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Cell Injury, Repair, Aging and Apoptosis

Gene Silencing of Complement C5a Receptor Using siRNA for Preventing Ischemia/Reperfusion Injury

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Ischemia/reperfusion (I/R) injury in organ transplantation significantly contributes to graft failure and is untreatable using current approaches. I/R injury is associated with activation of the complement system, leading to the release of anaphylatoxins, such as C5a, and the formation of the membrane attack complex. Here, we report a novel therapy for kidney I/R injury through silencing of the C5a receptor (C5aR) gene using siRNA. Mice were injected with 50 μg of C5aR siRNA 2 days before induction of ischemia. Renal ischemia was then induced through clamping of the renal vein and artery of the left kidney for 25 minutes. The therapeutic effects of siRNA on I/R were evaluated by assessment of renal function, histopathology, and inflammatory cytokines. siRNA targeting C5aR efficiently inhibited C5aR gene expression both *in vitro* and *in vivo*. Administering C5aR siRNA to mice preserved renal function from I/R injury, as evidenced by reduced levels of serum creatinine and blood urea nitrogen in the treated groups. Inhibition of C5aR also diminished *in vivo* production of the pro-inflammatory cytokine tumor necrosis factor-α and chemokines MIP-2 and KC, resulting in the reduction of neutrophils influx and cell necrosis in renal tissues. This study demonstrates that siRNA administration represents a novel approach to preventing renal I/R injury and may be used in a variety of clinical settings, including transplantation and acute tubular necrosis. ***(Am J Pathol 2008, 173:973–980; DOI: 10.2353/ajpath.2008.080103)***

Ischemia reperfusion (I/R) injury occurs in organ transplantation and other medical settings, causing a characteristic pattern of injury to organs and tissues. I/R induces endothelium cell perturbance of signal pathways and expression of molecules. Many toxic metabolic products accumulate in I/R-injured kidney, resulting in renal dysfunction associated with many life-threatening conditions and disease. Although the intracellular and molecular mechanisms involved in the development of renal I/R injury are complex and not yet fully understood,[1] I/R injury is the main cause of the acute tubular failure. Recent studies in animals have demonstrated a pivotal role for the complement system in mediating renal I/R injury.[2,3] Activation of the complement pathway results in the release of the anaphylatoxins C3a and C5a and formation of the membrane attack complex, which induce chemokines and mediate neutrophils activation and infiltration, leading to renal cell injury, apoptosis, and necrosis.[3] The biological activities of C5a are mediated through its binding to the ubiquitous C5a receptor (C5aR), a G-protein-coupled seven transmembrane domain receptor.[3] In animal models, renal I/R injury can be abrogated by treatment with the complement inhibitors, such as anti-C5 antibodies and C5a receptor antagonists,[4,5] or by genetic manipulation of C3 in knockout mice,[6] or by gene silencing C3 with C3-specific siRNA.[7]

Small-interfering RNA (siRNA) is a powerful tool used to silence gene expression in mammalian cells at the post-transcriptional level. siRNA specifically inhibits gene expression with high efficiency.[8] Previously, other groups and we have successfully delivered siRNA into kidney/liver tissues by systemic administration for prevention of kidney/liver warm I/R injuries.[7,9–12]

In this study, we report for the first time that efficient silencing of C5aR, the central component of the complement activation cascade, can be achieved using siRNA, and furthermore, results in the inhibition of complement activation and prevention of renal I/R injury.

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Materials and Methods

Mice

CD1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained under strict pathogen-free conditions. All mice were male of 6 to 8 weeks old. All experiments were performed in accordance with the *Guide for the Care and Use on Animals Committee Guidelines*.

C5aR siRNA Design

Two target sequences of C5aR gene were selected. The oligonucleotides containing sequences specific for C5aR (#1: 5′-GATCCCGTTTAGAGTGAGCAGAGGCAACTTCAAGAG-AGTTGCCTCTGCTCACTCTAAATTTTTTCCAA A-3′ and 5′-AGCTTTTGGAAAAAATTTAGAGTGAGCAGAGGCAACTC-TCTTGAAGTTGCCTCTGCTCACTCTAAACGG-3′; #2: 5′-GATCCCGTCAGAAACCAGATGGCGTTTGTTCAAGAGAC-AAACGCCATCTGGTTTCTGATTTTTTCCAA A-3′ and 5′-AGCTTTTGGAAAAAATCAGAAACCAGATGGCGTTTGTC-TCTTGAACAAACGCCATCTGGTT TCTGACG G-3′) were synthesized and annealed. A C5aR siRNA expression vector, which expresses hairpin siRNA under the control of the mouse U6 promoter and cGFP genes, was constructed by inserting pairs of annealed DNA oligonucleotides into a pRNAT-U6.1/Neo siRNA expression vector that had been digested with BamH I and HindIII (Genescript, Piscataway, NJ).

