CHAPTER 7

IN PREPARATION

Recombinant human C1 inhibitor fails to reduce Mannan-binding lectin-mediated tubular injury in a rat model of renal ischemia/reperfusion

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ABSTRACT

Ischemia/reperfusion injury (IRI) is a key event in kidney transplantation and is accompanied by activation of the complement cascade. In addition, a novel and complement-independent role for Mannan-binding lectin (MBL), initiator of the lectin pathway of complement, was established in the pathogenesis of renal IRI. Recombinant human C1-inhibitor (rhC1INH), a serine protease inhibitor, inhibits complement activation and through its differential glycosylation might also directly interact with MBL. Therefore, application of rhC1INH might be a potential therapeutic option to prevent or reduce renal IRI.

Here, we explored the therapeutic application of rhC1INH in a rat model of renal IRI and studied whether rhC1INH is able to attenuate MBL-mediated tubular injury. We demonstrate that rhC1INH was functionally active in rat serum and blocks both classical and lectin pathway activation *in vitro*. However, the therapeutic administration of rhC1INH *in vivo* did not attenuate renal dysfunction, tubular injury, inflammatory cell influx and complement cascade activation following reperfusion in rats. Kinetic studies revealed that rhC1INH was mostly cleared from the circulation within 2 hours, complement activity was only briefly reduced and circulating MBL levels were unaffected. In addition, we demonstrated that rhC1INH did not block the MBL-mediated cytotoxicity towards human tubular cells *in vitro*.

Taken together, we demonstrate that rhC1INH is not protective in a rat model of renal IRI and does not rescue human tubular cells from MBL-mediated cytotoxicity *in vitro*.

INTRODUCTION

Ischemia caused by inadequate local blood supply, is an inevitable event in kidney transplantation. Restoration of blood flow to ischemic tissue during the transplantation procedure paradoxically exacerbates tissue damage by initiating a cascade of inflammatory events including endothelial dysfunction, formation of reactive oxygen species, neutrophil sequestration and complement activation, which all contribute to post-ischemic injury (1-3).

Numerous clinical and experimental studies have shown that renal IRI has a major impact on short- and long-term graft survival after kidney transplantation and usually accounts for delayed graft function and associated morbidity and mortality in the clinic (3-5). Complement activation is a key feature of renal ischemia/reperfusion injury (IRI) and has been demonstrated both in the clinical setting (6;7) as well as in experimental models (8-10).

The complement system is a complex cascade of around thirty plasma proteins that can be activated via either the classical pathway (CP), lectin pathway (LP) or alternative pathway (AP)(11). The CP is activated by binding of C1q to e.g. antigen-antibody complexes or apoptotic cells (12) which lead to activation of the associated serine proteases C1r and C1s. The LP is activated by binding of Mannan-binding lectin (MBL) or ficolins to sugar moieties which lead to activation of the MBL-associated serine proteases (MASPs). Activated serine proteases C1r/C1s or MASPs then cleave C4 and C2, which result in the generation of the classical C3 convertase. The alternative pathway (AP) is continuously activated at a low level (so-called tickover), does not require C4 and is tightly regulated by membrane-bound and soluble complement regulators. Furthermore, properdin can act as a focal point of AP-mediated complement activation upon binding to its ligand (13). All three pathways converge at the level of complement factor C3, which is cleaved and subsequently deposits as C3b on target cells resulting in opsonization and clearance by phagocytic cells. Further activation of the complement cascade leads to generation of the membrane attack complex (MAC) C5b-9 and lysis of target cells.

C1 esterase inhibitor (C1INH), a physiologic serine protease inhibitor belonging to the serpin superfamily, acts as a major inhibitor of complement system activation and prevents uncontrolled activation (14-16). It is an acute phase protein with a mean plasma level of 250 µg/ml, and is increasing up to 2.5-fold during inflammation. C1INH can inhibit both the classical and lectin pathway, and upon complement activation, C1INH binds to activated MASP or C1r and C1s to generate MASP(C1INH) (17) and C1rC1s(C1INH)2 complexes (18-20), which subsequently dissociate from the MBL or C1q molecule, respectively. These complexes are then rapidly cleared from the circulation.

