Protection of Renal Ischemia Injury using Combination Gene Silencing of Complement 3 and Caspase 3 Genes

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Background. Ischemia/reperfusion (I/R) injury occurs in clinical kidney transplantation, which results in graft dysfunction and rejection. It has been documented that I/R injury is associated with complement activation and renal cell apoptosis. The purpose of this study was to develop a strategy to prevent I/R injury using small interfering RNA (siRNA) that target complement 3 (C3) and caspase 3 genes.

Methods. siRNA-expression vectors were constructed to target C3 and caspase 3 genes. Gene silencing efficacy was assessed using real-time polymerase chain reaction. In vivo gene silencing was performed by hydrodynamic injection with C3 and caspase 3 siRNA. Renal I/R injury was induced through clamping the renal vein and artery for 25 min. I/R injury was evaluated using kidney histopathology, blood urea nitrogen (BUN), serum levels of creatinine, and survival.

Results. Effective gene silencing was first confirmed in vitro. Notably upregulated expression of C3 and caspase 3 genes was observed from 2 to 48 hr after I/R injury, which were effectively and specifically inhibited by C3 and caspase 3 siRNA. In comparison with control mice, serum levels of creatinine and BUN were also significantly decreased in C3 and caspase 3 siRNA-treated mice. Furthermore, the therapeutic effect of siRNA was assessed in a severe, lethal I/R injury experiment, in which siRNA treatment significantly reduced mortality. Tissue histopathology showed an overall reduction in injury area in siRNA-treated mice.

Conclusions. This is the first demonstration that renal I/R injury can be prevented through silencing the complement gene and apoptosis gene, highlighting the potential for siRNA-based clinical therapy.

Keywords: Gene silencing, Small interfering RNA, Ischemia reperfusion injury, Complement 3, Caspase 3, Kidney.

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Renal ischemia/reperfusion (I/R) injury occurs in clinical kidney transplantation. This type of kidney injury subsequently results in graft dysfunction and chronic rejection. It has been documented that I/R injury is associated with complement activation (*1*) and renal cell apoptosis (*2*). Both anaphylatoxin (C3a, C5a)-dependent and membrane attack complex-dependent mechanisms have been proposed as means by which the complement cascade induces tissue injury in animal models of renal I/R injury (*3–7*).

Caspases are intracellular cysteine proteases that play a central role in mediating apoptosis. In animal models, renal I/R injury can be abrogated by treatment with complement inhibitors, such as anti-C5 antibodies and C5a receptor antagonists (*8*, *9*). Many inhibitors of apoptosis have been used for reducing organ damage. There is, however, no clinically accepted therapy that ameliorates or prevents cellular injury after renal ischemia (*10–12*).

Gene silencing using small interfering RNA (siRNA) is a newly developed method that is more potent and specific in suppressing gene expression than other conventional methods (*13*). A recent study has proven that systemic administration of siRNA targeting caspase 3 and caspase 8 can prevent murine liver ischemia (*14*). Our previous study demonstrated that treatment with complement 3 (C3) siRNA was capable of protecting mice from renal I/R injury (*15*).

The current study explored a therapy of I/R injury through simultaneously inhibiting complement activation and apoptosis pathways, using siRNA that target C3 and caspase 3 genes.

MATERIALS AND METHODS

C3 and Caspase 3 siRNA Design

The target sequences 5′-CTGTGCAAGACTTCCTAAAGA-3′ (specific to C3) and 5′-GGATCTATCTGGACAGTAGTT-3′ (specific to caspase 3) were selected. The oligonucleotides containing sense and antisense of the target sequences and loop sequence, were synthesized, annealed, and constructed into a pRNAT-U6.1/Neo siRNA expression vector, which had a cGFP gene and a U6 promoter driving to express shRNA (Genescript, Piscataway, NJ).

In Vitro Silencing of the C3 and Caspase 3 Genes

L929 cells were transfected with C3 siRNA or caspase 3 siRNA using lipofectamine 2000 (Invitrogen). The vehicle

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alone and scrambled (nonsense) siRNA were used as negative controls. Briefly, cells were plated into 12-well plates (2×10^5 cells per well) and allowed to grow overnight, to reach 90% confluence. Cells were transfected with 2 μg of C3 siRNA, caspase 3 siRNA, or negative control siRNA plasmids in serum-reduced medium for five hr, then incubated in complete medium for 24 hr. RNA was extracted from the transfected cells 24 hr after transfection.

