Nontransgenic Hyperexpression of a Complement Regulator in Donor Kidney Modulates Transplant Ischemia /Reperfusion Damage, Acute Rejection, and Chronic Nephropathy

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Complement activation during ischemia and reperfusion contributes to the development of tissue injury with severe negative impact on outcomes in transplantation. To counter the effect of complement, we present a strategy to deliver a novel complement regulator stabilized on cell surfaces within donor organs. The membrane-bound complement regulator is able to inhibit complement activation when the donor organ is revascularized and exposed to host-circulating complement. Application of this construct to donor kidneys protected transplanted tissues from ischemia/reperfusion injury and reduced the deposition of activated complement and histological signs of damage under conditions in which a nontargeted control construct was ineffective. Treatment of donor organs in this way improved graft performance in the short and long term. An analysis of the immune response in allograft recipients showed that reducing graft damage at the time of transplantation through complement regulation also modulated the alloresponse. Additionally, the results of perfusion studies with human kidneys demonstrated the feasibility of targeting endothelial and epithelial surfaces with this construct, to allow investigation in clinical transplantation. (*Am J Pathol* 2003, 163:1457–1465)

Ischemia/reperfusion (I/R) injury is inherent in transplantation because every donor organ by definition is devoid of blood for a period of time. The tissue damage caused is in part dependent on the length of ischemia and also mediated by complement.¹ This has been shown for several models of ischemia, for example in the gut, kidney, and skeletal muscle, using both complement-deficient mice and complement-inhibiting agents.^{2–4}

To address the difficulties of preventing I/R damage, we have treated donor organs with a novel, modified

membrane-binding complement regulator. Our approach is designed to prevent complement activation specifically within a transplanted organ. The ligands for complement regulators are strictly the active fragments of complement components present only at the site of complement activation. It is now recognized that the kidney itself is a significant source of complement components.⁵ Recent experimental work has suggested that such local synthesis of complement has great influence on local tissue injury in the transplanted kidney⁶ and that local production in the kidney, for example of the pivotal component C3, which links the activation and terminal cascades of complement, is increased in response to I/R injury.⁷ In addition, ischemia/reperfusion injury and its contributory factors have a serious negative impact on acute rejection and the long-term results in clinical transplantation.⁸

We have modified a form of human complement receptor type 1 (CR1, CD35) as a means to reduce I/R injury in transplanted kidneys. Human CR1 is found on the surface of almost all peripheral blood cells, but has a limited distribution on other cell types. It is, however, a potent inhibitor of complement activation, having decay accelerating activity for the disassembly of the complexed C3 convertases of the classical and alternative activation pathways, and co-factor activity for Factor I, which inactivates C3b (Figure 1a).⁹ The active site in CR1 was initially reported to reside in the first four short consensus repeats (SCRs) in each long homologous repeat¹⁰ but is probably bounded by the first three domains—at least in long homologous repeat (LHR)-A.¹¹ Each of the functions of CR1 have been identified in other complement control proteins, but the presence of multiple functions in CR1 makes it an ideal candidate for therapeutic tissue engineering.¹²

To confer ability to localize the soluble SCR1-3 fragment on target cell surfaces, a membrane-binding moiety

Supported by the Guy's and St. Thomas' Kidney Patients Association (to J. P.) and Adprotech Ltd.

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Accepted for publication June 25, 2003.

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Figure 1. Comparative structure of human CR1 and m-pSCR(1-3). **a:** Human CR1 exists on the cell surface as a string of short consensus repeat (SCR) domains attached via a transmembrane portion to a short cytoplasmic domain, each SCR being of 60 to 70 amino acids. Several allotypes exist, differing in the number of SCRs. The most common form, allotype A, consists of 30 SCRs and exhibits a further level of internal homology in the form of long homologous repeats (LHRs) of every seven SCRs totaling 235 kd. **b:** m-pSCR(1-3) consists of recombinant SCR(1-3) of LHR-A of CR1 attached to the membrane-binding tag. The tag consists of a myristoyl tail chemically linked to a synthetic positively charged peptide whose terminal cysteine enables attachment of the SCR(1-3) of CR1. This water-soluble molecule is ~23 kd in size and retains the known complement regulatory mechanisms of intact CR1 as well as the ability to bind cells. The construct is designed to insert itself into the hydrophobic core of the lipid bilayer of cell membranes and binding is stabilized by the basic peptide region allowing presentation of the functional moiety, in this instance human SCR(1-3) of CR1.