Renal IRI is associated with extensive loss of the cortico-medullary proximal tubular epithelial cells (PTEC), an event often referred to as acute tubular necrosis (ATN). Recently, we demonstrated that therapeutic inhibition of MBL with a monoclonal antibody was protective against renal IRI and more in-depth studies revealed a direct cytotoxic effect of MBL on tubular epithelial cells independent of complement activation (18). Upon reperfusion of the ischemic kidney, MBL from circulation leaks into the interstitium where it is internalized by tubular cells and contributes to tubular injury. Unfortunately, to date no anti-MBL therapy or other treatments for renal IRI in the clinic exists.

Recently however, several clinical possibilities of pharmacologic inhibition of complement, including C1INH have become available (21). C1INH might be a possible therapeutic candidate to prevent renal IRI given that C1INH regulates vascular permeability (22) and might prevent leakage of MBL in the interstitial compartment following reperfusion. Secondly, C1INH targets the MASPs,

which might be involved in the cytotoxic effect of MBL on tubular cells. Thirdly, complement that is activated at a later stage following reperfusion leading to exacerbation of tissue injury, might be inhibited.
Recombinant human C1INH (Ruconest®), which recently has been registered for treatment of acute attacks in hereditary angioedema, is differentially glycosylated (23;24) with increased oligo-mannose structures and therefore, compared to plasma-derived C1INH, might include an additional inhibitory effect towards MBL recognizing mannose residues.
In the present study, we explored the therapeutic application of rhC1INH (Ruconest®) in a rat model of renal IRI and studied whether rhC1INH is able to attenuate MBL-mediated tubular injury. We demonstrate that rhC1INH is not protective in a rat model of renal IRI and does not rescue human tubular cells from MBL-mediated cytotoxicity *in vitro*.

METHODS

Animals

The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments. Eight-week-old male Lewis rats (200–250 gram) purchased from Harlan (Horst, The Netherlands), were housed in standard laboratory cages and were allowed free access to food and water throughout the experiments. Unilateral ischemia was induced by clamping of the left renal pedicle for 45 minutes using a bulldog clamp (Fine Science Tools, Heidelberg, Germany). During clamping the contralateral kidney was removed. Sham-treated rats had identical surgical procedures except for clamping of the left kidney but including removal of the contra-lateral kidney. Before induction of ischemia, rhC1INH (Ruconest®, 625 U/kg, equivalent to 100 mg/kg; Pharming Group NV, Leiden, The Netherlands) or as a control human serum albumin (HSA; 100 mg/kg) was infused intravenously. Blood samples were collected at 1, 2, 5 or 24 hours following reperfusion and processed as serum. Kidneys were harvested 24 hours after reperfusion and subsequently animals were sacrificed.

Assessment of kidney function

Renal function was determined by measuring creatinine and urea in serum samples using standard autoanalyzer methods by our hospital research services.

Renal immunostaining

Rat kidney sections (4 μm) of snap-frozen kidneys were air dried and acetone-fixed. Kidney sections were stained with digitonin (DIG)-conjugated mAb

against CD68 (ED-1, macrophages) or goat pAb against rat KIM-1 (R&D systems, Abingdon, UK), followed by horseradish peroxidase (HRP)-conjugated sheep anti-DIG (Roche Diagnostics, Mannheim, Germany) or rabbit anti-goat (DAKO, Glostrup, Denmark), respectively. The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Rat C3 deposition was assessed by direct staining using a FITC-conjugated rabbit polyclonal to rat C3 (made in-house). Quantification of immunohistochemistry was performed in a blinded manner by assessing 10 consecutive high power fields (HPFs; magnification ×200) of the outer medulla and cortico-medullary junction on each section. Using image J software, the positive area (expressed in pixels) was quantified.

