leukocytes) was injected intravenously into the right jugular vein before the measurement time point. A saltwater immersion objective (SW ×250/0.6; Leitz) allowed magniﬁcation of approximately ×800. Observations were recorded by means of a charge-coupled device (CCD) video camera (model FK 6990; Colu, Perspectives Measurements, San Diego, CA) and stored on videotape (video recorder; AG-Panasonic) for ofﬂine evaluation. Quantitative assessment of the microcirculation included determination of the FCD and the number of adherent leukocytes in the postcapillary venules. These parameters were measured at 120 min after the start of reperfusion. FCD is deﬁned as the length of red blood cell perfused capillaries (cm) per observation area (cm²) (16). The FCD was determined by analysis of the videotapes using CAMA images analyzing system (9). Ten randomly selected regions of interest (400 × 300 μm) of the pancreas were evaluated. For quantiﬁcation of leukocyte-endothelial interaction, at least three postcapillary venules (<40 μm diameter and <150 μm length) per animal were recorded for 30 s. Adherent leukocytes were deﬁned as cells remaining stationary on the surface of the endothelium for the whole observation time of 30 s. The surface area of the vessel segments was calculated based on diameter measurement, assuming a cylindrical geometry of the vessels. Adherent leukocytes are given as cells per endothelial surface (mm²).

Assay of serum total hemolytic C activity. Blood was withdrawn through the arterial catheter and centrifuged (3,000 rpm, 4°C, 10 min) to obtain serum. The samples were then stored at −70°C until assayed. For an assay, 5 μl of serum were added to wells plated in agarose gel containing standardized sheep erythrocytes sensitized with hemolysin (kit RC001; The Binding Site, San Diego, CA). Plates were incubated for 18 h at 4°C and then for 1 h at 37°C. Areas of the zones of hemolysis around each well (radial immunodiffusion) were measured by imaging these zones with a CCD camera after scanning of the images. Their relative optical densities were calculated by using the software WinLab for analyzing electrophoretic gels. These values were converted to total hemolytic C activity (CH50) units (one unit being the amount of C that lyses 100% of the erythrocytes) by interpolation from calibration curves plotted using the manufacturer’s
standard, diluted from neat to 1:32 (minimum sensitivity, 32 CH₁₀₀₀ units).

Statistical analysis. All data are presented as means ± SD. Data analyses were performed by using a statistical software package (Sigma Stat 2.0; Jandel Scientific Software, San Rafael, CA). Data comparison between the groups were tested by Mann-Whitney U-test followed by a Bonferroni-Holm correction. Within each group, Friedman repeated-measures ANOVA on ranks followed by Dunnnett’s method was performed. P < 0.05 was considered to be statistically significant.

RESULTS

Macrohemodynamics and general data. There was no significant difference of MAP in sham operated animals during the whole observation time (Fig. 1). In both ischemia groups, MAP dropped significantly compared with baseline values and sham-operated animals at the early onset of reperfusion. In the sCR1-treated group, this drop of MAP could not be reversed completely and was more severe compared with initial control values during the whole reperfusion time. Whereas there were no significant changes in lactate values (Table 1) in the sCR1-treated and sham-operated animals, lactate values were significantly increased in the vehicle-treated ischemia group 120 min after reperfusion. Hematocrit and negative base excess values were significantly higher 120 min after reperfusion in both ischemic groups compared with sham-operated animals.

Microvascular permeability. At 90 min after reperfusion, vehicle-treated animals showed a significant increase (6.95 ± 1.56 × 10⁻⁸ cm/s; n = 7, P < 0.01) of rhodamin-labeled albumin permeability compared with the sham group (3.18 ± 0.7 × 10⁻⁸ cm/s; n = 7) (Fig. 2). sCR1 treatment resulted in significant (P < 0.01) reduction of permeability (1.77 ± 1.34 × 10⁻⁸ cm/s; n = 7) when compared with the vehicle-treated group. There was no significant difference of microvascular permeability between sham-operated and sCR1-treated animals undergoing ischemia.

**FCD.** FCD (Fig. 3) was reduced to 279 ± 15.7 cm⁻¹ (n = 7, P < 0.01) and 330 ± 3.7 cm⁻¹ (n = 7, P < 0.01) in vehicle- and sCR1-treated ischemia groups, respectively, compared with sham-operated animals (393 ± 8.9 cm⁻¹; n = 7). Treatment with sCR1 was associated with improved FCD (P < 0.01) compared with the vehicle-treated group.