In Vivo Gene Silencing and siRNA Treatment

In vivo gene silencing was performed using "hydrodynamic" injection method that has been described previously (*15*). In brief, 48 hr prior to I/R injury experiments, 50 μg of C3 and caspase 3 siRNA plasmid DNA were diluted in 1 ml of phosphate buffered saline (PBS) and injected intravenously into mice over a period of 10 seconds.

Renal I/R Injury Model

CD1 mice, six to eight weeks old, 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 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 min or 35 min. During the procedure, animals were kept well hydrated with warm saline and at a constant temperature (37°C). After 25 min of ischemia, the clamps were removed. The right kidney was resected.

Assessment of Renal Function

Blood samples were obtained from the inferior vena cava 24 hr after ischemia. Serum creatinine levels and blood urea nitrogen (BUN) were measured by the core laboratory at the London Health Sciences Centre in order to monitor renal function.

Histology Detection

At 24 hr postischemia, kidneys were dissected from mice, and tissue slices were fixed in 10% formalin, and then 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 examined in a blinded fashion by a pathologist. Histology changes in the cortex and medulla were examined.

Measurement of Renal C3 and Caspase 3 Messenger RNA Levels by Reverse Transcriptase and Quantitative Polymerase Chain Reaction

Total RNA was extracted from kidneys and cells using Trizol (Invitrogen). Total RNA was reverse-transcribed using oligo-(dT) primer and reverse transcriptase (Invitrogen). Primers used for the amplification of murine C3, caspase 3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: C3, 5′-CCCTGCCCCTTACCCCTTCATTC-3′ (forward), and 5′-CGTACTTGTGCCCCTCCTTA TCTG-3′ (reverse); caspase 3, 5′-CGGGGTACGGAGCTGGACTGT-3′

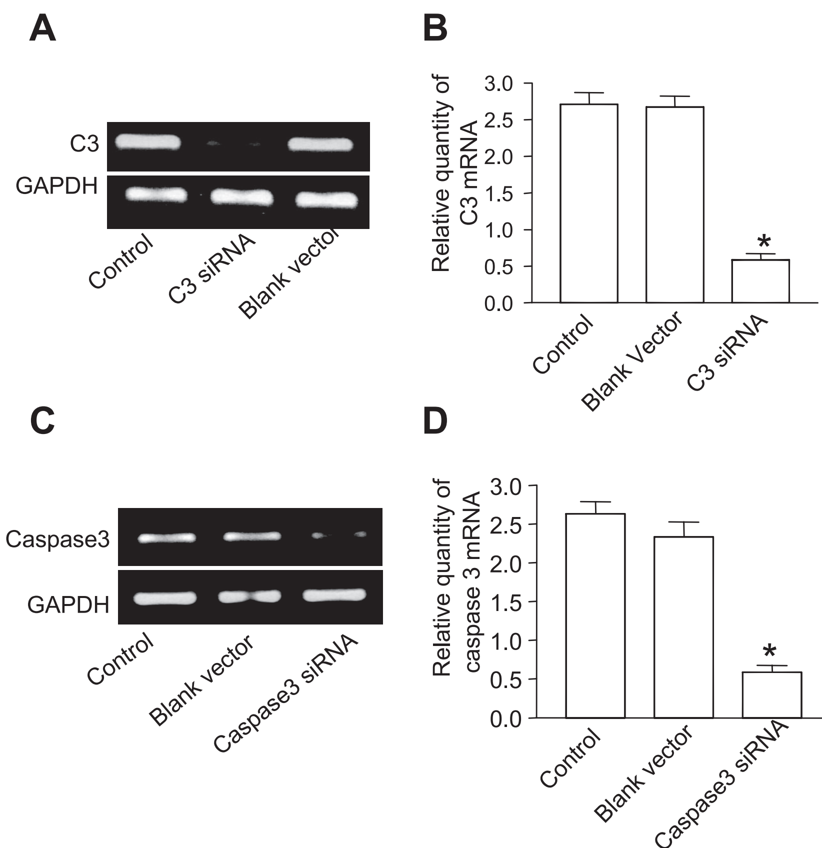

FIGURE 1. In vitro gene silencing. (A and B) Silencing C3 gene. L929 cell lines were transfected with C3 cDNA vectors using lipofectamine 2000 and cotransfected with C3 siRNA, empty vectors, or non-siRNA (control). Then 24 hr after transfection, cells were harvested to extract total RNA. Transcripts of C3 and GAPDH were determined using RT-PCR (A) and quantitative PCR (B). (C and D) Silencing caspase 3 gene. L929 cells were transfected with caspase 3 siRNA, empty vectors, or non-siRNA (control). Then 24 hr after transfection, expression of caspase 3 was detected using RT-PCR (C) and quantitative PCR (D). Data (A and C) is a representative of four independent experiments. Data (B and D) was expressed as means±SEM. Statistical significance as compared with siRNA vs. vector (*$P<0.05$).