In Vitro *Silencing of the C5aR Gene*

L929 cells were co-transfected with C5aR cDNA and C5aR siRNA using lipofectamine 2000 (Invitrogen Life Biotechnologies, Carlsbad, CA). Briefly, cells were plated into 24-well plates (10^5 cells per well) and allowed to grow overnight to reach 90% confluence. Cells were co-transfected with 0.5 μg C5aR cDNA and 2 μg C5aR siRNA or negative control siRNA plasmids in serum-reduced medium for 5 hours, and then incubated in complete medium for 24 hours. The vehicle alone and scrambled (nonsense) siRNA were used as negative controls.

Renal I/R Injury Model and siRNA Administration

CD1 mice, aged 6 to 8 weeks, were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and placed on a heating pad to maintain their body temperature during the surgery. Following abdominal incisions, renal pedicles were bluntly dissected and a microvascular clamp (Roboz Surgical Instrument, Washington, DC) was placed on the left renal pedicle for 25 minutes or 30 minutes. During the procedure, animals were kept at a constant temperature (37°C). Following ischemia, the clamps were removed, along with the right kidney. Then, the incisions were sutured, and the animals were allowed to recover with free access to food and water. Blood was collected and the left kidney was harvested for analysis 24 hours after reperfusion.

To investigate the efficacy of protection on mice renal I/R injury by siRNA, the mice were administrated with siRNA 48 hours before operation. 50 μg of C5aR siRNA plasmid DNA were diluted in 1 ml of PBS and injected into mice by tail vein by a "hydrodynamic" injection.[13]

Assessment of Renal Function

Blood samples were obtained from the inferior vena cava 24 hours postischemia. Serum creatinine levels were measured by the core laboratory at the London Health Sciences Center to monitor renal function. Blood urea nitrogen (BUN) also was measured.

Histology Detection

At 24 hours post-ischemia, kidneys were dissected from mice, and tissue slices were fixed in 10% formalin and processed for histology examination using standard techniques. Formalin tissue was embedded in paraffin and 5 μm sections were stained with H&E. These sections were double-blindly examined by a pathologist. The percentage of histology changes in the cortex and medulla was scored using a semiquantitative scale designed to evaluate the degree of infarction, tubular vacuolization, and cast formation on a five-point scale based on injury area of involvement. The scale is as follows: 0 = $<$10%; 1 = 10% to 25%; 2 = 25% to 50%; 3 = 50% to 75%; and 4 = 75% to 100%.

Myeloperoxidase Aassays

Kidney myeloperoxidase activity was evaluated by immunohistochemistry. Briefly, paraffin sections were deparaffinized and rehydrated. Then, the samples were incubated in a ready-to-use peroxidase blocking solution (Dako, Carpinteria, CA) to inhibit endogenous peroxidase. To block nonspecific background staining, the sections were incubated with 10% horse serum. The sections were then incubated with rabbit anti-human myeloperoxidase antibody at 1:100 dilution (Lab Vision, Fremont, CA), followed by incubation with EnVision$^+$ Rabbit-HRP (Dako). After incubating with the chromogenic substrate, the sections were counterstained with hematoxylin. The slides were examined under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) at $\times$400, and all analyses were performed by two pathologists blind to the group assignments. The staining of cytoplasmic myeloperoxidase in the neutrophils was evaluated, and the results were expressed as average percentage of myeloperoxidase -positive staining cells in 10 fields in the same section.

Western Blot

Cytoplasmic extracts were prepared from Ca5R-silenced and control siRNA-treated cells, which were mechanically released from tissue culture plates by scraping in cold PBS. Cells were collected by centrifugation (800 $\times$

g), and then resuspended in buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1% NP40, 1 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethylsulfonyl fluoride] with complete protein inhibitor (Roche Diagnostics, Laval, QC). Protein content was determined (Bio-Rad Laboratories, CA) and 50 μg of each cell lysate was resolved on 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Bio-Rad Laboratories), blocked with 5% fat-free milk (Carnation) and 3% bovine serum albumin in tris-buffered saline with 0.25% Tween-20, probed with a rabbit anti-mouse C5aR Ab (Sancruz, CA), and goat anti-mouse β-actin Ab (Sigma, Saint Louis, MO) according to the manufacturer's instructions, and visualized by an enhanced chemiluminescence assay (Amersham Pharmacia Biotech).