**Leukocyte-endothelium interaction.** Normothermic ischemia-reperfusion of the pancreas resulted in a significant (314 ± 87 mm⁻²; n = 7) increase of leukocyte adherence in postcapillary pancreatic venules (P < 0.01) compared with the sham-operated group (128 ± 47 mm⁻²; n = 7) (Fig. 4). Intravenous sCR1 treatment during reperfusion reduced this increase to values (163 ± 71 mm⁻²; n = 7) not significantly different from values of sham-operated animals.

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<table>
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<th>Parameter</th>
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<th>Reperfusion, 120 min</th>
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<tr>
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Values are means ± SD; *P < 0.05 vs. sham; †P < 0.05 vs. baseline; sCR1, recombinant soluble complement receptor 1.

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Fig. 1. Mean arterial pressure before (−60 min) and after (up to 120 min) reperfusion in animals with sham operation (●), 60-min pancreatic ischemia and 3 ml/kg vehicle infusion (○), and 60-min pancreatic ischemia and 15 mg/kg recombinant soluble complement receptor 1 (sCR1) infusion (∗). Values are means ± SD; *P < 0.05 vs. sham; †P < 0.05 vs. −60 min.

Fig. 2. Microvascular permeability of rhodamin-labeled albumin 90 min after the onset of reperfusion. Values are means ± SD. *P < 0.01 vs. sham and ischemia + sCR1. There were no significant differences between sCR1 and sham.
Serum CH_{100}. Total hemolytic complement activity given as the percentage of baseline values was significantly reduced in the vehicle-treated animals undergoing ischemia compared with the sham-operated group (Fig. 5). SCRI treatment resulted in less reduction of hemolytic activity without significant difference compared with sham-operated and vehicle-treated animals.

DISCUSSION

This study provides evidence that complement activation plays a pivotal role in ischemia-reperfusion-induced pancreatitis. With the use of a method for measurement of macromolecular permeability in pancreatic tissue by intravital epifluorescence microscopy, we could show the prevention of increased microvascular permeability and edema formation by administration of sCR1 during the early reperfusion time. Furthermore, the activation of leukocyte-endothelium interaction as well as the impairment of functional capillary perfusion was significantly reduced by intravenous injection of sCR1 during the onset of reperfusion.

The recombinant soluble form of CR1 (TP10), has proven to block the complement pathway being a cofactor of degradation of classical and alternative C3 convertase and enabling degradation of C3b to the inactive form iC3b. SCRI is considered to block alternative and classical complement pathway on a very early level. Next to its therapeutic effectiveness in other models of ischemia-reperfusion injury as in liver (10), heart (31), intestine (32), kidney, and lung (15), its efficacy on the postischemic pancreas was unknown. Complement activation by ischemia-reperfusion is partly dependent on the generation of hydroxyl radicals as shown by the prevention of radical generation in an intestine model of experimental ischemia-reperfusion injury (25). Furthermore, it has been shown that complement may mediate ischemia-reperfusion-induced tissue damage by nitration and attenuation of superoxide dismutase (13). In contrast to other organs, the pancreas reacts on ischemia-reperfusion stimulus with circulatory liberation of digestive enzymes like trypsin (11), which are considered to be additional potent complement-activating factors (19). It is quite controversial whether all components of the activated complement system are proinflammatory noxes in the reaction of the pancreas. A hyperexpressing C5a mouse had less inflammatory tissue damage after induction of acute pancreatitis, leading to the conclusion that this single complement might be protective by an anti-inflammatory effect (2). This might be because complex single components of the complement system have their specific tasks and differences. Furthermore, application of sCR1 to the cerulein model of acute pancreatitis failed to ameliorate pancreatic edema or lung damage (30). Both cited studies used a pancreatitis model involving cerulein as an inflammatory stimulus to the pancreas, which is very much different from the ischemia-reperfusion damage our results show. In postischemic pancreatitis, blocking the complement system on a very early step of the cascade resulted in an amelioration of pancreatic microcirculatory reperfusion damage.

