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Renal Urokinase-Type Plasminogen Activator (uPA) Receptor but not uPA Deficiency Strongly Attenuates Ischemia Reperfusion Injury and Acute Kidney Allograft Rejection

Faikah Gueler,† Song Rong,§ Michael Mengel, † Joon-Keun Park, * Julia Kiyana, Torsten Kirsch,* Inna Dumler,* Hermann Haller,* and Nelli Shushakova2*‡

Central mechanisms leading to ischemia induced allograft rejection are apoptosis and inflammation, processes highly regulated by the urokinase-type plasminogen activator (uPA) and its specific receptor (uPAR). Recently, up-regulation of uPA and uPAR has been shown to correlate with allograft rejection in human biopsies. However, the causal connection of uPA/uPAR in mediating transplant rejection and underlying molecular mechanisms remain poorly understood. In this study, we evaluated the role of uPA/uPAR in a mice model for kidney ischemia reperfusion (IR) injury and for acute kidney allograft rejection. uPAR but not uPA deficiency protected from IR injury. In the allogenic kidney transplant model, uPAR but not uPA deficiency of the allograft caused superior recipient survival and strongly attenuated loss of renal function. uPAR-deficient allografts showed reduced generation of reactive oxygen species and apoptosis. Moreover, neutrophil and monocyte/macrophage infiltration was strongly attenuated and up-regulation of the adhesion molecule ICAM-1 was completely abrogated in uPAR-deficient allografts. Inadequate ICAM-1 up-regulation in uPAR−/− primary aortic endothelial cells after C5a and TNF-α stimulation was confirmed by in vitro experiments. Our results demonstrate that the local renal uPAR plays an important role in the apoptotic and inflammatory responses mediating IR-injury and transplant rejection. The Journal of Immunology, 2008, 181: 1179–1189.

Urokinase-type plasminogen activator (uPA) is a multifunctional molecule that serves either as a proteolytic enzyme or as a signal-inducing ligand. The urokinase receptor (uPAR; CD87) was originally identified as a proteinase receptor for uPA, directing pericellular proteolysis. However, accumulating data clearly demonstrate that uPAR can also activate a variety of intracellular signal pathways via its lateral interaction with different cell surface proteins such as integrins, growth factor receptors, and G-protein-coupled membrane proteins. These interactions enable the uPA/uPAR system not only to control pericellular fibrinolytic and proteolytic activities, but also to modulate cell adhesion, migration, proliferation, and differentiation (1, 2). Moreover, the important role of the uPA/uPAR system has recently been demonstrated in both innate and adaptive immune-mediated responses (3). The uPA/uPAR system modulates Ag processing and presentation (4), lymphocyte activation (5), generation of pro- and anti-inflammatory signals (6), activation of intracellular signaling pathways (7), cytotoxicity (8), cell adhesion (9), and migration (10–12), all of which are critical steps in cell-mediated immune responses. Furthermore, uPA potentiates neutrophil activation (7) and superoxide production (13). Recently, we were also able to demonstrate an important link between the uPA/uPAR and complement system in the regulation of immunological responses in kidney (14) and lung tissues (15).

Renal ischemia reperfusion (IR) injury after transplantation leads to acute renal failure, which profoundly affects both early and long-term allograft function (16). Accumulating evidence demonstrates that prolonged cold ischemia time is a strong risk factor for unfavourable outcome after allogenic kidney transplantation (17, 18) and suggests that the severity of IR injury to the allograft determines its immunogenicity and subsequent graft fate (19). The mechanisms underlying IR damage of kidney tissue seem to be multifactorial and interdependent. The oxygen supply to the tissue by reperfusion leads to the generation of reactive oxygen species (ROS) exceeding the protective anti-oxidative capacity of kidney cells. Oxidative stress is known to be a major apoptotic stimulus in allograft nephropathy (20). Furthermore, IR injury stimulates the components of innate immune response, such as complement activation and up-regulation of multiple proinflammatory genes including chemokines, cytokines, cytokine receptors, and adhesion molecules. This inflammatory response induced by innate mechanisms early after transplantation is markedly amplified by the subsequent adaptive response (16). Therefore, IR injury initiates and induces the alloimmune response leading to acute and chronic allograft rejection (21, 22).