Rat serum complement activity measurements

Functional activity of the lectin and classical pathway were assessed using plates coated with mannan or IgM, as previously described (25), with minor modifications. Rat sera drawn at different time points after reperfusion or normal rat serum mixed with rhC1INH (Ruconest®, Pharming Group NV) were 1/50 diluted in GVB++ (veronal buffered saline, 0.1% gelatin, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% Tween 20) and incubated for 60 min at 37°C to allow C3 deposition in the well. The plates were washed with PBS/0.05%Tween and incubated with dig-conjugated mouse anti-rat C3 antibody (ED11, made in-house (26)), followed by HRP-conjugated sheep anti-DIG (Roche Diagnostics). After washing, C3 deposition was quantified with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; from Sigma-Aldrich; 2.5 mg/ml in 0.1M Citrate/Na_2HPO_4 buffer, pH 4.2) in the presence of 0.01% H_2O_2, for 30–60 min at room temperature. The OD at 415 nm was measured using a microtiter plate reader (Biorad).

Rat MBL ELISA

MBL levels in rat serum were assessed using a sandwich ELISA. In short, Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated with a mAb against rat MBL (P7E4, kindly provided by G.L. Stahl Harvard Medical, School, Boston, USA) followed by rat serum incubation. Rat MBL binding was detected using DIG-conjugated mAb against rat MBL (P7E4). After washing, MBL binding was quantified with ABTS; (Sigma-Aldrich). The OD at 415 nm was measured using a microtiter plate reader (Biorad).

MBL-MASP complex ELISA

Human MBL-MASP complexes after MBL purification were assessed using a sandwich ELISA. In short, Nunc Maxisorb plates were coated with a pAb against MASP-2 (kindly provided by R. B. Sim, University of Oxford, Oxford, U.K.) followed by sample incubation. MBL-MASP complex binding was detected

using DIG-conjugated mAb against human MBL (3E7, Hycult Biotecht, Uden, Netherlands). After washing, MBL-MASP complex binding was quantified with ABTS; (Sigma-Aldrich). The OD at 415 nm was measured using a microtiter plate reader (Biorad).

RhC1INH ELISA

Circulating levels of rhC1INH before, 1, 2, 5, and 24 hours after reperfusion were assessed using a sandwich ELISA as previously described (24).

Cell culture

Immortalized human PTEC (HK-2) were grown as previously described (27).

Fluorescence microscopy

HK-2 cells were fixed using cold methanol and stained with mAb against human MBL (mAb 3E7; Hycult Biotech) followed by Alexa-488 conjugated goat anti-mouse IgG (Molecular Probes). Nuclei were stained using Hoechst (Molecular Probes, Leiden, The Netherlands).

Viability assays

Cell viability was assessed using propidium iodide (PI; Molecular Probes, Leiden, The Netherlands). Following incubation with PI, cells were washed and permeabilized using cold methanol and nuclei were stained using Hoechst. Viable cell count was assessed by counting Hoechst-positive, but PI-negative cells using fluorescence microscopy.

Preparation of human MBL and MASP-free MBL

MBL was purified from human recalcified plasma as previously described (28). To yield a MASP-free MBL preparation, MBL was further purified by gel filtration using a Sepharose 6B column as previously described (29). In short, the purified MBL preparation was dialysed against 0.1 M acetic-acid containing 0.2 M NaCl and 5 mM EDTA at pH 5. Subsequently, the dialysed sample was subjected to size exclusion chromatography on a Superdex 200. This procedure results in dissociation of purified MBL–MASP complexes which were subsequently separated by size exclusion.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and subjected to nonparametric statistical analysis using one- and two-factor ANOVA or Mann-Whitney test using GraphPad Prism software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

