The Cytokine-adhesion Molecule Cascade in Ischemia/Reperfusion Injury of the Rat Kidney

Inhibition by a Soluble P-selectin Ligand

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Abstract

Ischemia/reperfusion (I/R) injury associated with renal transplantation may influence both early graft function and late changes. The initial (≤ 7 d) events of warm and in situ perfused cold ischemia of native kidneys in uninephrectomized rats were examined. mRNA expression of the early adhesion molecule, E-selectin, peaked within 6 h; PMNs infiltrated in parallel. T cells and macrophages entered the injured kidney by 2–5 d; the associated upregulation of MHC class II antigen expression suggested increased immunogenicity of the organ. Th1 products (IL-2, TNFα, IFNγ) and macrophage-associated products (IL-1, IL-6, TGFβ) remained highly expressed after 2 d. To examine directly the effects of selectins in I/R injury, a soluble P-selectin glycoprotein ligand (sPSGL) was used. Ischemic kidneys were perfused in situ with 5 μg of sPSGL in UW solution; 50 μg was administered intravenously 3 h after reperfusion. E-selectin mRNA remained at baseline, leukocytes did not infiltrate the injured organs throughout the 7-d period, and their associated products were markedly inhibited. Class II expression did not increase. No renal dysfunction secondary to I/R occurred. The early changes of I/R injury may be prevented by treatment with soluble P- and E-selectin ligand. This may reduce subsequent host inflammatory responses after transplantation. (*J. Clin. Invest.* 1997. 99:2682–2690.)

Key words: P-selectin • E-selectin • cytokine • adhesion molecule • P-selectin glycoprotein ligand

Introduction

Impaired or delayed kidney function after engraftment is one of the most common sequelae of renal transplantation, occurring in as many as 50% of cadaver grafts in some series (1). As this complication is rare in living donor kidneys, regardless of histocompatibility differences, the condition has been thought to result from a variety of circumstances, including the state of the donor and the effects of warm or cold ischemia, storage, and reperfusion injury (2, 3). Although the majority of the renal changes are reversible, hospital stay is increased, administration of immunosuppression is more difficult, dialysis is prolonged, and cost is amplified. There is also evidence that the incidence of both acute rejection and diminished long-term graft survival is higher in such organs than in those which function immediately (4). It seems likely that the events surrounding initial ischemia/reperfusion (I/R)[1] may trigger upregulation of MHC antigens; the potentially increased graft immunogenicity may promote host cellular infiltration and expression of cell products (5). This nonspecific inflammatory response may interact with the cytokine-adhesion molecule cascade characteristic of immune injury.

The early dynamics after I/R injury which are potentially involved in subsequent graft changes have not been defined completely. This insult presumably causes elevation of P-selectin, which is rapidly translocated to endothelial cell surfaces within 5 min of revascularization of the organ, initiating steps leading to tethering of PMNs to the vascular intima (6). Local production of IL-1β and/or TNFα by these leukocytes induces P- and E-selectin expression on endothelium which continues the cascade of events which increase cell adherence and infiltration of the injured tissues (7, 8). In this study, the relationship between the induced expression of E-selectin and cellular and molecular kinetics has been examined. This molecule is associated with infiltration of PMNs which themselves may trigger the activities of other leukocyte populations and expression of their products. We have assessed the role of a soluble form of P-selectin glycoprotein ligand (sPSGL) in inhibiting selectin activity and subsequent events in the damaged organs. It appears that successful blockade of the earliest steps in leukocyte adhesion (i.e., rolling) is effective in diminishing I/R injury. This manipulation may reduce later changes which contribute potentially to subsequent host alloresponsiveness.

Methods

Animals. Inbred male Lewis (LEW) rats (Harlan Sprague-Dawley, Indianapolis, IN) of 250–300 g were used throughout the experiments.

Experimental design and operative technique. Three experimental models were examined. The initial events developing after cold I/R were examined in detail with particular reference to inflammatory molecules, cellular infiltration, and expression of associated products. Patterns occurring after warm ischemia were used for comparison.

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1. *Abbreviations used in this paper:* ICAM, intercellular adhesion molecule; I/R, ischemia/reperfusion; PSGL-1, P-selectin glycoprotein ligand-1; RANTES, regulated upon activation, normal T cell expressed and secreted; RT-PCR, reverse transcriptase-PCR; sPSGL, soluble form of P-selectin glycoprotein ligand-1; UW, University of Wisconsin solution.

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The inhibitory effects of a soluble P-selectin ligand administered both in the cold perfusate and to the animal after reconstitution of the circulation were assessed. Uninjured kidneys in uninephrectomized rats acted as controls. Three animals in each group were examined for each time period.

The right kidney of normal LEW rats was removed and the left kidney was isolated. For the studies of warm ischemia, the left renal artery and vein were occluded for 45 min then released; the organs do not recover if the injury is prolonged to 60 or 90 min. The abdominal incision was closed and the animals returned to their cages. To produce cold ischemia, the aorta was occluded proximal and distal to the left renal artery, and the left renal vein clamped with a vascular clip near its junction with the inferior vena cava. The aorta was then catheterized with a silicone tube (Silicone Medical Grade Tubing, 0.02 inch inside diameter, 0.037 inch outside diameter; Baxter Scientific Products, Boston, MA) and a drainage vent made in the renal vein distal to the vascular clip. The isolated kidney was then perfused slowly (2–3 min) with 10 ml of iced perfusion solution consisting of 83.33% by volume of University of Wisconsin (UW) solution (K_2HPO_4, 3.38 mg/ml; KH_2PO_4, 8.16 mg/ml; KCl, 1.23 mg/ml; $NaHCO_3$, 0.45 mg/ml), 8.33% by volume of albumin (Albutein; Alpha Therapeutic Corporation, Los Angeles, CA) and 8.33% by volume of 50%-dextrose injection, (Abbott Laboratories, North Chicago, IL). The vascular pedicle was then clamped at the level of the hilum for a total ischemic time of 45 min. During that time, the involved vessels were repaired with 7-0 monofilament suture (Proline; Ethicon Inc., Somerville, NJ) and the other clamps released.