Edema formation is one of the most obvious signs of microcirculatory derangement during acute pancreatitis. Ac-
According to the Starling hypothesis, fluid transport across the endothelial barrier is driven by the hydrostatic and oncotic pressures inside and outside of the microvessel wall (23). An increased permeability of the microvascular wall for albumin results in a decreased intravascular oncotic pressure. Due to this decreased intravascular oncotic force, fluid can easily be extravasated, resulting in edema of the organ, hemoconcentration, and fluid loss from systemic circulation that triggers development of hypovolemic shock state. Furthermore, the increased hydrostatic pressure of the tissue could further compromise microvascular perfusion and hinder oxygen diffusion, due to the increased space between microvessels. Although newer data from ex vivo and mathematical experiments suggest that convective flux across a capillary barrier is independent from different variables, like the local protein concentration underneath the endothelial glycocalix (8), the Starling model is probably the most suitable for explanation and determination of edema formation in the in vivo experiment. Methods so far used in estimation of edema formation, capillary leakage, and macromolecular permeability, e.g., Evans blue extravasation and organ wet/dry ratio, are invasive and thus difficult to apply together with quantitative microvascular analysis. They provide no specific information of the specific role of albumin, which is probably the most important oncotic component of the plasma. By applying a method developed for intravital microscopy of tumor microcirculation (29), we could show that activation of the complement system during the early onset of reperfusion is a crucial event of the initiation of the edema formation in posts ischemic pancreatitis.

In the present study, we could observe an increase of the functional capillary perfusion during the reperfusion by sCR1 treatment. Although we found a significant difference between both ischemic groups compared with the control group, FCD is not significantly different at the end of the experiment when microcirculatory data are measured. Therefore, in our opinion, the existing tendencies of differences of MAP during reperfusion cannot explain the differences seen in FCD and microvascular permeability after 90 and 120 min. Capillary “no-reflow” is a crucial hallmark of microcirculatory rearrangement of the pancreatic ischemia-reperfusion damage (7, 27). Because erythrocyte-perfused capillaries are very important for oxygen supply to the tissue, this parameter gives valuable information on tissue viability. By measuring pancreatic tissue oxygen pressure in patients during the early reperfusion period after pancreas transplantation, it could be shown that the increase of oxygen pressure during reperfusion correlates negatively with the highest concentration of C-reactive protein (1). This gives an indirect proof that diminished oxygen supply during reperfusion is a very important factor for the development of the early reperfusion injury.

Leukocyte-endothelium interaction is a crucial event in ischemia-reperfusion-induced pancreatitis. Complement activation results in formation of proinflammatory components like C3a, C5a, iC3b, and the membrane attack complex C5b-9. These components are associated with promotion of leukocyte activation via upregulation of CD18/11b (33), ICAM-1 (26), and selectin adhesion molecules (14). Activated leukocytes can further release proteases like cathepsin G and neutrophil elastase, which are considered to have proinflammatory potential. Using the same animal model, we have previously shown that inhibition of such proteases by a recombinant serpin could effectively reduce the posts ischemic capillary perfusion deficit (28), suggesting the active role of leukocytes in posts ischemic pancreatitis. It has been shown that the development of pancreatic injury in severe pancreatitis is associated with the increased endothelial expression of ICAM-1 (24). With the use of serum obtained from rats after induction of acute necrotizing pancreatitis, Hartwig et al. (6) have shown that trypsin-generated complement activation participates in the upregulation of the integrin MAC-1 on neutrophils, which, in turn, is known to enable the firm adhesion of phagocytes to endothelium by binding to ICAM-1. The addition of sCR1 reduced this up-regulation of MAC-1. Our experiments showed the reduction of leukocyte adhesion in postcapillary venules of the pancreas by the injection of sCR1 after normothermic ischemia and reperfusion. Therefore, complement activation may well account for the adhesion of activated leukocytes in postcapillary venules, probably by an ICAM-1-dependent mechanism in posts ischemic pancreatitis.

Total hemolytic complement activity was reduced after ischemia-reperfusion of the pancreas in vehicle-treated animals, which might be a sign of complement activation in the pathophysiological setup of ischemia-reperfusion-induced pancreatitis. sCR1 treatment resulted in a reduction of microcirculatory reperfusion damage. Hemolytic complement activity was still reduced after sCR1 treatment compared with baseline values, which is similar to the data obtained after kidney transplantation and sCR1 treatment in rats (18). Unfortunately, the cited paper shows no data of complement activity in the transplantation group not receiving sCR1, making the interpretation and comparison of the significant reduction of hemolytic activity in the vehicle-treated control group very difficult. The measured parameter might thus resemble, on the one hand, increased consumption of complement components by posts ischemic pancreatitis and, on the other hand, a sustained blocking of the complement system by sCR1, which would be expected to be more pronounced during the first 30 min after sCR1 injection.

In conclusion, the data presented are the first proof that blocking the complement system by sCR1 in the early reperfusion period is an effective approach for preservation of microcirculatory disturbances such as increased macromolecular permeability, capillary perfusion failure, and leukocyte adherence. Thus sCR1 might become a therapeutic option for prevention of reperfusion injury in the clinical setting of pancreas transplantation.

ACKNOWLEDGMENTS

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REFERENCES


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