RhC1INH is functionally active in rat serum

Before therapeutic application rhC1INH (Ruconest®) in a rat model of renal IRI, functionality of rhC1INH in rat serum was tested *in vitro*, using a complement activation assay assessing classical and lectin pathway activity. Preincubation of rat serum with rhC1INH resulted in a dose-dependent inhibition of C3 deposition (Fig 1). The lectin pathway was more effectively inhibited by rhC1INH than the classical pathway (Fig 1B), with an IC50 of 1 and 5 U/ml, respectively. Maximum inhibition of lectin pathway activation was achieved at 10 U/ml. Therefore, a therapeutic dose of 625 U/kg was chosen for *in vivo* application in rats, corresponding to an estimated end concentration of at least 10 U/ml rhC1INH in circulation.

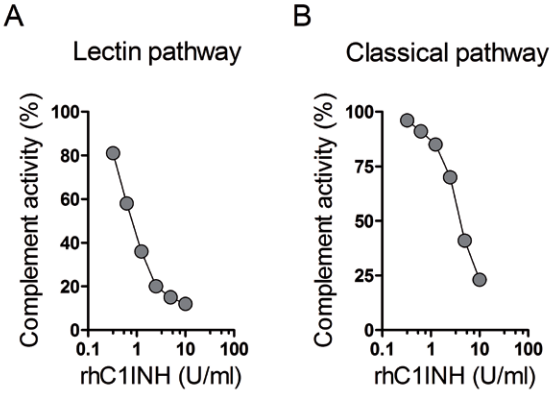

Figure 1. RhC1INH inhibits complement activation in rat serum. Preincubation of rat serum with rhC1INH resulted in a dose-dependent inhibition of C3 deposition on mannan- (A) and IgM-coated plates (B). C3 deposition as a measure of complement activity was calculated as a percentage of C3 deposition in wells with untreated rat serum.

RhC1INH does not preserve renal function after ischemia/reperfusion

Five minutes before induction of renal ischemia, rats (n=6) were infused with rhC1INH (625 U/kg) or as a control human serum albumin (HSA). Induction of ischemia for 45 min followed by reperfusion resulted in a significant renal dysfunction in control-treated rats as measured by serum levels of creatinine (Figure 2A) and urea (Figure 2B). Treatment with rhC1INH did not protect against renal dysfunction. Rats treated with rhC1INH even tended to show a higher increase in creatinine and urea (Fig 2B) compared to control treated animals at 24 hours of reperfusion.

RhC1INH does not reduce C3 deposition, cellular influx and tubular injury

Rats were sacrificed at 24 hours of reperfusion and deposition of C3 was assessed. While kidneys from sham-operated rats showed a C3 staining with a typical half-moon distribution surrounding tubular cells, following IRI there

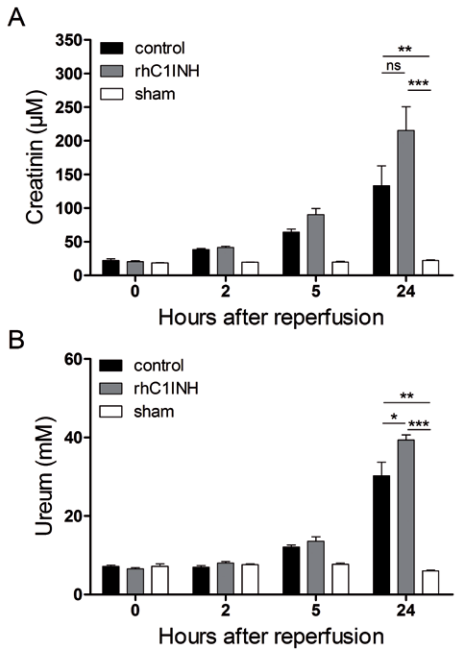

Figure 2. RhC1INH does not preserve renal function after ischemia/ reperfusion. Before induction of unilateral ischemia and removal of the contralateral kidney, rats received rhC1INH (625 U/kg) or as a control human serum albumin (100 mg/kg). Before and following reperfusion, blood samples were drawn at consecutive time points to assess renal function by measuring serum creatinine (A) and urea (B) levels. Data are shown as mean ± SEM, n = 6 per group. The comparison between groups is indicated by asterisks. *p<0.05; **p<0.01; ***p<0.001.