In the treatment group, 1 ml of sPSGL (5 μg/ml) in 1 ml UW solution was perfused slowly (2–3 min) into the left kidney after perfusion with 9 ml of perfusion solution, and the vascular pedicle was clamped for 45 min; this allowed sPSGL to block P-selectin during the actual period of ischemia. Additional material (50 μg) was then injected intravenously into the animal after 3 h of reperfusion to block E-selectin before its peak at 6 h (data not shown). This protocol was determined by numerous preliminary experiments with various sPSGL doses to assess the most effective blockade of PMN infiltration and E-selectin expression (data not shown). Blood samples (0.5 ml) for creatinine determination were obtained from the tail vein at 0, 1, 2, and 3 d after reperfusion. Creatinine was measured by a modified Jaffe's reaction on an autoanalyzer (911; Hitachi, Indianapolis, IN).

sPSGL. sPSGL.I316 (Genetics Institute, Cambridge, MA) is a recombinant soluble form of P-selectin glycoprotein ligand-1 (PSGL-1) (9). The mammalian expression vector pED (10) was liganded to a cDNA encoding sPSGL.I316 which comprises the mature extracellular domain of PSGL-1, truncated at the isoleucine molecule at position 316. This vector was stably transfected and amplified in a DHFR-Chinese hamster ovary cell line stably transfected with vector pMT4neo expressing both the cDNA encoding an α(1, 3/1, 4) fucosyltransferase (11) and a cDNA encoding core 2 β-1,6-*N*-acetylglucosaminyltransferase (12). The secreted sPSGL.I316 was purified from serum-free Chinese hamster ovary cell conditioned medium by application to a toyopearl column (QAE 500C; TosoHAAS, Montgomeryville, PA). The column was washed using 5 column vol of 25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer. After sixfold concentration by ultrafiltration/diafiltration using tangential flow membrane unit (Millipore Corp., Bedford, MA) and 1 M NaCl/25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer, the concentrated material was applied to a hydroxyapatite column (Pharmacia Biotech, Piscataway, NJ) equilibrated with 1 M NaCl/25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer, washed with 15 column vol of 150 mM NaCl/25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer and eluted using 30 mM $NaPO_4$/150 mM NaCl, pH 7.4. The eluate was run through a Sephacryl S-300 HR sizing column equilibrated in PBS, pH 7.2. Collected fractions were analyzed by SDS-PAGE using Alcian blue/silver staining (Fig. 1). sPSGL-containing fractions were assayed for selectin binding activity as described (13). Endotoxin levels were determined to < 2 endotoxin U/mg.

Protease-treated sPSGL. sPSGL.I316 (2.5 mg/ml) was incubated with or without 10 μg/ml mocarhagin (Sigma Chemical Co., St. Louis, MO) in PBS/1 mM $CaCl_2$ at 37°C for 6 h (14). Mocarhagin, a protease isolated from cobra venom, was inactivated by adding EDTA to 2 mM and heating to 85°C for 5 min. Buffer with only mocarhagin was also included as an additional control. Cleavage of amino-terminal 10 amino acids of mature sPSGL was confirmed by Western blot using monoclonal PSL275 (Genetics Institute) which binds to the amino-terminal portion of mature PSGL-1 (15), or monoclonal PSL1H3 (Genetics Institute), which binds to an epitope proximal to the carboxyl terminus of sPSGL.I316 (Fig. 2). Inactivation of P-selectin binding was confirmed by binding to soluble P-selectin in competitive ELISA format.

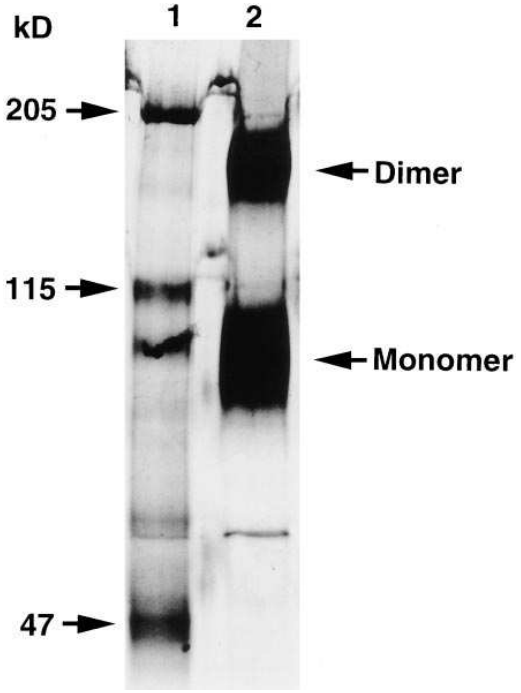

Figure 1. Nonreducing 4–20% polyacrylamide-SDS gel of 1 μg purified recombinant sPSGL.I316. The gel is developed with Alcian blue and silver stain. The purified sPSGL glycoprotein appears as both a dimer and monomer exhibiting an average approximate relative molecular mass of 190 and 95 kD, respectively. Lane *1*, size marker; lane *2*, sPSGL.

Immunohistology. Representative portions of the kidneys were snap-frozen in liquid nitrogen at 0, 3, 6, and 12 h and at 1, 3, 5, and 7 d after operation. The contralateral kidneys removed from the animal at the time of ischemic injury were used as controls. Sections (4 μm) were fixed in acetone for 10 min, air-dried, and stained individually with mouse anti–rat monoclonal or polyclonal antibodies. PMNs were stained with a rabbit anti–rat PMN FITC-conjugated gamma globulin (Inter Cell Technologies, Inc., Hopewell, NJ). Stained cells were counted under a fluorescence microscope (UFX-IIA; Nikon, Garden City, NY) and expressed as cells per glomerulus (×400, $>$ 40 fields counted/specimen, 2–3 specimens/kidney). The specimens were also stained with ED-1 for macrophages, W3/25 for $CD4^+$ cells, OX-8 for $CD8^+$ cells, and OX-3 for MHC class II (Bioproducts for Science, Inc., Indianapolis, IN). The sections were then interacted with rabbit anti–mouse IgG by the alkaline phosphatase, anti–alkaline phosphatase method and counterstained with hematoxylin (16). Stained cells were then counted on an ocular grid and marker positive cells expressed as mean±standard deviation (M±SD) of cells per field of view (cells/FV, ×400, $>$ 30 fields counted/specimen, 2–3 specimens/kidney). For morphology, formalin-fixed sections were stained with periodic-acid-Schiff.