Immunohistochemistry for C5aR, C3, and C9 Deposition in Kidney Tissue

Kidneys were snap-frozen in optimal cutting temperature compound (Sakura, Finefeck, Torrance, CA) and stored at −80°C. Five μm frozen sections were cut with cryostat. Sections were fixed in cold acetone for 10 minutes and allowed to air dry, followed by blocking with 10% normal horse serum in PBS. The primary antibodies, rabbit anti-mouse C5aR(Santa Cruz Biotechnology, CA), C3 (Hycult Biotechnology, Netherlands), and rabbit anti-rat C9 (kindly gifted by Dr. P. Morgan, University of Wales College of Medicine, Cardiff, UK), which cross-reacts with mouse C9,[14] were added to the sections for 1 hour at room temperature, respectively. Sections were then rinsed with PBS and treated with a ready-to-use peroxidase-blocking solution (DakoCytomation, Carpinteria, CA). After washing with PBS, the slides were incubated with rabbit EnVison$^+$ HRP (DakoCytomation) for 30 minutes. Sections were washed and developed with ready-to-use diaminobenzidine for 1 to 3 minutes and counterstained with hematoxylin (DakoCytomation). The semiquantitative method was applied to measure the C5a, C3, and C9 positive staining. Isotype sera and omitting primary antibody were used as negative controls.

Measurement of Renal C5aR, Tumor Necrosis Factor-α, KC, and MIP-2 mRNA Levels by Quantitative Real-Time PCR

Total RNA was extracted from kidneys and cells using Trizol (Invitrogen Life Biotechnologies) and reverse-transcribed using the oligo-(dT) primer and reverse transcriptase (Invitrogen Life Biotechnologies). Primers used for the amplification of murine C5aR, tumor necrosis factor (TNF)-α, KC, MIP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: C5aR, 5′-GAAGCGGCAACCTGGGGATGT-3′ (forward) and 5′-AGGAAACGGTCGGCACTAATGGTA-3′ (reverse); TNF-α, 5′-CTCCCTCCAGAAAAGACACCAT-3′ (forward) and 5′-ATCACCCCGAAGTTCAGTAGACAG-3′ (reverse); KC, 5′-CGCTCGCTTCTCTGTGCA-3′(forward) and 5′-ATTTTCTGAACCAAGGGAGCT-3′ (reverse)[5]; MIP-2, 5′-TGCCGGCTCCTCAGTGCTG-3′ (forward) and 5′-AAACTTTTTGACCGCCCTTGA-3′(reverse)[5]; and GAPDH, 5′-TGATGACATCAAGAAGGTGGTGAA-3′ (forward) and 5′-TGGGATGGAAATTGTGAGGGAGAT-3′ (reverse).

Real-time PCR reactions for C5aR, TNF-α and GAPDH were performed to examine gene expression in the Stratagene MX 4000 multiplex quantitative PCR system using the SYBR Green PCR Master mix (Stratagene) and 100 nmol/L of gene-specific forward and reverse primers. The reaction conditions were 10 minutes at 95°C, 15 seconds at 95°C, 1 minute at 58°C, and 1 minute at 72°C (40 cycles). Samples were normalized using the housekeeping gene GAPDH, and a comparative C_T method was used for the analysis.

Semiquantitative PCR was used to detect MIP-2 and KC gene expression. PCR was performed in a total reaction volume of 25 μl, in the presence of 0.2 mmol/L dNTP, 0.2 μmol/L of each primer, 0.15 mmol/L $MgCl_2$, and 1 U Tap DNA polymerase (Invitrogen, Life Biotechnologies). For each gene, the following PCR conditions were used: MIP-2 and KC, 95°C for 40s, 55°C for 40s,72°C for 40s repeating 35 cycles; GAPDH, 95°C for 30s, 58°C for 30s,and 72°C for 30s, repeating 30 cycles. The PCR products were run on 1.5% agarose gel and estimated densitometry of ethidium bromide-stained using a CCD