was designed for posttranslational chemical attachment to SCR1-3 (Figure 1b).^{13,14} This was based on a linear array of membrane-binding units, each of a different specificity and assembled into a synthetic peptide termed MSWP1¹⁴ based on the myristoyl electrostatic switch¹⁵ for membrane localization. The myristoylated peptide membrane-binding product of this process, termed m-pSCR1-3 (Figure 1b), is a soluble, extrinsically administered protein with membrane-binding properties capable of intrinsically regulating complement activation on cell surfaces and able to exert a local and persistent anti-inflammatory effect.¹⁶

Here we report the ability of the membrane-targeted complement regulator to bind to rat and human kidney, and to reduce antigen-independent and antigen-dependent injury of rat donor kidney in subsequent transplantation studies.

Materials and Methods

Design of a Recombinant Low-Molecular Weight Complement Regulator

The gene coding for the N-terminal SCR1-3 of LHR-A of CR1 with a codon for an additional C-terminal cysteine was constructed as described by Dodd and colleagues,¹⁷ and a plasmid, pDB1081-1 constructed as described in Mossakowska and colleagues¹¹ for transformation into the expression host *Escherichia coli* BL21 (DE3). The protein product of the expression vector was

purified and shown to inhibit antibody-sensitized red blood cell lysis by human serum, to inhibit C3a release, and to function as a co-factor for Factor I.¹⁷ The analysis of activity revealed that the 21-kd SCR1-3 fragment was active against both the classical and alternative pathways and appeared to be correctly folded.^{11,13,17}

Membrane-Binding Reagents

The membrane-localizing thiol-reactive agent *N*-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-(S-2-thiopyridyl)-Cys-carboxamide was prepared by solid-phase synthesis as described previously.¹¹ The corresponding reduced (free cysteine) form lacking SCR1-3 and used as a control agent in this study was designated "m-p" (myristoylated peptide).

Construction of a Membrane-Binding Complement Regulatory Molecule, m-pSCR(1-3)

A plasmid encoding SCR1-3cys of CR1 was constructed and expressed in *E. coli* BL21 (DE3) as described by Mossakowska and Smith,¹² purified by the method of Dodd and colleagues,¹⁷ coupled to the membrane-binding tag m-p, and isolated as described.¹³ The product had M_r ~24 kd by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a 50% inhibitory concentration in a classical pathway hemolytic assay of ~0.15 nmol/L. using a commercially available antibody-sensitized sheep red blood cell preparation (Diamidex, Miami, FL) and a dilution of human serum of 1:400.¹¹

Kidney Transplantation and Treatment with m-pSCR(1-3)

Animals were purchased from Harlan Olac (Bicester, UK) and maintained with standard rat chow and water *ad libitum*. All animal experiments were conducted within the restrictions of a Home Office License for animal experimentation. Anesthesia was performed with isoflurane administered through a vaporizer. The left donor kidney was dissected and the aorta ligated cephalad and caudad to the renal artery. A portex tube catheter (0.5-mm outside diameter) (Portex Ltd, Hythe, UK) was inserted between the ligatures and the kidney was perfused with 5 ml of Soltran Kidney Perfusion Solution (Baxter Health Care, UK) containing 200 μ g of m-pSCR1-3 (40 μ g/ml) throughout a period of 5 minutes. The kidney was then placed in ice-cold saline for 30 minutes. Orthotopic renal transplantation was performed using the method of Fabre and colleagues¹⁸ and microvascular clips releasing the blood flow were removed once 60 minutes of warm ischemia had elapsed. Syngeneic transplantation was performed using DA rats. The 7-day experiment used groups of six rats, and the 20-week experiment used groups of five and six control and treated rats, respectively. The allograft experiment with Fischer F344 (RT1^{lv1}) rat donors and

Lewis (RT¹) rat recipients used groups of six control and seven treated rats.