Recently, up-regulation of the uPA/uPAR system has been demonstrated under hypoxia/re-oxygenation conditions in vitro (23, 24). Moreover, uPA/uPAR activation has been shown to correlate with allograft rejection in a human biopsy study (25, 26) implying a probable involvement of this system in acute and chronic allograft rejection. However, the causal connection of uPA/uPAR in

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3 Abbreviations used in this paper: uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; IR, ischemia reperfusion; ROS, reactive oxygen species; RT, room temperature; DHE, dihydroethidium; PFA, paraformaldehyde; MAEC, mouse aortic endothelial cell; PBMC, peripheral blood mononuclear cell; MO, monocytes/macrophages; PMN, polymorphonuclear leukocyte; ATN, acute tubular necrosis; C5aR, receptor for C5a anaphylatoxin; MO, macrophage/monocyte; WT, wild type.

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mediating of IR injury and transplant rejection as well as the possible molecular mechanisms for uPA/uPAR mediated initiation and perpetuation of inflammatory reaction after transplantation remain unexplored.

The aim of our study was to assess the role of the renal uPA/uPAR system in renal IR injury and in allogenic kidney transplant rejection. We demonstrate that uPAR but not uPA deficiency of the allograft protected from IR injury and acute allogenic transplant rejection. As underlying mechanisms we could elucidate impaired susceptibility of the uPAR-deficient allografts to IR-induced ROS-mediated apoptosis and reduced expression of adhesion molecules, leading to impaired migration of host monocytes/macrophages and neutrophils into uPAR+/− allografts.

Materials and Methods

Animals

The uPA- and uPAR-deficient mice (uPA−/− and uPAR−/−), generated as previously described (27), were a gift from Peter Carmeliet and Mieke Dewerchin (Center for Transgene Technology and Gene Therapy, University of Leuven, Belgium). uPA/uPAR deficiency was verified by PCR genotyping. The uPA−/− mice on a C57BL/6 background and the corresponding WT controls (WT1), as well as uPAR−/− mice on a mixed C57BL/6 (75%) × 129 Sv (25%) background and their WT littermate controls (WT2) served as kidney donors (H2k). BALB/c (H2d) mice served as recipients in kidney transplantation experiments and were supplied by Charles River Laboratories. The animals were bred under pathogen-free conditions in the animal facility of PhenoN GmbH (Hannover, Germany) and cared for in accordance with our institution’s guidelines for experimental animals. All experiments were approved by the animal protection committee of the local authorities. Mice weighing 25–30 g were used for all experiments. For IR injury, 7–12 mice were used in each group (8 uPAR−/− mice, 7 WT2 mice, 10 uPA−/− mice, and 12 WT1 mice). For transplant experiments 6 mice for each group were used for the survival study and additional 6 mice for each group and each time point were sacrificed at 4 h, at day 1, and at day 6 after transplantation for histological and molecular analysis.

Renal ischemia reperfusion injury

Renal IR-injury was induced in homozygous male uPA−/−, uPAR−/−, WT1, and WT2 mice by bilateral clamping of both renal pedicles. The animals were anesthetized with isoflurane. After median laparotomy, renal pedicles were bluntly dissected and a nontraumatic vascular clamp was applied for 35 min. At 24 h, posts ischemia kidney function was estimated by serum creatinine measurement using an automated method (Beckman Analyzer).

Kidney transplantation

For transplant experiments, homozygous female uPA−/−, uPAR−/−, WT1, and WT2 mice were used as kidney donors and female BALB/c (H2b) mice served as recipients. Vascularized kidney transplantation was performed as described previously (28). In brief, the animals were anesthetized with isoflurane, and the left donor kidney attached to a cuff of the aorta and the renal vein with a small caval cuff and the ureter were removed en bloc. After left nephrectomy of the recipient, the vascular cuffs were anastomosed to the recipient’s abdominal aorta and vena cava, respectively, below the level of the native renal vessels. The ureter was directly anastomosed to the bladder (29). The times of cold and warm ischemia of allografts were 60 and 30 min, respectively. The right native kidney was removed through flank incision either on the day of transplantation or four days later. After transplantation, kidney function was estimated at designated time points (ranging from 24 h to several weeks) by serum creatinine level measurement. The general physical condition of the animals was monitored for any evidence of rejection.