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(forward) and 5′-AATTCCGTTGCCACCTTCCTGTT-3′ (reverse), and GAPDH, 5′-TGATGACATCAAGAAG GTGGTGAA-3′ (forward) and 5′-TGGGATGGAAATTGTGAGG GAGAT-3′ (reverse). Polymerase chain reactions (PCRs) were performed under the following conditions: 95°C for 30 sec, 58°C for 30 sec, and then 72°C for 30 sec (30 cycles).

Real-time PCR reactions were performed using SYBR Green PCR Master mix (Stratagene) and 100 nM of gene-specific forward and reverse primers with the same sequences as reverse transcriptase (RT)-PCR. The PCR reaction conditions were 95°C for 10 min, 95°C for 30 sec, 58°C for one min and 72°C for 30 sec (40 cycles).

Statistical Analysis

Data are expressed as means±SEM. Statistical comparisons among groups were performed using one-way analysis of variance (Newman-keuls). Statistical significance was determined as $P<0.05$.

RESULTS

Gene Silencing of C3 and Caspase 3 In Vitro

Gene silencing has been successful in our previous study, which used synthesized siRNA (*16*). In order to achieve long-lasting gene silencing in vivo, we constructed vectors that express hairpin siRNA sequences specific to C3 and caspase 3, respectively. To determine the gene silencing efficacy of these siRNA vectors, we cotransfected C3 cDNA vectors together with C3 siRNA vectors. After cotransfection, C3 expression was markedly decreased (more than fivefold) when compared with control vector transfected L929 cells (Fig. 1, A and B).

In a parallel experiment, we assessed caspase 3 siRNA vectors in L929 cells that express high levels of caspase 3 (Fig. 1, C and D). Twenty-four hours after gene silencing, C3 expression was significantly decreased as determined by RT-PCR (Fig. 1C). Approximately 80% of gene silencing efficacy