Analysis of Transplant Recipients

At regular intervals after transplantation blood samples were collected for analysis of blood urea nitrogen as previously described.¹⁹ Kidney transplants were harvested at various time intervals and portions of tissue were placed in 4% formal saline for standard histological analysis, or frozen in liquid nitrogen for subsequent immunohistochemical staining, using antibodies against C3d (DAKO, Glostrup, Denmark), C5b-9 (Quidel, CA), CD3 (Becton Dickinson, Abingdon, UK), and CD45 (Becton Dickinson, Abingdon, UK), and for morphometric image analysis as previously described.²⁰

Analysis of m-pSCR(1-3) Binding to Rat Kidney Cells

Immortalized rat renal endothelial cells²¹ or primary cultured rat tubular epithelial cells⁷ were incubated with varying concentrations of m-pSCR1-3 in phosphate-buffered saline (PBS) for 40 minutes at 4°C. For immunocytochemistry cells were grown on glass coverslips and for flow cytometry cells were detached from plastic culture flasks and aliquots of 5×10^5 cells exposed to m-pSCR(1-3). For immunocytochemistry cells were washed twice in PBS and then fixed in cold acetone and stained using Cy3-labeled 3E10 [mouse anti-human SCR1-3 monoclonal antibody (mAb); provided by Adprotech Ltd.] for 30 minutes at 4°C, mounted in Fluoromount (Sigma), and examined using a fluorescence microscope.

For flow cytometry, cells were washed twice in PBS and then incubated with Cy3-labeled 3E10 for 30 minutes at 4°C, washed twice, and fixed in 1% paraformaldehyde in PBS. The samples were analyzed using a FACscan flow cytometer (Becton Dickinson).

Mixed Lymphocyte Reaction

Responder and stimulator lymphocytes were prepared as previously described.⁶ Cellular proliferation was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). This is a colorimetric method in which methyl tetrazolium salt (MTS) is reduced into a soluble formazan product. The absorbance at 490 nm provides an index of the number of viable proliferating cells, which was determined at days 1, 3, 5, and 7 of the assay.

Perfusion of Human Tumor Nephrectomy Specimens

Human kidneys were obtained, with research ethics committee approval and patient consent, from patients undergoing nephrectomy for the removal of renal cell carcinoma in which there was a defined tumor mass recognized by

preoperative imaging. Organs were placed on ice and perfused via the renal artery with 1 L of ice-cold Soltran solution with ($n = 4$) or without ($n = 1$) m-pSCR1-3 at 80 μ g/ml. Tissue samples were taken at frequent time intervals (approximately every 2 hours) for 24 hours, and processed for histological analysis, for immunohistochemistry, and by enzyme-linked immunosorbent assay to determine the presence of m-pSCR(1-3).

Statistics

Student's t-test or repeated measures analysis of variance was used where appropriate to determine significance between treatment groups; $P < 0.05$ was taken as significant.

Results

m-pSCR(1-3) Binds to Rat Kidney Endothelial and Epithelial Cells

We incubated cultured rat glomerular endothelial cells with m-pSCR1-3 and assessed binding by immunocytochemistry using a labeled mouse anti-human SCR1-3 mAb (3E10) (Figure 2, a and b). We found that m-pSCR(1-3), but not the untagged SCR1-3 bound in a dose-dependent manner (Figure 2, c and d). Binding of m-pSCR1-3 also occurred on cultured rat tubular epithelial cells (data not shown). In a hemolytic assay using antibody-sensitized sheep erythrocytes, the addition of the m-p membrane-binding tag to SCR1-3 produced a substantial (~100-fold) increase in inhibitory activity compared to untagged SCR1-3 (unpublished data).¹³

m-pSCR(1-3) Reduces Acute Complement-Mediated I/R Injury

The routine flushing of donor kidneys with perfusion fluid before transplantation presents an ideal opportunity to introduce m-pSCR1-3 into donor organs. We used this approach in a rat model of syngeneic renal transplantation. We perfused the donor graft with ice-cold kidney perfusion solution containing either m-pSCR1-3 or a control substance, before exposing the donor tissue to 30 minutes of cold ischemia and 60 minutes of warm ischemia. This schedule was chosen because it produced histological and functional damage followed by partial recovery in untreated transplants.