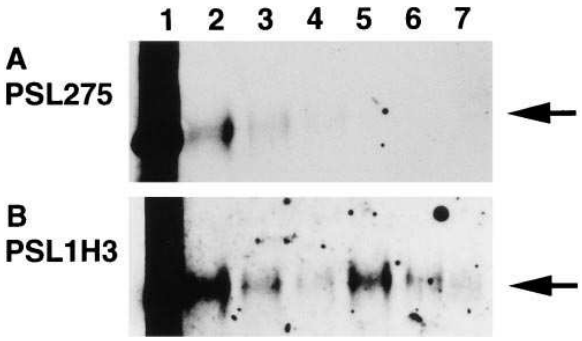

Figure 2. Western blot of various amounts of recombinant sPSGL. (*A*) Undigested (lanes *2–4*) or digested with mocarhagin (lanes *5–7*) using amino-terminal specific mAb PSL275. Amounts of sPSGL loaded onto each lane are 100 ng (lanes *2* and *5*), 50 ng (lanes *3* and *6*), 25 ng (lanes *4* and *7*), respectively. Lane *1*, size marker. (*B*) Same as *A* using a proximal to carboxyl-terminal specific mAb PSL1H3. Note that protease-digested sPSGL (lanes *5–7*) has lost its amino terminus for P-selectin binding (*A*).

Reverse transcriptase polymerase chain reaction (RT-PCR). The expression of various adhesion molecules, cytokines, and growth factors was examined by RT-PCR (17–19) at 0, 3, 6, 12, 24, and 48 h and at 3, 5, and 7 d. Total RNA of kidney specimens was extracted by the guanidine isothiocyanate method (Ultraspec RNA kit; Biotecx Laboratories, Inc., Houston, TX) (20). The total RNA was air-dried, resuspended in diethyl pyrocarbonate–treated water, and the approximate quantity of RNA was determined with an OD 260 reading. The purity of the RNA was assessed with an OD 260/280 ratio, confirming all samples > 1.8. Total RNA (2.5 μg) was used for first-strand cDNA synthesis employing 1.2 μg of oligo dT_{12-18} and the Superscript reverse transcriptase method according to supplier-recommended conditions (GIBCO-BRL, Gaithersburg, MD). Nonlooping nonoverlapping oligonucleotide primer pairs from separate exons were prepared for each gene studied by Clontech (Palo Alto, CA) or Genosys Biotechnologies (The Woodlands, TX). The specific primers (IL-2, IL-2R, IL-4, IFNγ, IL-1, IL-6, TGFβ, TNFα, RANTES, MCP-1, and β-actin) were used as described (21). The sequences for the E-selectin, C3, and C1 specific primer are as follows: E-selectin: upstream primer, 5′-TCTTTAAGCTCAAGGAAT-3′; downstream primer, 5′-TACCGATCGTGCAGGTCA-3′; C3: upstream primer, 5′-CGGAATCGATTGCCTATT-3′; downstream primer, 5′-CCGTCTTGGTCCAACTTA-3′; C1: upstream primer, 5′-GTCCGATTCGCGTTGCA-3′; downstream primer, 5′-TAACGCATTTCATCGGAC-3′.

The competitive PCR for quantification of mRNA was performed as described (19). For each 25-μl amplification, 2.5 μl of first-strand cDNA product was used. 2–16-fold serial dilutions of known quantities of PCR MIMICS were added to the PCR reaction containing constant amounts of sample target cDNA. PCR products (5 μl) were run on 1.5% agarose gel and stained with ethidium bromide, then gene-specific bands were visualized with ultraviolet light (Fig. 3). The quantities of MIMICS and target cDNA were compared using a PC SCANJET with analysis by Adobe Photoshop software (Adobe Inc., Mountain View, CA). Tissues were tested at all time points for an individual cytokine mRNA transcript as well as for the control β-actin mRNA transcript. Scanner analysis of photographs of the DNA-stained agarose gels was evaluated by the band intensity comparison of β-actin expression versus each cytokine expression in computer image analysis. Each PCR reaction was repeated twice in all three kidney specimens in each time group and were not appreciably

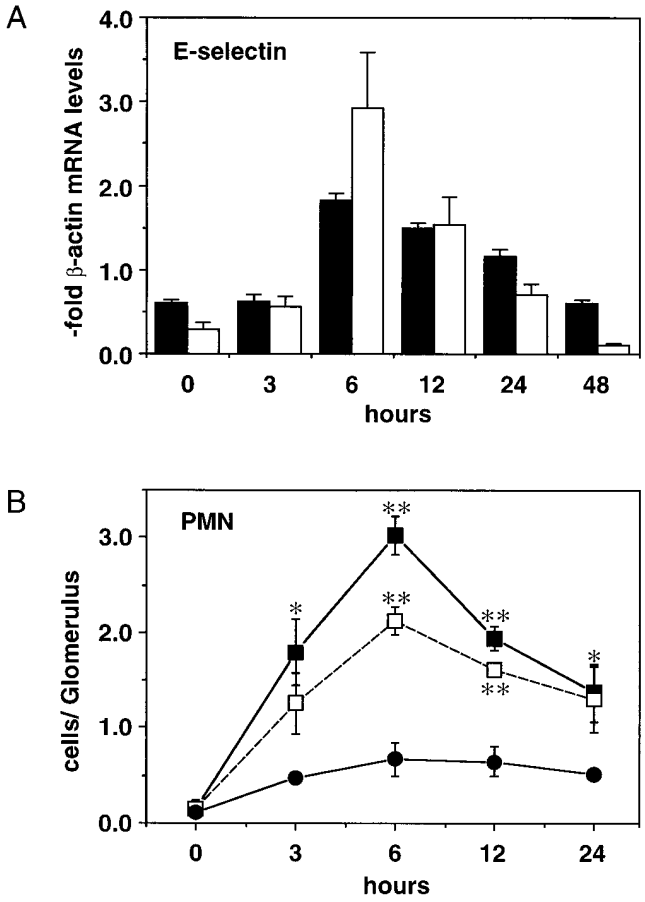

Figure 4. Patterns of E-selectin mRNA expression and PMN infiltration in rat ischemic kidneys are shown. (*A*) E-selectin mRNA expression is upregulated both in warm (■) and cold (□) ischemia. (*B*) PMNs infiltrate the organs in parallel. $^*P < 0.05$; $^{**}P < 0.01$ compared with control kidneys (●). Data are expressed as mean±SEM.