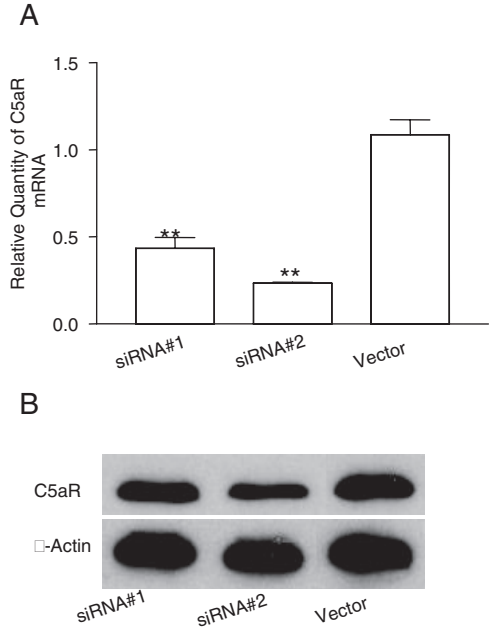

Figure 1. siRNA inhibits C5aR expression *in vitro.* **A:** Gene expression detected by quantitative PCR. L929 cell lines were cultured *in vitro* and were co-transfected with C5aR cDNA and two different sequences of C5aR-specific siRNA using lipofectamine 2000. 24 hours after siRNA transfection, cells were collected to extract total RNA. Transcripts of C5aR and GAPDH from silenced and non-silenced cells were determined using quantitative real-time PCR. Vector was used as a calibrator sample, and the C5aR gene was normalized to GAPDH, a housekeeping gene. Relative quantity of C5aR mRNA was expressed as mean ± SEM. Statistical significance as compared with sequence #1 and #2 versus vector (**) was denoted at as $P < 0.05$. **B:** Gene expression detected by Western blot. L929 cells were co-transfected with C5aR cDNA and C5aR siRNA as described above. 48 hours after transfection, cells were collected to extract protein. 50 μg of total protein from the lysate was resolved on 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane. The membranes were immunoblotted with a rabbit anti-mouse C5aR Ab, and goat anti-mouse β-actin Ab as an internal control of protein amount.