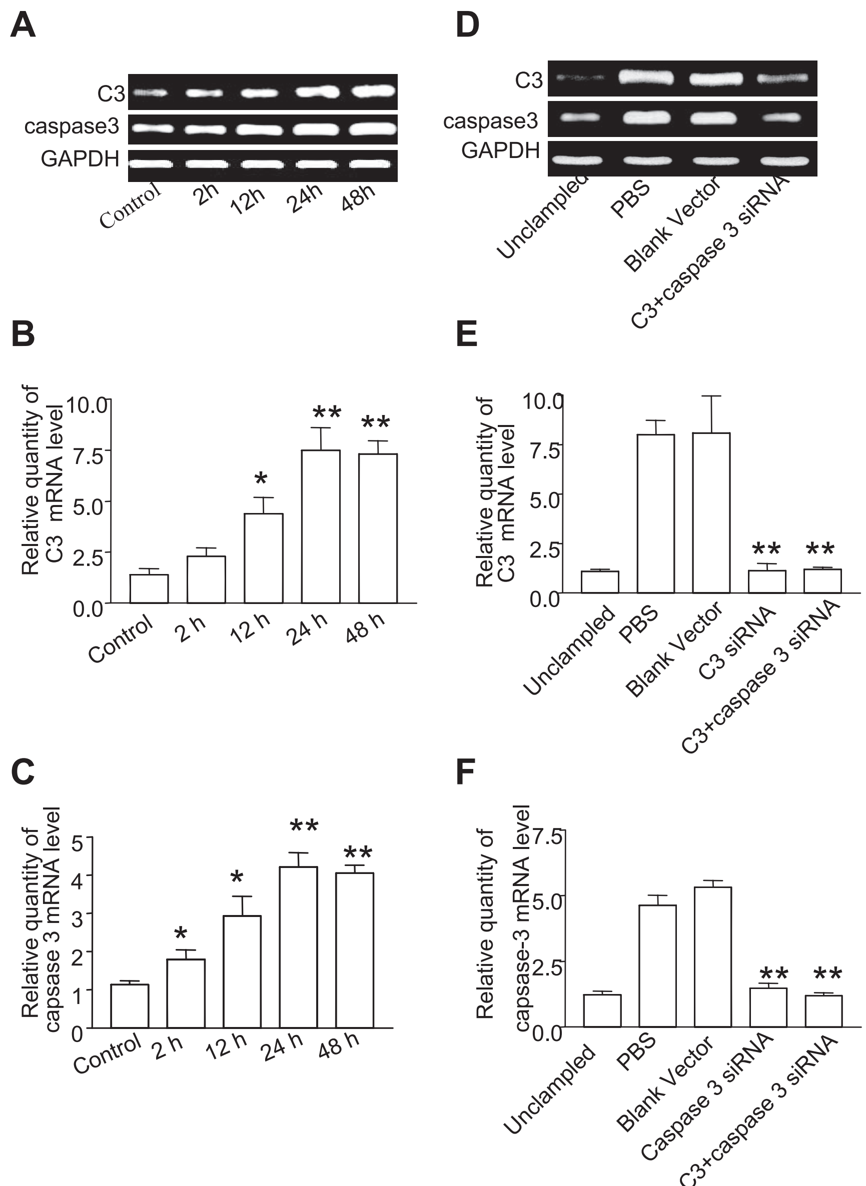

FIGURE 2. Upregulated gene expression by I/R and gene silencing in vivo. (A–C) Upregulated expression of C3 and caspase 3 genes in the kidney after I/R injury. Left kidneys of mice (n=4) were subjected to clamping for 25 min as described in Materials and Methods. Kidneys were harvested at indicated time points after clamping. The expression of C3 (A and B) and caspase 3 (A and C) was detected by RT-PCR (A) and real-time PCR (B and C). (D–F) C3 and caspase 3 gene silencing in vivo. Mice (n=8 each group) were pretreated with 50 μg of C3 siRNA and caspase 3 siRNA, or empty vectors for 48 hr followed by I/R experiments. Kidneys were harvested 24 hr after I/R for determination of C3 (D and E) and caspase 3 (D and F) gene expression using RT-PCR (D) and real-time PCR (E and F). Data (A and D) is representative of four independent experiments. Data (B, C, E, and F) is expressed as means±SEM. Statistical significance as compared with different time point vs. control in data (B and C), siRNA treatment vs. PBS or blank vector. (*$P<0.05$; **$P<0.001$).

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was seen in real-time PCR (Fig. 1D). This data suggests that potent gene silencing can be achieved using siRNA expression vectors.

Gene Silencing of C3 and Caspase 3 In Vivo

It has been reported that I/R injury is associated with complement activation (*3–7*) and apoptosis (*2*). Accordingly, we examined the dynamic changes of C3 and caspase 3 gene expression in kidney tissue after I/R injury. We used a warm ischemic murine model, in which renal arterial flow was blocked by clamp for 25 min, and then released. Renal expression, of C3 and caspase 3, was determined at the level of messenger RNA (mRNA) by RT-PCR (Fig. 2A) and real time PCR (Fig. 2, B and C). Both C3 and caspase 3 mRNA levels were elevated two hr after I/R injury, and reached the highest levels at 24 and 48 hr. These data are in agreement with an earlier report in which it was shown that C3 is upregulated in kidneys after enduring a longer period of I/R injury (*7*, *15*).

We next tested in vivo gene silencing in kidneys using the "hydrodynamic" injection method (*15*, *17*). Mice were treated with 50 μg of C3 siRNA and caspase 3 siRNA 48 hr prior to I/R experiments. C3 and caspase 3 expression was markedly suppressed in treated kidneys as detected by RT-PCR (Fig. 2D) and real-time PCR (Fig. 2, E and F).

Prevention of I/R Injury by Silencing C3 and Caspase 3

As complement activation and apoptosis have been proven to be involved in the pathogenesis of I/R, we postulated that silencing these two pathway components would prevent renal I/R injury. In siRNA-treated versus untreated mice, we measured BUN and serum creatinine to monitor renal dysfunction after I/R injury. Control mice, which were treated with either PBS or empty vectors, showed significantly increased BUN and serum creatinine levels, suggesting severe renal injury had occurred (Table 1). In contrast to this, siRNA-treated groups, using single siRNA or combination C3 and caspase 3 siRNA, effectively protected renal function in I/R injury (Table 1).