Allograft pathology

Kidney grafts were harvested 24 h and six days after transplantation and one half of each allograft was immediately fixed in buffered formalin and embedded in paraffin. Sections of 3 µm were stained with hematoxylin-eosin and periodic acid-Schiff stain, and evaluated according to the updated Banff classification (30) by a nephropathologist, who was masked to the experimental groups. Immunohistochemistry was performed using the following primary Abs: rat anti-mouse monocytes/macrophages (F4/80; Serotec), polyclonal rabbit anti-mouse active caspase 3 (BD Pharmingen), monoclonal anti-mouse neutrophils (Gr-1, a gift from Prof. Hoffmann, Hannover Medical School, Hannover, Germany), monoclonal rat anti-mouse T lymphocytes (CD4 and CD8; BD Pharmingen), rabbit polyclonal anti-mouse CD25 (Santa Cruz Biotechnology), rabbit polyclonal anti-mouse uPAR (Santa Cruz Biotechnology). For indirect immunofluorescence, nonspecific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories) for 30 min. Thereafter, sections were incubated with the primary Ab for 1 h. All incubations were performed in a humid chamber at room temperature (RT). For fluorescent visualization of bound primary Abs, sections were further incubated with Cy3 conjugated secondary Abs (Jackson Immuno Research Laboratory) for 1 h. Specimens were analyzed using a Zeiss Axiosplan-2 imaging microscope with the computer program AxioVision 3.0 (Zeiss). Analysis of inflammation was done by semiquantitative scoring of the infiltrating cells in 10 randomly chosen, nonoverlapping fields of view on original magnification (×200). Score: 0, no; 1, weak; 2, moderate; 3, high; and 4, very high numbers of infiltrating cells. For CD4 and CD25 expression, absolute cell numbers were counted in 20 nonoverlapping view fields each specimen. Active caspase-3 expression in the outer strip of the outer medulla was scored as follows: 0, < 10%; 1, 10 – 30%; 30 – 50%; 4, > 50% of the tubuli affected. ICAM-1 expression in the cortex was scored as follows: 0, < 10%; 1, 10 – 30%; 30 – 50%; 4, > 50% of the glomeruli affected. The analysis was done without knowledge of the animal assignment.

Mixed lymphocyte reactivity (MLR)

Priming of allograft-specific T cells from kidney graft recipients was investigated by performing MLR assay based on the measurement of BrdU incorporation during DNA synthesis. The responder spleen cells obtained from naive BALB/c mice or from WT2 or uPAR−/− allograft recipients at day 6 after transplantation were treated with ammonium chloride solution (Cell Systems) to lyse erythrocytes, washed three times, and resuspended at 3 × 10^6 cells/ml in complete RPMI 1640 medium (Life Technologies) supplemented with 10% FCS (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 100-µl aliquots were delivered in triplicate to the wells of a 96-well, flat-bottom tissue culture plate. Stimulator cells were prepared from the spleens of syngeneic (i.e., BALB/c) and allograft donors (i.e., WT2, uPAR−/−). After lysis of erythrocytes the stimulator cells were treated with 50 µg/ml mitomycin C for 30 min at 37°C, washed and resuspended in culture medium at 3 × 10^6 cells/ml, and 100-µl aliquots per well were added to responder cells and cultured for 48 h. Afterward, BrdU was added and 18 h later responder cell proliferation was quantified using colorimetric Cell Proliferation ELISA kit (Roche Diagnostics GmbH) in accordance to the manufacturer’s instructions. Syngeneic stimulator cells were used as background controls and were subtracted from allosponses.

ELISpot assay

Priming of allograft-specific T cells from kidney allograft recipients was also tested by enumerating of IFN-γ-producing T cells using a mouse IFN-γ ELISpot kit (BD Biosciences) in accordance to the manufacturer’s instructions. Spleen cell suspensions were prepared from naive BALB/c mice or from allograft recipients at day 6 after transplantation and used as responder cells. Spleen cells from WT2 or syngeneic BALB/c mice were prepared and treated with mitomycin C for use as stimulator cells in the assay as described above. Responder and stimulator cells were cultured together for 24 h at 37°C in 5% CO2. The resulting spots were counted using the A.E.L VIS 4-Plate ELISpot reader with the A.E.L VIS ELISpot analysis software.