was a significant increase in tubular C3 deposition (Fig 3A). Although rhC1INH was able to inhibit C3 deposition *in vitro* (Fig 1), administration *in vivo* did not reduce tubular deposition of C3. Also influx of macrophages (Fig 3B), which was apparent in the control treated animals, was not reduced in the rhC1INH-treated group. Expression of kidney injury molecule (KIM)-1, an early marker for tubular injury, was strongly expressed at 24 hours following reperfusion (Fig 3C) and was also not reduced by application of rhC1INH.

Kinetics of rhC1INH in rats are unfavorable for effective protection

Assessment of complement activity in circulation revealed that 1 hour following reperfusion both the lectin and classical pathway were blocked efficiently by rhC1INH. However, this inhibitory effect was only short, since at 2 hours of reperfusion, complement activity of both lectin (Fig 4A) and classical (Fig 4B) pathway was restored to basal levels. This prompted us to study the pharmacokinetics of rhC1INH *in vivo*. Measurement of rhC1INH levels following administration revealed a fast clearance of rhC1INH from circulation. One hour after reperfusion still sufficient rhC1INH levels (Fig 4C) for lectin and classical pathway inhibition in circulation (Fig 4A, B) were detected (10 U/ml) as was also calculated from the assay assessing rhC1INH functionality in rat serum *in vitro* (Fig 1). In contrast, rhC1INH levels at 2 hours were reduced by more than threefold and 5 hours after reperfusion almost undetectable. From this, a half-life of approximately 30 minutes could be calculated. The data suggest that the

rhC1INH concentrations at 2 and 5 hours of reperfusion were not sufficient for effective complement inhibition in the circulation.

RhC1INH does not inhibit MBL-mediated tubular cell death

Recently we showed that inhibition of MBL by a monoclonal antibody is protective against renal IRI in rats (30). In addition, we showed that MBL directly induces tubular injury independent of complement activation. Assessment of circulating MBL levels after rhC1INH administration demonstrated that rhC1INH did not affect the levels of MBL in circulation (Fig 4D). Lectin pathway activity at 1 hour reperfusion was efficiently blocked, indicating that rhC1INH only targets and dissociated the MASPs from MBL, but did not affect MBL itself. To assess whether rhC1INH was able to prevent MBL-mediated tubular injury, human tubular epithelial cells were incubated with purified MBL *in vitro* (Fig 5A), which resulted in an extensive induction of cell death within 24 hours. Preincubation of MBL with a dose response of rhC1INH (10-1000 µg/ml, Fig 5A, B), however, did not protect tubular cells from MBL-mediated cell death.

RhC1INH is a protease inhibitor and irreversibly binds to and inactivates MBL-associated serine proteases (MASP)-1 and -2. To study whether these MASPs,