In addition to renal function, we also evaluated survival of the mice after I/R injury. Because most mice with renal failure die by the fourth day, long-term survival was defined as survival beyond eight days. While survival rates of control groups was 20%, treatment with C3 siRNA or caspase 3 siRNA protected the mice from lethal I/R injury with survival rates of 60% (Table 1). The combination of these two siRNA provided even greater protection from lethal I/R injury as 90% of mice survived.

Histological Changes in I/R Injury After Silencing C3 and Caspase 3 Genes

To confirm the treatment effects by siRNA, we further examined histopathological changes in the I/R injured kidneys. In comparison to kidneys from normal mice (Fig. 3A), control mice treated with PBS (Fig. 3B) or empty vectors (Fig. 3C) demonstrated severe tissue damage, including tubular infarction, vacuolization, cast formation, and neutrophil infiltration (Fig. 3, B and C). In contrast, mice pretreated with a combination of C3 siRNA and caspase 3 siRNA showed significant attenuation of all pathological changes. The injury area in the kidneys was also dramatically decreased from 75% (in control mice) to less than 10% in the mice treated with siRNA (Fig. 3).

DISCUSSION

Ischemia reperfusion injury is the primary cause of acute renal failure, both in native kidneys and in allografts. It is also of major importance in long-term renal graft survival as injured kidneys are more likely to experience chronic rejection (*18*). The results of this study represent an initial demonstration that administration of C3 siRNA and caspase 3 siRNA protects the kidney against I/R injury, thus improving renal function.

It is now well established that the process of ischemia and subsequent reperfusion initiates a complex, interrelated sequence of events that results in kidney injury and failure (*19*). Our results further prove that I/R injury upregulates complement 3 and caspase 3 gene expression in kidneys as early as two hr after I/R injury, and also confirms that complement activation and apoptosis pathway are both involved in renal I/R injury.

Complement is a complex cascade of more than 30 proteins that are activated in an orderly manner. The cascade has three initiating arms, including classical, lectin, and alternative pathways. Each pathway produces enzymatic complexes,

TABLE 1. siRNA protects kidneys from I/R injury[a]

Treatment	BUN mmol/L	*P* value[b]	Creatinine μmol/L	*P* value[b]	Survival[c]	*P* value[b]
Not clamped	7.8±1.7		22.0±5.2		100.0%	
PBS control	30.3±11.7		131.8±35.9		20.0%	
Vector control	34.9±8.7	>0.05	134.3±56.9	>0.05	20.0%	>0.05
Caspase 3 siRNA	19.2±6.4	<0.05	54.0±18.3	<0.001	60.0%	<0.001
C3 siRNA	12.5±4.8	<0.001	32.6±10.9	<0.001	60.0%	<0.001
Caspase 3+C3 siRNA	11.7±3.9	<0.001	33.1±6.5	<0.001	90.0%	<0.001

[a] Mice were treated with 50 μg siRNA *i.v.* or empty vectors 48 hrs prior to I/R injury experiment. Twenty-four hrs after I/R, blood was collected to determine levels of BUN and serum creatinine.

[b] Data shown are means±SEM. *P* values were compared with PBS-treated control groups using one-way ANOVA (Newman-keuls).

[c] Mice were treated with 50 μg siRNA *i.v.* or empty vectors 48 hrs prior to I/R injury experiment. The survival of mice (n=10 each group) was observed by the eighth day after I/R injury.

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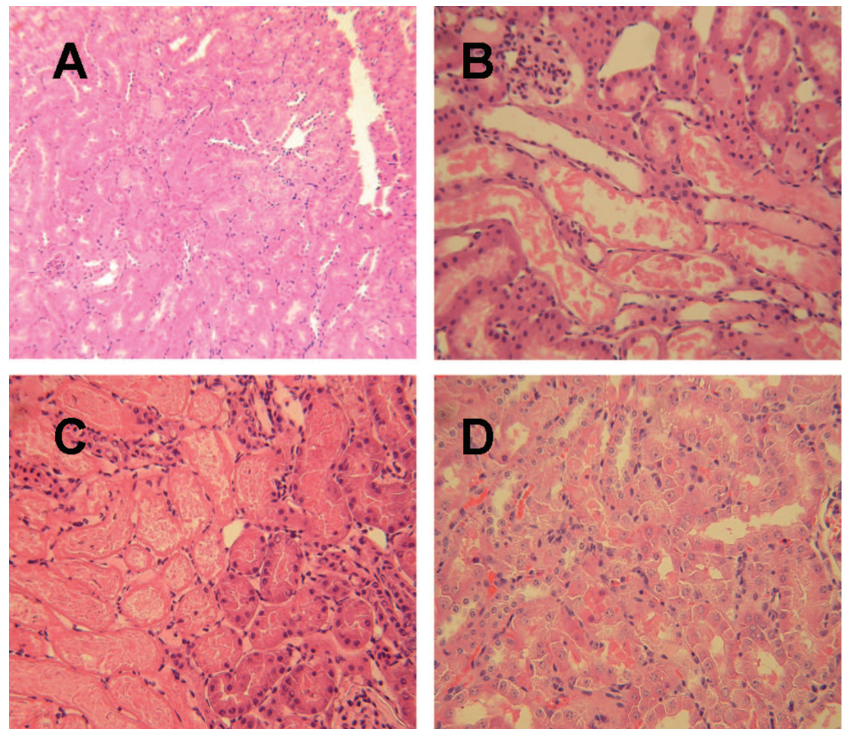