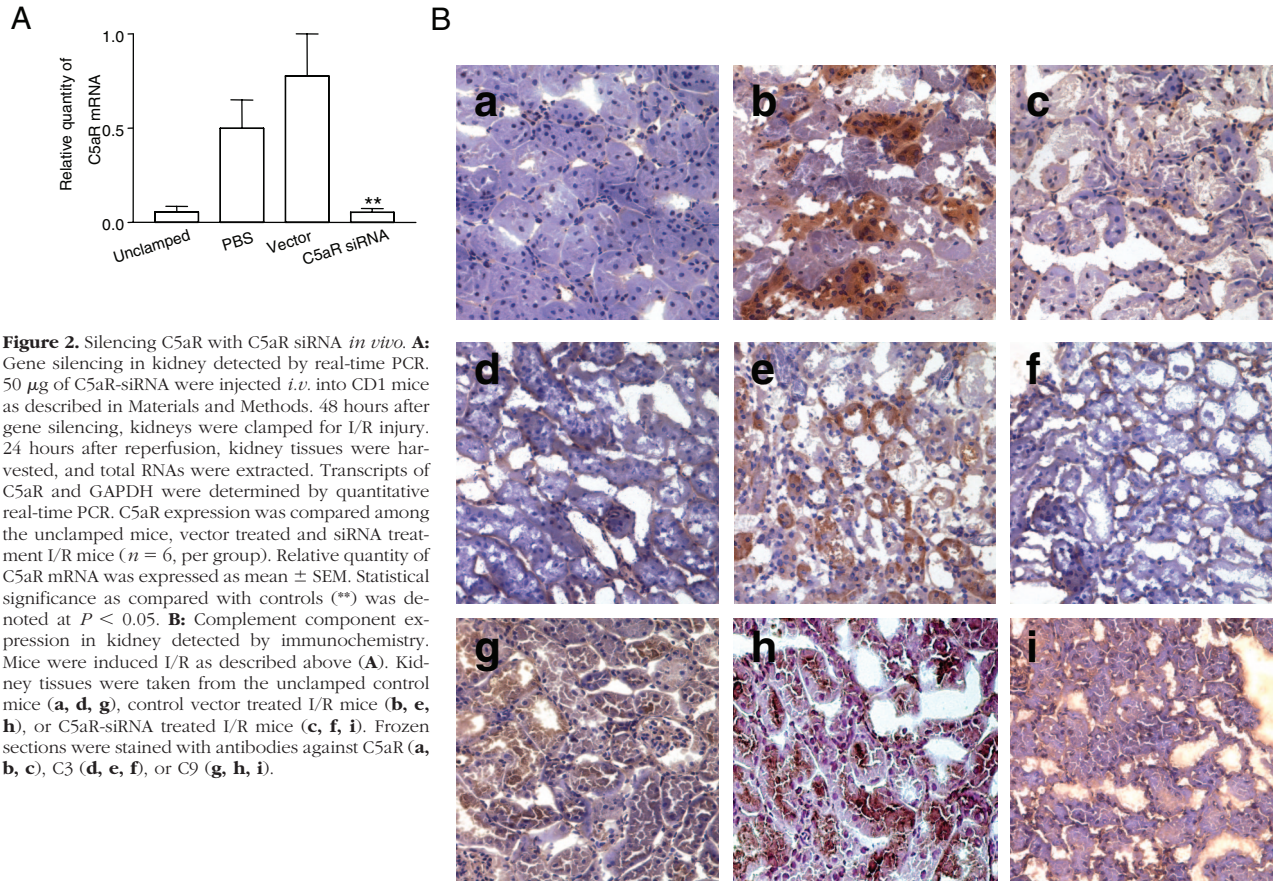

Figure 2. Silencing C5aR with C5aR siRNA *in vivo*. **A:** Gene silencing in kidney detected by real-time PCR. 50 μg of C5aR-siRNA were injected *i.v.* into CD1 mice as described in Materials and Methods. 48 hours after gene silencing, kidneys were clamped for I/R injury. 24 hours after reperfusion, kidney tissues were harvested, and total RNAs were extracted. Transcripts of C5aR and GAPDH were determined by quantitative real-time PCR. C5aR expression was compared among the unclamped mice, vector treated and siRNA treatment I/R mice ($n = 6$, per group). Relative quantity of C5aR mRNA was expressed as mean ± SEM. Statistical significance as compared with controls (**) was denoted at $P < 0.05$. **B:** Complement component expression in kidney detected by immunochemistry. Mice were induced I/R as described above (**A**). Kidney tissues were taken from the unclamped control mice (**a, d, g**), control vector treated I/R mice (**b, e, h**), or C5aR-siRNA treated I/R mice (**c, f, i**). Frozen sections were stained with antibodies against C5aR (**a, b, c**), C3 (**d, e, f**), or C9 (**g, h, i**).

camera and Imagemaster VDS software (Bio-Rad). Relative MIP-2 and KC expressions were calculated by comparison of band intensities of the PCR products. GAPDH gene expression was used as an internal control.

Statistical Analysis

Data are expressed as means ± SEM. Statistical comparisons between groups were performed using a One-way analysis of variance test. Histopathology data were analyzed using χ^2 analysis. Survival was analyzed by rank-log test. Statistical significance was determined as $P < 0.05$.

Results

Silencing C5aR in Vitro *Using siRNA*

To knock down C5aR expression, we designed two targeting sequences specific to C5aR gene (C5aR-siRNA). The targeting sequences were cloned into the shRNA

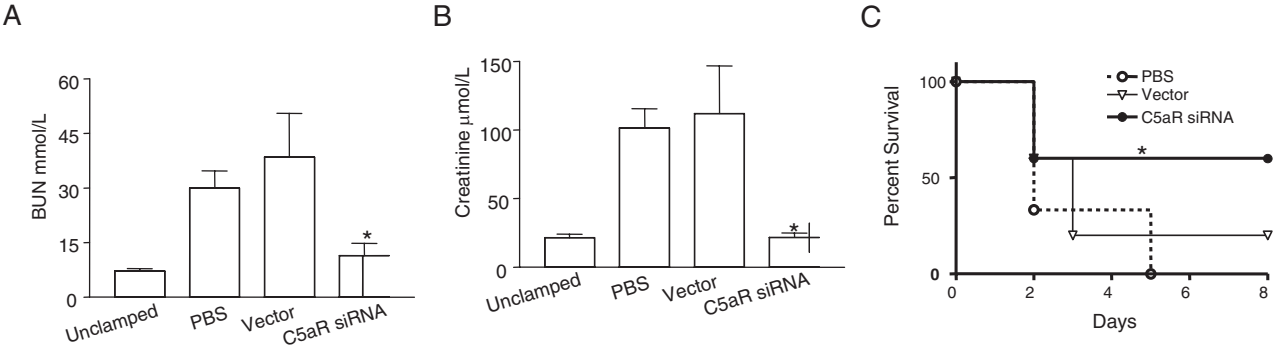

Figure 3. siRNA reduces the effect of renal ischemia and reperfusion on renal function. Renal pedicles were clamped for 25 minutes. Blood was collected from unclamped mice or mice ($n = 8$, per group) with I/R injury that were treated with PBS, vectors or C5aR-siRNA. BUN (**A**), serum creatinine (**B**), and survival (**C**) ($n = 10$, per group) were determined as described in Materials and Methods. Ischemia time was 30 minutes for survival study. Data shown are mean ± SEM (*$P < 0.05$).

Table 1. The Level of BUN and Creatinine in Blood*

	PBS	Vector control	C5aR siRNA	*P* value
BUN (mmol/L)	89.30 ± 27.60	97.33 ± 70.00	41.60 ± 23.23	$P < 0.05$
Creatinine (mol/L)	233.0 ± 32.70	213.0 ± 24.11	112.0 ± 50.23	$P < 0.05$

*Renal I/R was induced by clamping renal artery and vein for 30 minutes. Twenty-four hours after I/R, blood was collected from the mice treated with PBS, vectors, or C5aR-siRNA. BUN and serum creatinine were determined as described in Materials and Methods. Data shown are mean ± SEM.

expression vector that encodes a green fluorescent protein reporter gene as described previously.[7] To validate gene silencing efficiency, we co-transfected the C5aR siRNA with C5aR cDNA into L929 cells that do not express C5aR gene. The silencing efficiency was assessed at the level of mRNA using real-time PCR. Figure 1A shows the results of two different sequences (#1 and #2) of siRNA and their effects on C5aR expression in C5aR-siRNA treated versus empty vector treated control. Results show that sequence #2 of C5aR-siRNA displayed the most potent silencing efficacy (87.5% ± 0.05 of control vector). The gene silencing effect of C5aR siRNA was confirmed at the protein level by Western blot (Figure 1B). Hence, we used this siRNA in all subsequent experiments in this study.