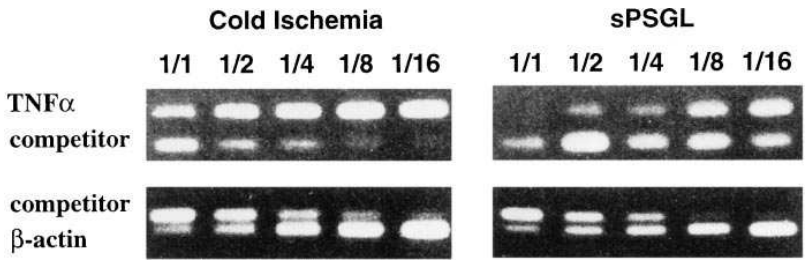

Figure 3. Examples of semiquantification by competitive RT-PCR assay. TNFα mRNA expression in a cold ischemic kidney and in a kidney at 3 d after sPSGL treatment is compared. Expression of TNFα and β-actin control mRNA were quantitated by the amount of competitive template that most nearly equaled the amplification of cellular cDNA. Compared to β-actin expression (equivalent point is 1/2), TNFα expression of cold ischemia is 2.0 (equivalent point is 1/1), and that of sPSGL-treated animal is 0.25 (equivalent point is 1/8) in this sample.

different from one another. This method has been noted as accurate as scintillation counting of ^{32}P-labeled PCR products (22).

Statistical analysis. The results are expressed as arithmetic means (±SEM). Statistical comparisons between groups were performed by Student's *t* test. The difference was considered to be significant when $P < 0.05$.

Results

Cellular, molecular, and morphological changes after I/R injury. Inflammatory changes became apparent in the single injured kidneys in uninephrectomized animals within a few hours after I/R. The expression of E-selectin mRNA peaked at 6 h after both warm ischemia and cold ischemia and was still upregulated at 12 h before declining to baseline (Fig. 4 *A*). Rapid migration of PMNs into the organ occurred in parallel, more intense in warm than in cold injury, with the cells localizing preferentially in and around glomeruli as well as throughout the interstitium. Few PMNs were ever noted in the uninjured kidneys of uninephrectomized controls (Fig. 4 *B*).

Within 24 h of the warm I/R insult, intercellular adhesion molecule (ICAM)-1 mRNA expression peaked and remained upregulated for 48 h before declining. Cold perfusion substantially inhibited expression of this adhesion molecule (Fig. 5 *A*). The transcripts of the complement component, C3, were highly upregulated by 12 h after warm ischemia, somewhat less in the cold ischemia kidneys. C1 remained minimally expressed (Fig. 5 *B*).

Lymphocytes and macrophages, as determined by immunohistology, began to infiltrate the affected organs coincident with ICAM-1 expression at 1–3 d. Cold perfusion decreased but did not abolish the cellular infiltrate (Fig. 6). ED-1$^+$ macrophages entered the kidneys after 24 h and remained obvious throughout the 7-d follow-up period. Relatively large numbers of CD4$^+$ leukocytes infiltrated the organs in a similar pattern, peaking at 5 d; CD8$^+$ lymphocytes were a minor feature of this injury. Importantly, MHC class II expression, noted in the interstitium, collecting tubules, and on periglomerular cells, was upregulated in parallel with the presence of CD4$^+$ cells.

Cytokine mRNA production increased substantially after the I/R insult (Fig. 7). Most T cell–derived cytokines (IL-2 and IL-4) peaked between 3 and 5 d, at which time numbers of infiltrating CD4$^+$ lymphocytes were obvious; IL-2R and TNFα, expressed by activated T cells, increased in parallel. RANTES, a macrophage chemoattractant derived from T cells and other cell populations, remained upregulated in a more sustained fashion throughout the follow-up period; its presence correlated with the broad peak of infiltrating macrophages (Fig. 7 *A*). IFNγ increased slowly, but seemed less of a feature in the process than the other T cell factors. The macrophage associated products, IL-6 and TGFβ, had become highly expressed within 3 d; IL-1 increased gradually (Fig. 7 *B*). Little expres-

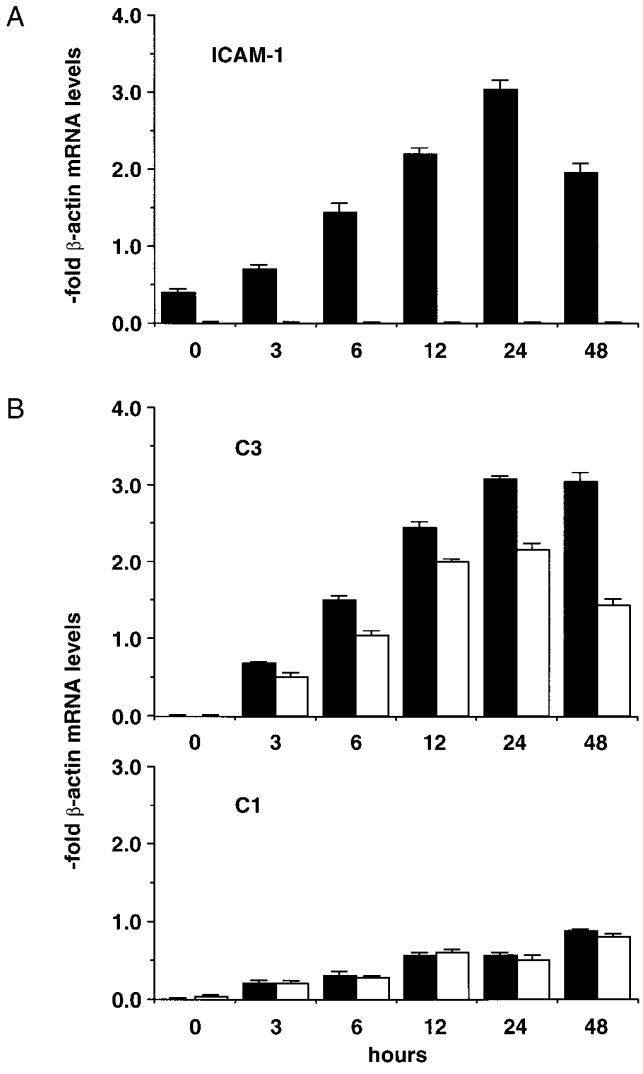

Figure 5. ICAM-1 and complement mRNA expression after warm and cold ischemia. (*A*) High mRNA expression of ICAM-1 in warm ischemia (■) and negligible expression in cold perfused organs (□) is noted. (*B*) C3 is highly expressed after both warm and cold ischemia; C1 is minimally expressed.