The distribution of m-pSCR1-3 in the donor kidney is shown in Figure 3; a to c. The pattern of staining was consistent with binding of m-pSCR1-3 to the glomerular capillary and peritubular capillary walls and the tubular epithelium at its basal surface. Histological analysis at 24 hours after transplantation showed reduced signs of acute tubular necrosis (tubular attenuation and cellular swelling, and associated neutrophil infiltration) in m-pSCR1-3-treated grafts (Figure 3, d and e). At 24 hours after transplantation tissue myeloperoxidase activity, taken here as a measure of neutrophil activity, was sig-

Figure 2. Membrane binding of m-pSCR(1-3). **a:** m-pSCR(1-3) was detected using Cy3-labeled anti-SCR(1-3) mAb (3E10) on cultured transformed rat renal endothelial cells exposed to 2.5 μ g/ml of m-pSCR(1-3). The dome of the cell is outside the focal plain of the photomicrograph giving the appearance of a central dark area. **b:** Staining was not present on endothelial cells incubated with SCR(1-3) lacking the m-p tail. **c:** Dose-dependent cell-surface binding to rat endothelial cells *in vitro* detected by FACS in the presence of the membrane-binding tag. **d:** No binding occurred at any concentration of SCR(1-3). Original magnification, $\times 1000$ (**a**).

nificantly reduced in m-pSCR1-3-treated grafts compared to control grafts (Figure 3f). Deposition of the complement split product C3d (not shown) and the membrane attack complex C5b-9 was absent or weak in m-pSCR1-3-treated grafts, whereas staining was more intense and widespread in control-treated grafts (Figure 4). These results indicate that m-pSCR1-3 treatment reduced the cleavage of C3 and the subsequent formation of C3d and C5b-9. The amount of tissue-bound m-pSCR1-3 detected by immunohistochemistry declined after 24 hours, so that on day 7 it was undetected in treated tissues.

Renal function in recipients of m-pSCR1-3-treated grafts was significantly better throughout the first week after transplantation compared to controls (Figure 5a). Among control grafts, including those treated with the untagged complement regulator, SCR(1-3), there was no significant difference in function. Therefore both functional and structural improvement required the inhibitor to be bound to treated cell surfaces via the m-p moiety.

m-pSCR(1-3) Treatment of Donor Organs Improves Long-Term Graft Function

We next investigated whether reducing the acute complement-mediated I/R injury could improve the long-term

Figure 3. Immunohistopathology of perfused donor kidneys Isolated rat kidneys were flushed with 5 ml of kidney perfusion solution containing 200 μ g of m-pSCR(1-3). The tissue distribution of m-pSCR(1-3) is shown after immunofluorescent staining with Cy3-labeled 3E10. **a:** Glomerulus with capillary loops stained for m-pSCR(1-3). **b:** Basolateral surfaces of tubules (T) and peritubular capillary wall (C) outlined by the staining for m-pSCR(1-3). **c:** The cortex of a control kidney perfused with carrier solution alone and stained with Cy3-labeled 3E10. Glomerulus (G) and tubules (T) are negative. H&E staining of illustrative donor kidney sections 24 hours after transplantation showed well-preserved architecture if perfused with m-pSCR(1-3) (**d**) whereas carrier-only perfused kidneys (**e**) showed marked tubular flattening (ATN) and some infiltration of neutrophils (N). Tissue myeloperoxidase activity 24 hours after transplantation (**f**) showed significantly reduced MPO activity in m-pSCR1-3-treated grafts $\blacksquare$ compared with control-treated grafts perfused only with carrier solution $\blacksquare$ ($P = 0.03$). MPO levels in SCR(1-3) $\square$ and m-p $\boxtimes$ -treated grafts were not significantly different from controls. Sham-treated animals $\boxplus$ had a laparotomy 24 hours before MPO measurement ($n = 4$ /group).

outcome of isografts. This is important because early graft injury may be a driver of late graft failure.²² In this experiment we followed m-pSCR1-3- or control-treated ischemic isografts for up to 20 weeks after transplantation. We found that m-pSCR1-3 treatment reduced the degree of functional (Figure 5b) and histological graft injury throughout the period of study, such that by 20 weeks m-pSCR1-3-treated grafts showed only mild features of cellular swelling (Figure 5, c and e), whereas control grafts demonstrated widespread swelling and intimal proliferation of the arteriolar walls (Figure 5, d and f). Thus, transient initial treatment of the donor organ had a durable and beneficial effect.