C5aR-siRNA Inhibits C5aR Up-Regulation in Kidney Induced by I/R Injury

The level of C5aR gene expression in I/R injury kidneys were detected by real-time PCR and immunohistochemistry staining. The results showed that the expression of C5aR in kidney tubular epithelial cells was significantly increased both at the mRNA (Figure 2A) and protein (Figure 2B, b) level by I/R, thereby confirming that the complement pathway was involved in renal I/R injury.

To monitor *in vivo* distribution of siRNA in renal tissue, we constructed a vector that encodes both siRNA sequence and a sequence of green fluorescent protein reporter gene. This co-expression system allows us to track siRNA expression and distribution of siRNA by detecting green fluorescence. Using this reporter system, we have previously demonstrated that systemic administration, using "hydrodynamic" injection method,[13] effectively delivered siRNA into kidney tissues.[7,9,10] Accordingly, we delivered C5aR-siRNA into kidneys using the same method and investigated C5aR siRNA *in vivo* silencing. Mice were treated intravenously with 50 μg of C5aR-siRNA, PBS, or the control vectors 48 hours before I/R injury induction. The expression of C5aR in kidneys was detected by real-time PCR. C5aR expression induced by I/R in the kidney was remarkably suppressed after treatment with C5aR-siRNA ($P < 0.05$ vs. PBS treated, vector-treated group) (Figure 2A). Inhibition of C5aR was also observed by immunohistochemical staining with reduction of C5aR deposition in kidneys from siRNA-injected mice (Figure 2B, a–c). The C3 (Figure 2B, d–f) and C9 (Figure 2B, g–i) deposition in the kidney was also found decreased in the C5aR siRNA-treated mice in comparison to the control mice.

C5aR-siRNA Protects Kidney Function from I/R Injury

Given that siRNA appeared to block the up-regulation of C5aR and, hence, the complement pathways in the mice that were exposed to I/R injury, we hypothesized that siRNA would also prevent renal injury caused by the complement activation in I/R. We measured BUN and serum creatinine levels 24 hours after reperfusion to examine renal function. The levels of BUN (Figure 3A) and creatinine (Figure 3B) both increased significantly in the I/R injury group as compared with the unclamped controls. However, treating C5aR-siRNA before induction of I/R injury significantly prevented an increase of BUN and serum creatinine induced by I/R injury (Figure 3A and 3B).

To explore further the protective effects of C5aR-siRNA treatment on the I/R suffering mice, we examined the survival of mice after more severe I/R injury in which the kidney was clamped for 30 minutes. As shown in the Table 1, 30-minute ischemia induced more severe I/R injury, which resulted in lethally increased level of BUN and creatinine. Within the PBS-treated control mice or vector-treated control mice, only two of the ten mice survived up to the 8th day (Figure 3C). In contrast, siRNA administration significantly prevented renal from lethal I/R injury with decreased the level of BUN from 97.33 mmol/L to 41.6 mmol/L and creatinine from 213 μmol/L to 112 μmol/L as compared with control vector. siRNA treatment improved the survival of the mice after I/R injury, with 70% survival rate ($P < 0.001$ vs. PBS and vector control).

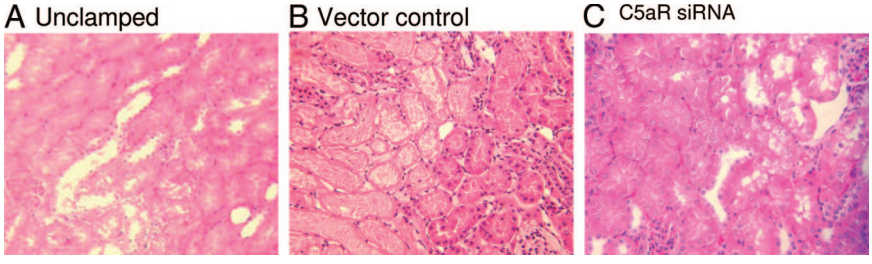

Figure 4. C5aR siRNA protect renal structure from I/R injury. Mice were treated with siRNA, and I/R experiments were performed as described in Figure 3. 24 hours after I/R, kidney tissues were harvested and stained with H&E. Normal unclamped kidney (**A**); control vector-treated I/R kidney (**B**); C5aR-siRNA treated I/R kidney (**C**).