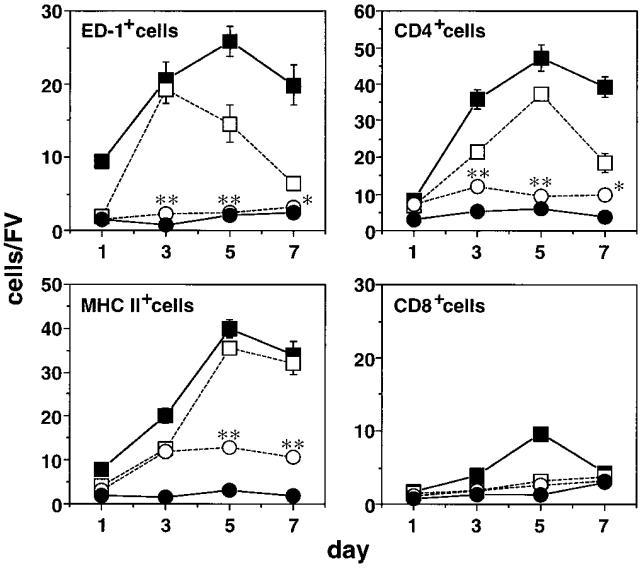

Figure 6. Cell infiltration into kidneys after warm (■) and cold (□) ischemic injury is increased. Treatment with sPSGL (○) inhibits cell migration, similar to that in uninephrectomized controls (●). *$P < 0.05$; **$P < 0.01$ compared with sPSGL treatment and cold ischemia.

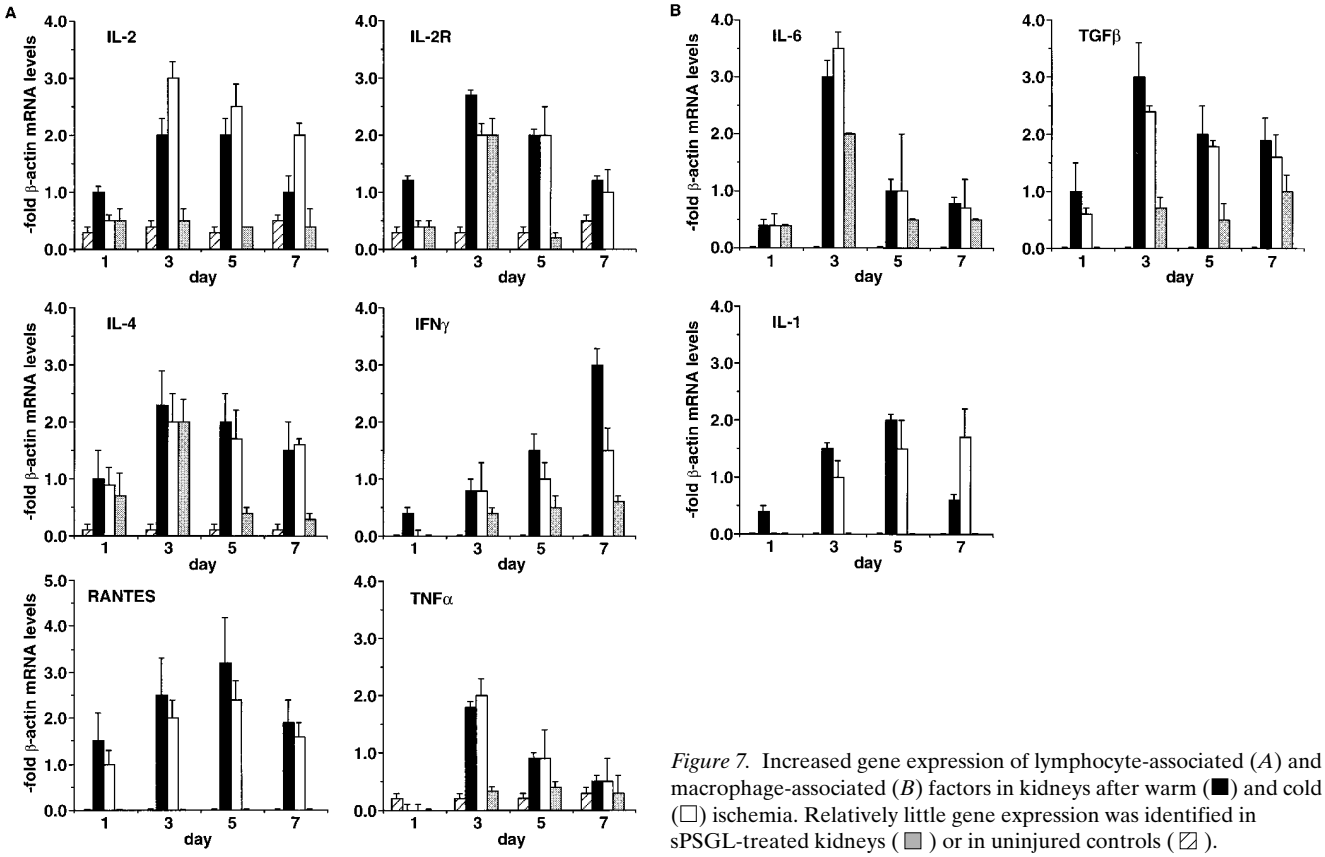

Figure 7. Increased gene expression of lymphocyte-associated (*A*) and macrophage-associated (*B*) factors in kidneys after warm (■) and cold (□) ischemia. Relatively little gene expression was identified in sPSGL-treated kidneys (▩) or in uninjured controls (▨).

sion of cells or cell products was identified in uninjured uninephrectomized control kidneys.