Figure 4. Membrane attack complex formation at day 1 after transplantation. Immunohistochemistry for C5b-9 membrane attack complex showing a normal, sham-operated kidney (**a**), a graft treated with perfusion solution alone (**b**), effect of treatment with perfusion solution containing untagged SCR(1-3) (**c**), effect of treatment with the m-p tag alone (**d**), and effect of treatment with m-pSCR1-3 (**e**). Control-treated grafts undergoing ischemia and reperfusion exhibited widespread tubular deposition of C5b-9. Grafts protected by m-pSCR1-3 showed markedly reduced deposition of C5b-9. Original magnifications, $\times 1000$.

m-pSCR(1-3) Treatment of Donor Kidney Reduces Allograft Rejection

To examine the effect of reduced I/R injury on the immunogenicity of the graft, we used the well-described Fischer F344 (RT^{lv1}) to Lewis (RT^l) renal allograft model.²³ The donor and recipient differ by a single MHC class I allele, resulting in a mild and spontaneously resolving acute rejection episode that peaks between 10 and 14 days after transplantation. The protocol for inducing graft ischemia and treatment with complement inhibitor or control solution was the same as before. On day 14 after transplantation, histological damage (Figure 6; a to d) and renal dysfunction (Figure 6e) in allografts treated with m-pSCR1-3 were reduced. This protective effect of m-pSCR1-3 was evident throughout the period of acute rejection.

To measure the degree of leukocyte infiltration, we performed immunohistological examination of common leukocyte (CD45) and specific T-cell (CD3) markers. Image analysis of the stained tissues at the peak rejection time indicated there was a reduction of infiltration by total

leukocytes and T cells in m-pSCR1-3-treated grafts (Figure 6f).

In addition we examined the anti-donor T cell proliferative response of the recipient on day 14 after transplantation. The T-cell response in recipients of m-pSCR1-3-treated grafts was reduced in comparison with recipients of control grafts (Figure 6g), indicating that the treatment of donor organs with the tagged complement regulator led to modulation of recipient T-cell stimulation.

m-pSCR(1-3) Binds to ex Vivo Perfused Human Kidney

To assess the feasibility of treating human organs, we obtained human tumor nephrectomy specimens in which the whole kidney had been removed on a short length of renal artery. We then perfused the organ with a solution containing m-pSCR1-3 and examined the distribution of m-pSCR1-3 in the nontumor tissue. Immunohistochemical analysis showed that m-pSCR1-3 localized, as in the rat studies, to the glomerular and tubular structures of the kidney (Figure 7, a and b). The presence of m-pSCR1-3 in blocks of tissue was confirmed by enzyme-linked immunosorbent assay (data not shown). Although the level of inhibitor detected by enzyme-linked immunosorbent assay was variable, suggesting that perfusion or tissue binding was not equal throughout the kidney, the inhibitor was detected in all areas sampled.

Discussion

Although there is strong circumstantial evidence that graft prognosis is influenced by the severity of donor organ injury sustained during the transplant procedure,²⁴ to date there have been few experimental reports to support this concept, much less a successful therapeutic strategy that prevents organ damage at the time of transplantation. We have developed a novel strategy to inhibit complement activation within donor organs used for transplantation. Our approach to treat donor kidney with membrane-targeted complement regulator had two important effects. Firstly, treatment increased the resistance of the donor kidney to acute I/R injury, which led to better long-term graft performance and reduced chronic damage. Secondly, the prevention of acute complement-mediated I/R injury lessened the immunogenicity of the graft in nonidentical transplant recipients. Our findings support the general notion that the inflammatory condition of the graft is a driver of antigen-independent and antigen-dependent injury. Moreover, they suggest that therapeutic manipulation of the donor organ is an effective means to prevent such injury.

I/R injury in clinical practice manifests most acutely as delayed graft function and may permanently decrease the function of transplanted organs. Increased morbidity and mortality associated with delayed graft function have been noted in clinical studies and represent a relationship between the condition of the engrafted tissue and the subsequent host response.^{25,26} In European studies

Figure 5. Functional and histological analysis of renal isografts. **a:** Blood urea nitrogen up to 7 days after transplantation in recipients of grafts treated with m-pSCR1-3 (■), perfusion solution only (◆), SCR(1-3) lacking the m-p tail (▲), or the m-p tag alone (▼) or in sham-operated rats undergoing nephrectomy without transplantation (●). Data are shown from day 3 because this was the point from which renal function was dependent on the transplanted kidney alone. Treatment with m-pSCR1-3 led to significantly improved renal function compared to any one of the control groups ($P < 0.001$; $n = 6$ animals/group). **b:** Blood urea nitrogen throughout 20 weeks after transplantation after perfusion of donor graft with perfusion solution alone (▲), or m-pSCR1-3 (●). Protection conferred by m-pSCR1-3 in the first week led to improved function throughout 20 weeks ($P < 0.001$; $n = 6$ m-pSCR1-3-treated grafts and $n = 5$ perfusion solution-treated controls). H&E staining of illustrative donor kidney sections showed well-preserved tubular structure in m-pSCR1-3-treated grafts at 20 weeks (**c**) compared to carrier-treated controls (**d**). Vessels also had a near normal appearance in the m-pSCR(1-3)-treated grafts (**e**), compared to carrier-treated grafts at 20 weeks in which arteriolar intimal proliferation was present (**f**). No elastic duplication was seen in untreated (**g**) or treated grafts (**h**). Original magnifications: $\times 400$ (**c, d**); $\times 1000$ (**e, f**).