FIGURE 3. Histological changes in I/R injury kidneys. Mice were treated with siRNA and I/R injury experiments were performed as described in Figure 2B. Then 24 hr after I/R, kidney tissues were harvested and sectioned, then stained with H&E. The tissue injury was determined and the necrosis of tubule and glomerulus was assessed, under high power (400×) over five fields. (A) Normal unclamped kidney. (B) PBS-treated I/R kidney. (C) Empty vectors-treated I/R kidney. (D) C3 siRNA and caspase 3 siRNA-treated I/R kidney.

C3 and C5 convertases. All three pathways result in C3 activation and merge into a common pathway that results in the formation of the membrane attack complex or C5b-9 (*15*, *20*), which results in cell necrosis. Caspases are intracellular cysteine proteases that mainly mediate cell death and inflammation (*21*). Caspase 3 is centrally important in both apoptotic and necrotic cell death (*21*). In this study, both complement and apoptosis pathways were markedly activated in I/R injury.

We and others have demonstrated that blocking the complement cascade by inhibiting central molecular C3 (*15*) or blocking the caspase chain by inhibiting caspase 3 is beneficial for renal function in I/R injury (*22*). However, the protection is limited only in the case of short I/R injury (*15*). In this study, we took advantage of siRNA, which can simultaneously silence multiple genes, to inhibit C3 and caspase 3, which resulted in protection of extended I/R injury. The capability of preventing lethal injury was increased by this combinational treatment to 90% survival rate, compared to only 60% in single siRNA treatment groups. Furthermore, the combination treatment significantly improved histopathology in the I/R kidney. The combination of C3 and caspase treatment greatly reduced the lesion area (less than 10%; Fig. 3) caused by I/R within treated kidney tissues, compared with 25% of area in kidneys treated with C3 siRNA (*15*) or caspase-3 siRNA (data not shown).

RNA interference (RNAi) with using siRNA is a newly-discovered process in which a double-stranded RNA (dsRNA) selectively inactivates homologous mRNA transcripts. The exogenous administration of siRNA is capable of blocking gene expression in mammalian cells without triggering the nonspecific panic response (*23*). Compared with other methods of silencing genes and their products, siRNA has the following significant advantages: 1) blocking efficacy is very potent (*24*); 2) targeting gene expression is specific to one nucleotide mismatch (*25*); 3) inhibitory effects can be passed over multiple cell cycles (*26*); 4) in vitro transfection efficacy is high (*16*) and can be expressed in a stable manner (*27*); 5) in vivo use is safer than antibody approaches; and 6) simultaneous targeting of multiple genes and multiple exon silencing is possible, leading to increased efficacy (*28*). The safety, specificity, and potency of therapeutic siRNA has now been firmly established. Therapeutically, siRNA has been used in many fields and has recently been applied to prevent ischemia/reperfusion injury. For example, intraportal administration of siRNA caspase-8 and caspase-3 inhibits the expression of caspase-8 and caspase-3, and attenuates warm I/R injury in the liver (*29*). siRNA targeting Fas or complement 3 genes has been shown to protect mice against renal I/R injury (*15*, *30*). Thus, siRNA provides an alternative therapy for preventing I/R injury, which can not 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 (*13*).

In summary, this study is the first to demonstrate that administration of C3 siRNA and caspase3 siRNA can inhibit complement and apoptosis activation. It provides a promising therapy for clinical application, both in the setting of renal transplantation and for other conditions associated with renal I/R injury.

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