The kidneys injured by ischemia were then assessed at day 3. Tubular necrosis was the most obvious effect of warm and cold ischemic injury (Fig. 8, *B* and *D*, respectively), with interstitial edema, denudation of tubular cells, and tubular casts compared to uninephrectomized controls (Fig. 8 *A*). Such changes were more severe after warm ischemia, but also surprisingly obvious after cold ischemia.

Effects of sPSGL on I/R injury. Cold ischemic organs were perfused with sPSGL to block P-selectin, and the animal was then given the material intravenously to inhibit E-selectin before its peak expression at 6 h. E-selectin expression in sPSGL-treated rats remained at baseline, comparable to that of β-actin controls (Fig. 9 *A*). At the same time, PMN infiltration into both glomeruli and interstitium was reduced by ~ 50% (Fig. 9 *B*). Neither protease-treated sPSGL with cleavage of P-selectin binding by mocarhagin nor buffer perfusion at 0 h and injection at 3 h affected E-selectin expression and PMN infiltration, emphasizing that the structural features of sPSGL are essential to its action.

Morphologically, sPSGL treatment improved renal damage substantially compared to the cold ischemia alone, with minimal evidence of tubular compromise or interstitial edema (Fig. 8 *C*). Immunohistologically, sPSGL reduced numbers of infiltrating ED-1$^+$ macrophages, CD4$^+$ T cells and MHC class II$^+$ cells toward baseline; indeed, these cells were virtually absent in the injured but treated organs and at the same level as kidneys in uninephrectomized controls (Fig. 6). Similarly, the mRNA expression of inflammatory cytokines was diminished, with IL-2, IFNγ, and RANTES completely inhibited by sPSGL treatment. IL-2R and IL-4 were less affected (Fig. 7 *A*). Expression of the macrophage-associated factors, IL-1, TNFα, TGFβ, and NOSi, was diminished, although IL-6 still showed some upregulation at 3 d (Fig. 7 *B*).

Effect of sPSGL on renal function after I/R injury. To ascertain whether the decrease in infiltrating cells and their products in the injured kidneys affected function, levels of plasma creatinine were monitored for 3 d after warm and cold I/R injury, and after treatment with sPSGL. Rats with ischemic kidneys showed significant elevation of plasma creatinine levels by 2 d compared with uninjured controls. Treatment with sPSGL protected against this transient dysfunction; no increase in creatinine was noted (Fig. 10).

Discussion

Increasing clinical evidence both from single centers and from United Network of Organ Sharing has supported a relationship between delayed initial graft function and acute rejection (1–3). The significance of this antigen-independent injury on later graft survival remains conjectural, with most series suggesting that its primary effects are limited to the first year after transplantation. However, it seems increasingly clear that initial dysfunction plus early acute rejection in combination interact to reduce long-term graft success substantially (4). This seemingly synergistic effect may be explained in practical terms by the difficulties in recognizing and treating acute rejections in patients with nonfunctioning grafts. It has also been noted in experimental models, including data from this study,

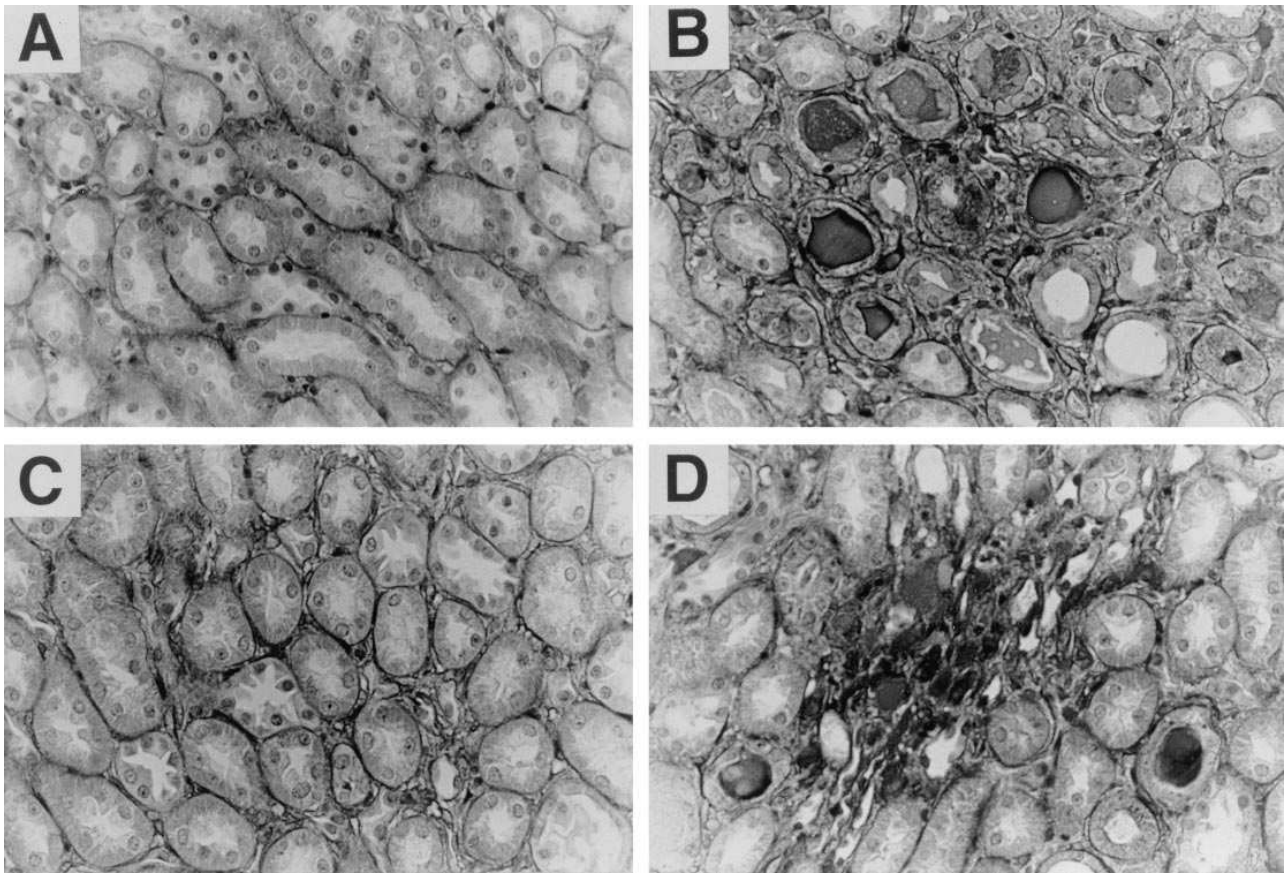

Figure 8. Histological features of ischemic kidneys (day 3). Significant tubular necrosis developed after both warm (*B*) and cold (*D*) ischemia. After sPSGL treatment (*C*) tubules are minimally damaged. (*A*) Control uninephrectomized kidney. Periodic-acid-Schiff staining, ×400.

that an ischemic insult may increase the immunogenicity of the organ by upregulating HLA class II antigens.