Table 2. Histological Score of Kidney Tissues

Tissue	Tubular vacuolization		Infarction		Cast formation		Neutrophil	
	Score*	*P* value	Score*	*P* value	Score*	*P* value	Score*	*P* value
Normal	0.5	>0.05	0	0	0		0	
Vector control	0.5	>0.05	1.67 ± 0.17	<0.05	1.67 ± 0.17	<0.05	0.5	<0.05
C5aR siRNA	0.5	>0.05	0.58 ± 0.15	>0.05	0.5 ± 0.13	>0.05	0.17 ± 0.11	>0.05

*0 <10% area; 1 = 10% to 25% area; 2 = 25% to 50% area; 3 = 50% to 75% area; 4 >75% area.

C5aR-siRNA Prevents Tissue Injury from I/R

To demonstrate further the beneficial effect of C5aR siRNA on renal I/R injury, we examined kidneys for indications of histopathology changes of I/R injury. The histopathology changes were scored (for five grades) based on the necrosis of tubular and glomerulus area by a blinded observer. Renal influx of neutrophils, an important feature of I/R injury-induced inflammation, was also assessed by counting the number of infiltrating cells present in the tissue sections. As compared with the unclamped kidneys, a much larger area of tubular infarction, vacuolization, and cast formation were observed in I/R injury kidneys (Figure 4). However, mice pre-treated with C5aR-siRNA demonstrated significant attenuation of pathological changes in the kidneys during I/R injury. I/R-injured tissues showed significantly higher scores on infarction, cast formation, and neutrophil infiltration (Table 2). In contrast, after siRNA treatment, the kidney tissues showed scores that were not significantly different from normal (Table 2).

C5aR-siRNA Inhibits I/R-Induced Cytokine and Chemokine Production

TNF-α is an important cytokine associated with I/R injury, which mediates the induction of chemokines and attracts neutrophils infiltration. In our previous study, we found that complement activation affects TNF-α production[7] and treatment with C3 siRNA prevented the expression of TNF-α induced by I/R. To determine whether C5aR siRNA affected renal tubular cell expression of TNF-α, we measured the expression of TNF-α in kidneys using real-time PCR. Renal expression of TNF-α was reduced by pre-treating the kidney with C5aR siRNA as compared with control siRNA group (Figure 5A).

C5a also reportedly stimulates chemokine secretions, such as KC and MIP-2, in I/R kidney.[5] Along these lines, we investigated the renal expression of the chemokine KC and MIP-2 after treatment with C5aR siRNA or control siRNA by semiquantitative PCR. As shown in Figure 5, B and C, the expression of KC and MIP-2 in the I/R injured tissues was 1.8- and 2.3-fold higher than that of unclamped tissues. Administration of C5aR-siRNA before induction of I/R prevented the up-regulation of KC and MIP-2 (Figure 5, B and C).

Discussion

It has been documented that complement activation is associated with I/R injury.[6] Prevention of kidney from I/R injury by blocking specific components of the complement activation cascade has been reported.[3,15,16] We previously showed that inhibiting C3 with siRNA protected renal from I/R injury.[7,9,10] In this study, we have demonstrated that silencing the C5aR gene using siRNA can inhibit the renal expression of C5aR and that successful delivery of C5aR-siRNA to the kidneys results in the prevention of renal I/R injury.

The complement system consists of more than 30 proteins that are activated in a sequential manner. Activation of the complement cascade generates C3a, C5a, and the membrane attack complex or C5b-9,[17] which destroy cells by membrane perforation. We observed that C5aR siRNA inhibited C5aR expression and effectively terminated activation of the downstream complement cascade. Interestingly, inhibition of C5aR in our renal model