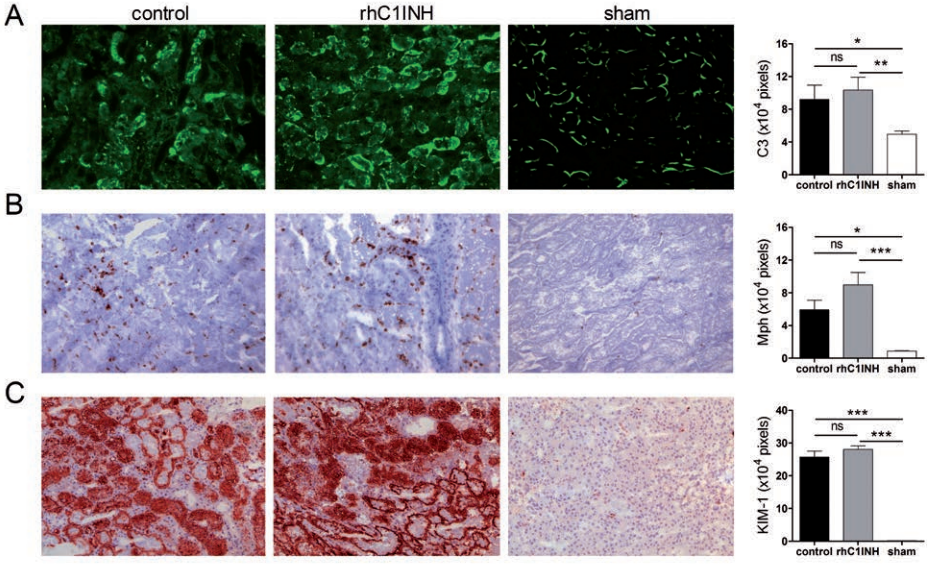

Figure 3. RhC1INH does not reduce C3 deposition, cellular influx and tubular injury. Renal sections from control- (A), rhC1INH-treated (B) or sham-operated rats 24 h following reperfusion were stained for C3 deposition (A) macrophages (Mph; B) or kidney-injury molecule (KIM)-1 (C). C3 deposition, Mph infiltrate and KIM-1 expression were quantified using digital image analysis. Data are shown as mean ± SEM (n = 6 per group). Original magnification ×200. The comparison between groups is indicated by asterisks. *p < 0.05; **p<0.01; ***p<0.001.

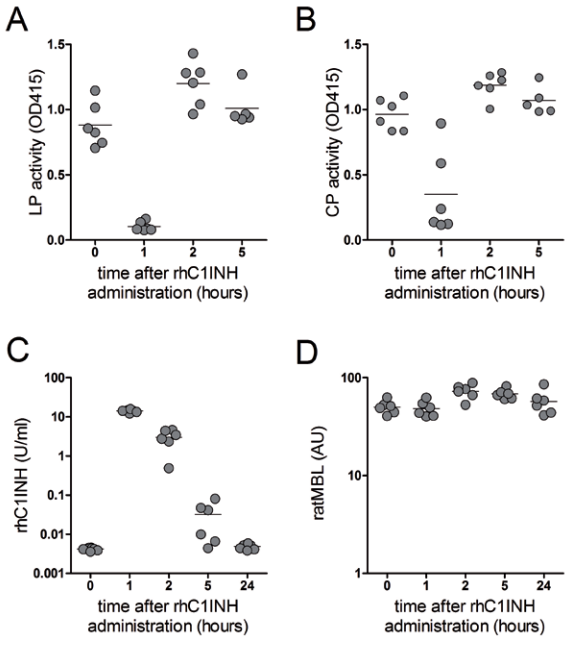

Figure 4. RhC1INH kinetics in rats are unfavorable for effective protection. Before induction of unilateral ischemia and removal of the contralateral kidney, rats received rhC1INH intravenously (625 U/kg). Before and following rhC1INH infusion, blood samples were drawn at consecutive time points to assess lectin (A) and classical (B) pathway activity and circulating levels of rhC1INH (C) and MBL (D).

which are co-purified with MBL, might be involved in MBL-mediated tubular cell death, blocking antibodies to MASP-2 were applied. Exposure of tubular cells to MBL in the presence of MASP-2 antibodies did not prevent MBL-mediated cell death (Fig 5A, B). Since this strategy did not target MASP-1, MASP-free MBL was purified. Using a MBL-MASP complex ELISA, absence of MBL-MASP complexes was confirmed (Fig 5C). Incubation of MASP-free MBL on tubular cells still affected tubular cells (Fig 5B), indicating that MASPs are not involved in MBL-mediated tubular injury.