Because detailed knowledge about the cellular and molecular dynamics occurring in the kidney after I/R injury is relatively limited (5), we have examined the early events occurring in a well defined model. It appears that adhesion molecules, infiltrations of cell populations, and their associated products are highly expressed after warm ischemia and are still obvious, albeit less intensely, after cold perfusion. This finding suggests that despite cold preservation of the organ before actual engraftment, the transplanted kidney may not be as protected as thought hitherto, and may harbor potential triggering mechanisms for later immunologic host events.

P-selectin is the key adhesion molecule involved in the earliest events in the adherence of circulating leukocytes in rolling to tissues in inflammatory states (23, 24). It is constitutively present in the membranes of alpha granules of platelets and the Weibel-Palade bodies of endothelial cells, and is translocated to the plasma membrane of these cells in response to various stimuli (25–28). It mediates the adhesion of PMNs or monocytes to activated platelets or endothelial cells (29, 30), and the rolling of leukocytes to activated endothelial cells (31). P-selectin also plays a role in inflammatory and thrombotic disorders, including I/R injury (32, 33), leukocyte adhesion to lung endothelial cells in rats after cobra venom factor infusion (34), and leukocyte accumulation in thrombogenic grafts (35). The molecule has an important role not only in earliest cellular responses but in chronic inflammation as well. Monocyte accumulation is diminished substantially at later time points after drug-induced peritonitis (36), and in T cell–dependent contact hypersensitivity responses in P-selectin knockout mice (37). P-selectin also mediates monocyte–microvascular interaction in rheumatoid synovitis (38).

Among several ligands for the selectin families (39), P-selectin's high-affinity counter receptor, PSGL-1, is probably the most extensively characterized (40). The effects of blocking antibodies to P-selectin also indicate that the binding of this molecule to its ligand is important in modulating I/R injury (32, 33).

Translocation of stored P-selectin to the plasma membranes of endothelial cells occurs early after activation. In contrast to P-selectin, which is already produced and stored within the endothelial cells, E-selectin is expressed de novo on endothelial cells only after the transcriptional induction of E-selectin mRNA by proinflammatory agents such as TNFα or IL-1β in vitro (7, 8, 41). Upregulation of E-selectin on endothelial cells of renal allografts has been seen in biopsy specimens which show acute rejection (42). In this study, we have used this induction of E-selectin transcription and the PMN infiltration into glomeruli as measures of the local inflammatory status of renal endothelial cells. The peak mRNA expressions of E-selectin and PMN infiltration at 6 h after reperfusion were used to determine the dosage protocol.

Several in vitro studies have demonstrated that PSGL-1

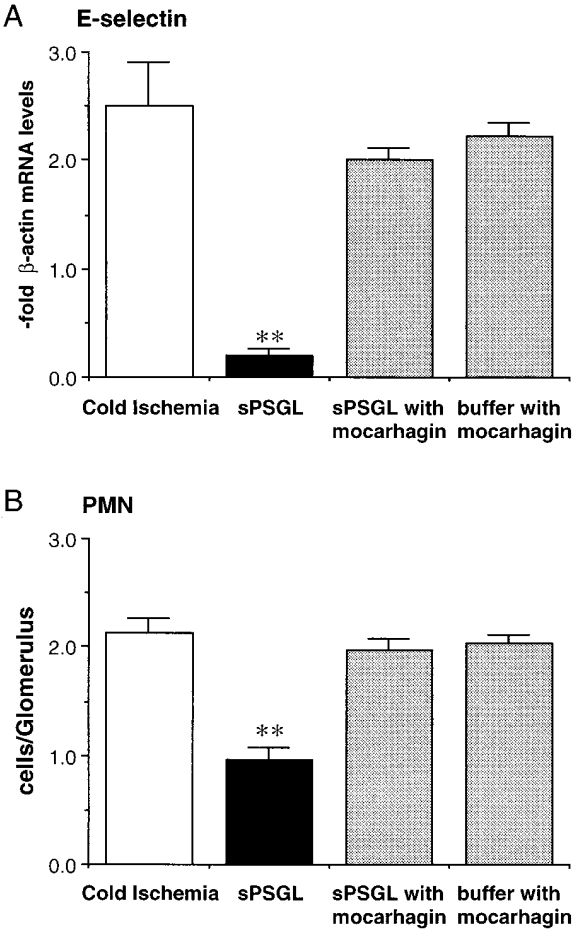

Figure 9. Inhibitory effects of sPSGL treatment on E-selectin mRNA expression and PMN infiltration. (*A*) E-selectin expression in cold perfused kidneys was inhibited by sPSGL without mocarhagin (■). No inhibition was observed after treatment with sPSGL with mocarhagin and buffer with mocarhagin (▨). (*B*) PMN infiltration in cold ischemia was also diminished with sPSGL without mocarhagin (■). No decrease in cell numbers was observed by sPSGL with mocarhagin and buffer with mocarhagin (▨). $^{**}P < 0.01$. Data are expressed as mean±SEM.