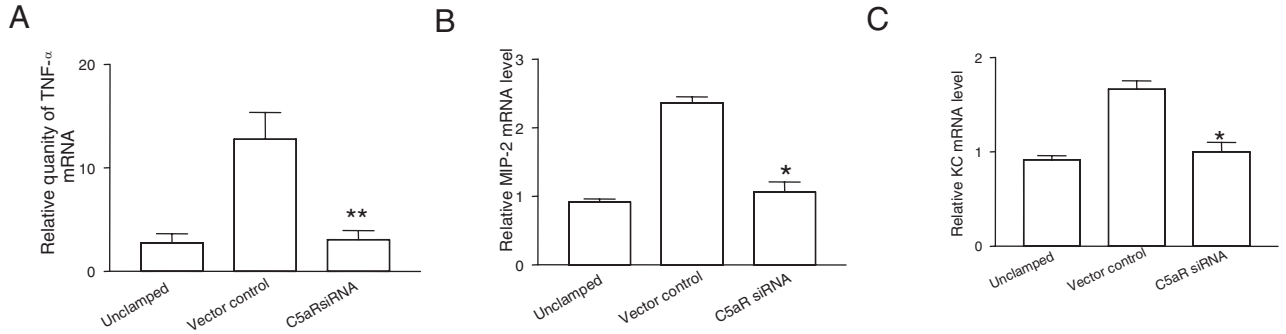

Figure 5. C5aR siRNA down-regulates TNF-α, MIP-2 and KC expression. 48 h after gene silencing, kidneys (n = 3, per group) were clamped for I/R injury. 24 h after reperfusion, kidney tissues were harvested and used to extract total RNA. Expression of TNF-α was determined by real time PCR (**A**). Expression of MIP-2 (**B**) and KC (**C**) was detected by semiquantitative PCR. Statistical significance as compared with controls was denoted at (*) $P < 0.05$; (**) $P < 0.01$.

of I/R injury also prevented extensive deposition of C3 (Figure 2, B [d–f]). Such prevention has also been reported in the case of C5 antibody treatment,[3] although the direct feedback mechanism between C5 and C3 activation is yet to be elucidated. In support to this notion, our previous study demonstrated that down-regulation of C3 expression with siRNA prevented membrane attack complex formation, resulting in deceased inflammatory cytokine secretion and neutrophils infiltration. This study also showed that inhibition of C5aR prevented inflammation in kidney. Blockade of the complement cascade could have reduced secondary cellular damage and inflammation. This may slow down the complement activation and subsequently prevent ongoing C3 deposition. The pathways and mechanisms that activate the complement system in the course of renal I/R injury remain to be elucidated.

I/R injury has demonstrated to be associated with the expression of pro-inflammatory cytokines, which can augment further injury. TNF-α is a potent inflammatory cytokine, expressed by renal parenchymal cells as well as mononuclear cells residing within the kidney, and contributes to neutrophil infiltration and kidney injury. We have previously demonstrated that inhibition of C3 by C3-specific siRNA reduced TNF-α expression in kidneys. Wada et al[18] also showed that inhibition of C5aR significantly inhibited local TNF-α expression after intestinal I/R injury. Our results in this study demonstrating the reduced expression of TNF-α following C5aR siRNA silencing suggest that TNF-α is linked to complement activation. Anaphylatoxin C5a induces chemokines and mediates neutrophils activation and infiltration. Additionally, blockage of C5aR with C5aR siRNA also resulted in the suppression of chemokine production, such as MIP-2 and KC in I/R, which is in agreement of previous findings.[5] Prevention of cytokine and chemokine molecules may lead to attenuation of neutrophils infiltration in the I/R tissues. Thus, beneficial effects of C5aR siRNA may be due in part to the attenuation of cytokines/chemokines in renal injury, as well as the complement cascade.

Both renal epithelial and endothelial cells are capable of complement synthesis,[19–21] and therefore, kidneys are well positioned to initiate complement activation by means of locally secreted components. Kidneys can express massive amounts of complement, as in the case of rejecting allografts.[22] On the other hand, using a mouse I/R injury model, we have demonstrated that the renal expression or deposition of C9 occurs early after reperfusion and is localized to cellular debris and injured tubular epithelial cells, which is consistent with our previous study[7,9] and is supported by the view that the complement pathway is crucially involved in renal I/R injury.[16,23–25] In our present study, we extend the link between renal I/R injury and complement by demonstrating that specific blockade of C5aR expression using siRNA can prevent renal I/R injury. While the role of complement as an important component of renal I/R injury has been supported by previous studies,[2,26–29] attempts to block renal I/R injury have been variably successful. Antibodies, directed at C5 mAb BB5.1 and C5aR, can inhibit complement activation and ameliorate I/R injury. However, these antibody treatments cannot prevent early stage complement up-regulation. Furthermore, these treatments require multiple administrations, which may limit therapeutic attractiveness.[3,5] siRNA provides an alternative therapy for preventing I/R injury, which cannot only be used in the late stage of I/R injury, but may also prevent early I/R injury through an siRNA-containing organ-storage solutions.[30]

In summary, the data presented here indicate that the administration of C5aR-specific siRNA is capable of reducing renal C5aR and C3 synthesis and preventing inflammation. These results further demonstrate that the systemic application of C5aR siRNA can prevent renal I/R injury. Consequently, the use of systemically delivered C5aR-siRNA may represent a novel approach to preventing complement-mediated renal damage, and may become therapeutically useful, both in the setting of renal transplantation and in other conditions associated with renal I/R injury.

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