DISCUSSION

In the present study we demonstrated that rhC1INH is not protective in a rat model of renal IRI. Therapeutic administration of functionally active rhC1INH *in vivo* did not attenuate renal dysfunction following reperfusion, nor did it inhibit influx of inflammatory cells, tubular injury or complement cascade activation.
Although rhC1INH inhibited complement activation *in vitro*, tubular C3 deposition at 24 hours following reperfusion was present in rhC1INH-treated animals at the same extent as control-treated animals. Recently, we and others have shown that activation of complement is a relative late event following reperfusion with the first signs of C3 deposition at 2 and 24 hours upon reperfusion in mice (7) and rats (30), respectively. In our rat model, kinetic analysis revealed that rhC1INH

only effectively inhibited complement activity in the first hour after reperfusion and that both classical as well as lectin pathway activity was fully restored in the next hour. This fast clearance of rhC1INH (Fig 4A) might explain the inability of rhC1INH to reduce complement deposition and complement-mediated injury. Castellano et al (6) showed that a comparable dose of rhC1-inhibitor infused in pigs significantly protected against renal IRI and reduced both lectin and classical pathway activation up to at least 240 minutes after infusion, indicating an important species difference in the clearance of and protection by rhC1INH. Another important difference between these two models is the presence of peritubular C4d staining in pigs following reperfusion (6), which is completely absent in rats (data not shown). This suggests that classical and/or lectin pathway activation by C1q and MBL leading to C4 deposition in the kidney following reperfusion is virtually absent in rodents. These data are in line with IRI studies in mice, in which C4-deficient animals are not protected (10). However, recently

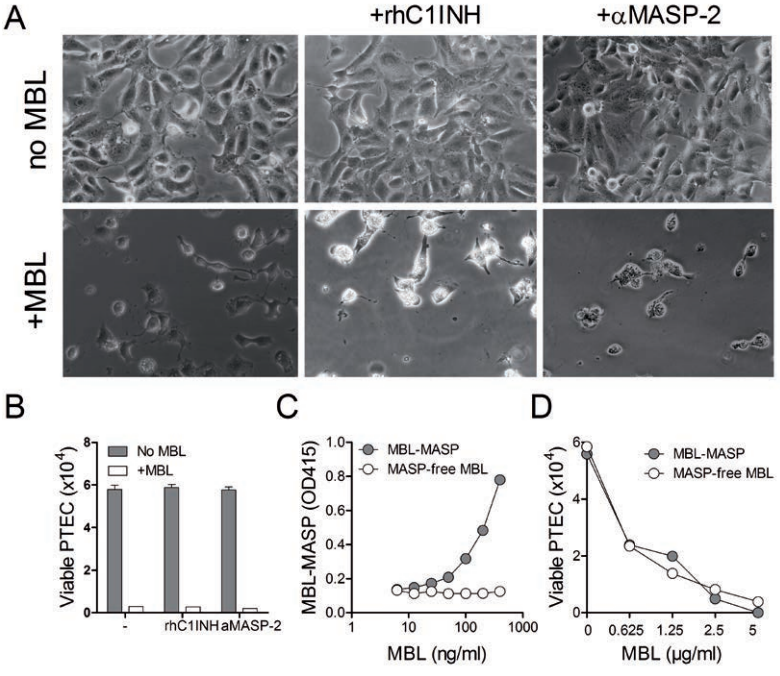

Figure 5. RhC1INH does not inhibit MBL-mediated tubular cell death. Human PTEC (HK-2) were incubated with or without 10 µg/ml human MBL (A, B) in the presence or absence of rhCINH (1 mg/ml) or blocking antibodies to MASP-2 (100 µg/ml) for 24 h. Subsequently, wells were photographed (A; magnification ×200) and quantified (B). To assess the contribution of MASPs in MBL-mediated cell death, MASP-free MBL was generated (C) and incubated (D) in a dose response on human PTEC. Viable cells were assessed by counting Hoechst-positive, but PI-negative cells in the well using fluorescence microscopy.