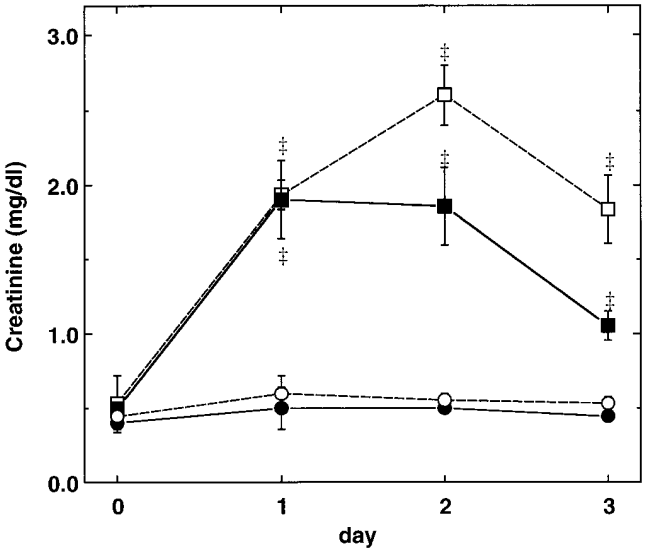

Figure 10. Increased plasma creatinine levels after warm (■) and cold (□) ischemia. Data are expressed as mean±SEM for each group ($n = 4$). $^{‡}P < 0.005$ compared with sPSGL treatment (○) and normal kidneys (●).

can serve as a ligand for all three selectin family members: P-, E-, and L-selectin (9, 43–46). A recombinant soluble form of PSGL-1, sPSGL, was used in these experiments to investigate the role of these molecules. Preliminary pharmacokinetic analysis estimated the postdistributional half-life of sPSGL in rats to be ~ 2 h (data not shown). The dosage protocol was designed to provide an adequate concentration of the blocking ligand during the ischemic period. Accordingly, a low dose of sPSGL was added to the final perfusion of cold UW solution to bind any P-selectin upregulated on renal endothelium during the 45-min ischemic period. Recent studies have shown that P-selectin expression on endothelial cells can also be regulated at the transcriptional level, with agents such as LPS, TNFα, or IL-3 inducing a later and more chronic expression of P-selectin (28, 47, 48). With this in mind, a second systemic dose was given 3 h after reperfusion to bind selectin molecules expressed at this later time point.

Because it is a specific ligand for selectins, sPSGL presumably acts by inhibiting the binding between endothelial cells (E-/P-selectin) and PMNs, and/or L-selectin–mediated PMN aggregation (9, 13, 15, 46, 48). In this study, sPSGL was found not only to block PMN infiltration but also to partially block the induction of E-selectin mRNA in the kidney. To confirm that this effect was associated with the blocking of P-selectin and not E-selectin, sPSGL was digested with the snake venom protease, mocarhagin. Mocarhagin-treated sPSGL loses the capacity to bind P-selectin, yet maintains the capacity to bind E-selectin (14). Animals treated with this protease-treated sPSGL failed to block both E-selectin induction and PMN influx 6 h after reperfusion (Fig. 9). This result strongly suggests that the mode of action for sPSGL on these early phase events is predominantly mediated by blocking P-selectin. One possible model of this finding is that by inhibiting the initial P-selectin–mediated tethering of leukocytes to endothelium, sPSGL diminishes the localized production of proinflammatory cytokines which induce E-selectin expression. This theory is supported by the low influx of PMN into tissues of P-selectin–deficient mice (49). Transplantation of hearts from such donors to wild-type recipients demonstrates a reduction of PMN infiltration and increase of graft survival (50), emphasizing the critical role of this selectin in the initial cell extravasation and infiltration. Moreover, PMN rolling is inhibited by anti–PSGL-1 antibody in vivo (51). It is unclear from the present data whether the later phase blocking effects which sPSGL treatment causes on the cytokine-adhesion cascade can be attributed to possible additional E- or L-selectin blockade by sPSGL. This will require further study. However, it is noteworthy that efforts by other investigators to ascribe a significant role for L-selectin in renal I/R injury, using L-selectin–deficient mice, have thus far failed (52).

De novo mRNA expression of ICAM-1 follows that of the selectins in the ischemic organs, peaking at 24 h in the warm ischemia model. I/R injury is reported to increase ICAM-1 expression (53). Cold I/R injury showed no de novo ICAM-1 expression compared to warm I/R injury, indicating the greater

influence of the warm injury. However, despite no ICAM-1 expression, sequential events still occurred, suggesting the effectiveness of alternative adhesion molecules in cold I/R. It has been reported that anti–ICAM-1 antibody can inhibit the transient renal dysfunction which occurs after warm ischemia (54). As noted in the present studies, sPSGL treatment also protects against this transient renal failure by interfering at an earlier phase of cell adhesion than anti–ICAM-1 antibody.

Activation of complement after I/R injury presumably increases the permeability of vascular walls (55). Activation of the complement cascade, whether the classical or alternative pathway, finally leads to the cleaving of C3, which plays a role in the adhesion of PMNs and monocyte/macrophages to endothelium (56). The presence of C3 mRNA has been identified in rejecting human kidney grafts (57). The rapidly increased expression of C3 in these experiments may contribute to cell infiltration into the injured kidney. The negligible mRNA expression of C1 after both warm and cold ischemia suggests this component is less important in this context.

Both MHC class II expression and subsequent cell migration to the injured kidney were suppressed by sPSGL. This putatively diminished immunogenicity of the organ may have important clinical ramifications, as it may decrease the incidence of acute rejection in grafts with delayed function (2). It has been reported that the kinetics of IFNγ induction of MHC class II genes occurs slowly, with mRNA levels peaking at 24–48 h (58). The pattern of IFNγ expression is well explained by the sequential expression of MHC class II in the present I/R model. Moreover, IFNγ can be potentiated by TNFα produced by PMNs and other cell populations (59). This IFNγ mRNA expression was also diminished by sPSGL. Other lymphocyte-derived cytokines inhibited by sPSGL include IL-2, TNFα, and RANTES (Fig. 7 *A*). RANTES may facilitate T cell and macrophage accumulation by direct chemotactic effects or by regulating the expression of macrophage products. The relatively prolonged expression of this factor after I/R injury has also been noted in a model of chronic allograft rejection (21). This and macrophage-associated cytokines (IL-1, IL-6, and TGFβ) were reduced after treatment.

These data all imply that minimal cytokine activity occurs once initial PMN adhesion, via P-selectin to graft endothelium which presumably triggers an inflammatory event, is blocked. Damage to the glomerulus and tubular cells caused by I/R is inhibited by blocking initial PMN adhesion. As a result, the transient renal dysfunction that follows ischemia can be prevented. These findings emphasize the role of various host cell populations and their products in the injury of I/R. Their early inhibition may potentially influence both short- and long-term survival of renal allografts.

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