delayed graft function affects as many as 20 to 30% of cadaveric renal transplants.²⁷ Moreover, the increasing discrepancy between numbers of patients listed for transplantation and potential donors has necessitated the use of so-called suboptimal donors, ie, older than 50 years old and/or organs that have experienced more than 24 hours of cold ischemia. At present no specific agent is used to reduce the susceptibility of transplanted organs to I/R injury, the risks and implications of which increase significantly with suboptimal donor organs.²⁷

Within minutes of I/R injury, activation and deposition of complement occurs, with subsequent up-regulation of cytokines and adhesion molecules²⁸ that mediate the infiltration of leukocytes. Leukocyte accumulation and adhesion to microvascular endothelium in postischemic tis-

ues is thought to be important through the release of toxic oxygen radicals and inflammatory cytokines.²⁹ Postischemic complement activation is thought to cause direct endothelial and epithelial tissue damage³⁰ and C5a-mediated neutrophil chemotaxis,³¹ which were reduced in the m-pSCR1-3-treated grafts in our study.

Soluble forms of naturally occurring complement regulators have been assessed in models of I/R injury.²⁻⁴ The soluble inhibitors currently available have been given systemically and have relatively short functional half-lives. This has necessitated the use of high doses that systemically abrogate the function of the complement system and that have the potential to lower host antimicrobial defenses. This is potentially undesirable for transplant recipients receiving other immunosuppressive

Figure 6. Effects of m-pSCR1-3 on renal allografts. Histological analysis of F344 to Lewis renal allografts at day 14 after transplant showed signs of cellular infiltration in all transplants. However, the degree of interstitial infiltration of mononuclear cells and evidence for tubulitis was less in m-pSCR1-3-treated grafts (**a** and **b**) compared to control transplants treated with perfusion solution only (**c** and **d**; $n = 8/\text{group}$). The m-pSCR1-3-treated grafts also showed significantly improved renal function compared to perfusion solution-treated controls (**e**) between days 4 and 7 ($P < 0.0001$), which was 24 hours after removal of the remaining native kidney. BUN levels <10 mg/dL were taken to be within normal range (for animals with two functioning kidneys). Morphometric analysis of antibody-stained frozen sections (**f**) showed reduced CD45 and CD3 staining in m-pSCR1-3-treated kidneys at day 14 after transplantation, compared to controls treated with perfusion solution only. Data are expressed in arbitrary units (pixels) and derived from 15 representative cortical fields from three transplanted kidneys in each group. Proliferation of spleen T cells isolated from transplant recipients at day 14 after transplantation (**g**) showed significantly reduced alloresponse in the recipients of m-pSCR1-3-treated kidneys compared to recipients of control-treated kidneys, analyzed throughout 7 days by MTS assay ($n = 4$ animals/group assayed in triplicate, in comparing the treatment group with either control group, $P < 0.01$). Original magnifications, $\times 250$ (**a–d**).

drugs. Attempts have been made to prolong the half-life of such reagents, for example by linking complement regulators to albumin,³² or to sialyl Lewis-x moieties to target endothelial surfaces.³³ The coupling of complement regulators to Ig domains has also been shown to prolong half-life,³⁴ but not to protect the kidney from I/R injury.³⁵ In our study, the untagged form of SCR(1-3) of CR1 similarly did not prevent I/R injury suggesting that the complement regulator must be bound to the target of complement activation at the site of tissue injury for it to be effective. Our therapeutic targeting strategy has overcome these difficulties.