it was shown in several mouse models of IRI, that MBL in association with MASP-2 (31) can bypass C4 and directly cleave C3 (32). Indeed, mice deficient for MBL were protected against renal IRI (33). Glycosylated tubular meprins, which bind MBL (34;35), were shown to be involved in the activation of complement in the kidney. Inhibition of MBL-MASP complexes by rhC1INH might therefore still be a therapeutic option, however the unfavorable fast clearance of rhC1INH does not warrant any protective effects in rodents.

We recently demonstrated an entirely new concept of lectin pathway participation in renal IRI. In a rat model we showed that MBL exerts direct cytotoxic effects to tubular epithelial cells in the complete absence of complement activation (30). Inhibition of MBL was almost completely protective against renal IRI, whereas downstream inhibition of complement was not. Because rhCINH targets the MBL-associated serine proteases (MASPs) (15), we examined whether MASPs might be involved in this MBL-mediated tubular cell death. However, blockade of MASP-2 or depletion of MASPs from MBL, did not abrogate the cytotoxic effect of MBL (Fig 5), suggesting that interference of rhC1INH with MASPs would not reduce the cytotoxicity of MBL towards tubular cells.

RhC1INH, in contrast to purified human C1INH, is known to be heavily glycosylated (23;24) and is therefore thought to have an additional inhibitory effect towards the lectin pathway of complement by directly binding MBL. Since rhC1INH was cleared quickly from circulation (Fig 4A), we considered whether this might affect circulating levels of MBL. This effect however was negligible since circulating MBL levels following reperfusion remained stable in the first hours following rhC1INH administration. Also *in vitro*, rhC1INH did not inhibit cytotoxic effects exerted by MBL towards human tubular cells, indicating that rhC1INH is not able to reduce the complement-independent effects of MBL.

Although an important function of C1INH is to regulate complement activation, it also inhibits proteases of the fibrinolytic, clotting, and kinin pathways. Deficiency of C1INH is associated with hereditary angioedema (22;36). Involvement of fibrinolytic and clotting pathways in renal IRI has been demonstrated (37-39), which also might be influenced by C1INH. In the past few years, it has become apparent that C1INH has additional anti-inflammatory functions independent of protease inhibition. These include interactions with leukocytes that may result in enhanced phagocytosis (40), with endothelial cells via E- and P-selectins that interfere with leukocyte rolling and in turn results in suppression of transmigration of leukocytes across the endothelium (41) and interactions with extracellular matrix components (42;43). In addition, C1INH has a direct anti-apoptotic activity on vascular endothelial and myocardial cells, associated with blocking of cytochrome c translocation and the inhibition of caspase-3 activation by normalization of the pro-apoptotic Bcl-2/Bax expression ratio

(44;45). Altogether, these effects contribute to the protective effect of C1INH in reperfusion injury. Treatment with C1INH has been successfully performed in animal IRI models in the heart (44;46), intestine (47), skeletal muscle (48), liver (49) and brain (50;51). However in all these models, purified C1INH from human plasma was used. This might explain the discrepancy observed with our renal IRI model, in which the recombinant form of C1INH was used. Pharmacokinetic studies in rats revealed that the recombinant form was cleared from circulation within two hours. Timing of rhC1INH administration before the initiation of ischemia was therefore not optimal, since most rhC1INH has been cleared from the circulation by the time that it was actually needed and complement activation was most evident. Furthermore, the complement-independent MBL-mediated cytotoxicity, which could not be inhibited by rhC1INH *in vitro*, may be more pronounced in the kidney than in other organs systems.
Taken together, we demonstrated that rhC1INH is not protective against renal IRI in rats and does not rescue human tubular cells from MBL-mediated cytotoxicity *in vitro*.

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