We have addressed two issues in the targeting of therapeutic complement regulators. Firstly we have considered where in the complement cascade therapeutic intervention is effective. Secondly, we have determined how to target complement inhibition to those tissues that bear damage. By using CR1 activity as the basis for intervention, we focused our approach on the inactivation of C3b, because this is the pivotal bridging step between the activation and terminal pathways. Recent studies of

I/R in C6-deficient mice have highlighted the importance of direct membrane damage caused by the membrane attack complex,³⁶ while the upstream release of the fragments C3a and C5a, which have anaphylactic and chemotactic properties, contributes to local inflammation. The CR1-derived molecule used in our novel construct is a structurally minimal component, retaining the activities of CR1 needed for breakdown of complexed C3b within C3 convertases, and thus for its inhibitory effect on the generation of C3a, C5a, and membrane attack complex.¹¹ The design of the membrane-binding tail exploits the membrane-inserting property of myristate, in combination with an electrostatic contribution to binding from a synthetic basic peptide sequence that interacts with negatively charged phospholipid headgroups, enabling binding to the cell surface. Combined with routine perfusion of the donor organ, this would permit organ-specific delivery and retention of m-pSCR(1-3). This two-site membrane interaction used in the targeting peptide exploits additive binding affinities of small molecules for spatially related components of cellular membranes¹⁴

Figure 7. Binding of m-pSCR1-3 to human kidney. Immunohistochemistry of human kidneys perfused with preservation solution containing m-pSCR1-3 showed (a and b) that the agent bound to glomeruli (G) and to tubules (Tu). c: The high-power view reveals a pattern of m-pSCR1-3 staining consistent with binding to the basolateral tubule surface (B). d: Human kidney perfused without m-pSCR1-3 showed no staining. Original magnifications: $\times 1000$ (a, b, d); $\times 2000$ (c).

and allows flexibility because the m-p tails can be modified to suit different applications. Site-directed regulators of complement activation have a wide variety of clinical applications beyond transplantation^{14,16} and this type of membrane localization strategy is also of wide applicability.

Our results show a significant antigen-independent component of injury late after transplantation that was preventable by modifying acute I/R injury. Additionally, there was an effect on the antigen-specific component of injury, suggested by the finding of reduced alloreactivity in the recipients of m-pSCR1-3-treated grafts. Thus the overall potential to improve graft function could reside in the benefit of reduced immunogenicity of the graft as well as reduced postischemic damage. A clinical corollary may be found in the results of live unrelated organ transplantation, in which ischemic times are minimized and consequently clinical outcomes are improved compared to cadaveric renal transplantation with equivalent degrees of MHC mismatch.³⁷

Complement activation is possibly one of a number of antigen-independent mechanisms that enhance the recognition of foreign tissue by the recipient immune system. Current debate on the role of nonspecific injury in the stimulation of T-cell immunity would suggest that reduced inflammation could attenuate specific immunity.³⁸ The results presented here support this concept, and suggest that intragraft inhibition of complement decreases the local inflammatory environment and reduces the formation of C3 fragments with immunoregulatory properties. Indeed, recent experiments using a mouse kidney allograft model found that donor organs with reduced capacity for the production of C3 elicit weaker alloimmunity,⁶

suggesting an important effect of local complement. Thus the transplanted kidney may be a particularly important target for therapeutic complement regulation.

To maximize the efficacy of therapeutic complement regulation, we introduced the complement regulator before complement activation, that is, before reperfusion of the transplant organ with recipient blood. By adding m-pSCR1-3 to the perfusion solution we were able to intervene in the pathogenesis of I/R injury. This method bypassed the inherent time delay for DNA transcription and protein translation required using gene transfection technologies to introduce site-specific protein expression. In organ transplantation this is particularly relevant because of the lack of notice clinicians receive before an organ transplant. The degree of protection we observed was achieved with a single application of a molecule that has been shown to be acceptable for use in humans.²⁰

In conclusion, we have shown that it is possible to modify the biological properties of donor kidney by targeting a complement regulatory protein to potential sites of tissue injury, thereby reducing the late consequences of inflammatory and immunological injury. The approach described here in a rat model has the potential to be applied to clinical evaluation, possibly in conjunction with other emerging therapies for transplant I/R damage, such as superoxide dismutase mimetics³⁹ and adhesion molecule blockade.⁴⁰ Transient modification of the donor organ without the need for systemic complement inactivation might have a significant advantage for kidney transplant recipients.

Acknowledgments

We thank Mr. Rodney Oldroyd, Mr. Ian Dodd, and members of the Development Team at Adprotech for their assistance and helpful discussions; and Dr. Louise Affleck for helping us with antibody reagents.

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