ROS generation by oxidation of dihydroethidium

The redox-sensitive fluorophore dihydroethidium (DHE) was used to evaluate O2− production in the kidney in situ (31). Frozen tissue Cryosections of 6 µM were incubated with 0.1 nM DHE dissolved in HEPES-Tyrod buffer solution (132 mM NaCl, 4 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 9.5 mM HEPES, and 5 mM glucose) for 12 min RT. After incubation, images were obtained using the Leica imaging system IM 500 (Ex, 520 nm; Em, 605 nm). Semiquantitative scoring was performed as follows: score: 0, no; 1, weak; 2; moderate; 3, high; and 4, very high intensity.

TUNEL-DAB assay

For TUNEL-assay (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) 2 µM sections of 4% paraformaldehyde (PFA)-fixed frozen tissue cryosections were performed on postfixed tissues, dehydrated through graded alcohols and xylene, embedded in paraffin, sectioned at 4 µM and dewaxed. Tissue sections were incubated with terminal deoxynucleotidyl transferase enzyme and incubated in a humidified chamber at 37°C for 1 h. After washing, the tissue was treated with FITC-labeled anti-digoxigenin, incubated for 60 min, and washed. Negative controls...
were prepared under the same conditions, with the omission of the terminal deoxynucleotidyl transferase enzyme. TUNEL pos. cell numbers were counted in 20 nonoverlapping view fields each specimen without knowledge of the animal assignment.

RNA extraction and real time quantitative PCR
Frozen kidneys were grinded in liquid nitrogen and total RNA was extracted using Trizol reagent (Invitrogen). For real-time quantitative PCR (qPCR), 1 µg of DNase-treated total RNA was reverse transcribed using Superscript II Reverse transcriptase (Invitrogen) and qPCR was performed on an SDS 7700 system (Applied Biosystems) using Rox dye (Invitrogen), Superscript II Reverse transcriptase (Invitrogen) and qPCR was performed using 1/20 of the cDNA synthesized from WT1, WT2, and uPA-deficient mouse. In contrast, uPAR deficiency led to markedly attenuated loss of renal function after IR injury (***, p < 0.001 vs WT2, 7–12 mice were investigated for each group). B, Representative specimens after TUNEL assay 24-h postischemia are shown (original magnification, X200). Because WT1 and WT2 mice demonstrated comparable results, representative data from WT2 kidney is shown. WT kidneys (a) and uPA-deficient kidneys (b) had increased numbers of apoptotic cells mainly in the tubular epithelium. In contrast, postischemic uPAR-deficient kidneys (C) showed almost no TUNEL positive cells. The results of quantification are presented as mean ± SEM in the lower panel (***, p < 0.001 vs WT2, six mice were investigated for each group).

Mouse aortic endothelial cell (MAEC) culture
Isolation of mouse aortic endothelial cells from 6- to 8-wk-old WT or uPAR−/− mice was performed as described previously (33). The cells were cultivated in medium consisting of endothelial cell growth medium 2 (Clonetics/Cambrex) and DMEM (1:1) supplemented with 20% FCS, 100 µg/ml endothelial cell growth supplement (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 0.5% nonessential amino acids, and 0.1 mg/ml heparin. MAEC at passage 3 were used for endothelial cell characterization and for all experiments. The endothelial nature of cells was confirmed by the typical cobblestone morphology of confluent monolayers, by Dil-Ac-LDL uptake and by surface expression of CD31 and CD106 analyzed by immunocytochemistry (data not shown).

MAEC immunocytochemistry
WT and uPAR−/− MAEC were seeded and cultured on glass coverslips. Serum-starved cells were fixed with 4% PFA in PBS for 20 min at RT. Nonspecific binding was blocked by 2 h incubation at RT with 3% BSA in PBS; the preparations were washed three times with PBS. Incubations with primary Ab (rat anti-mouse receptor for C5a anaphylatoxin (C5aR) clone 20/70 from Hycult Biotechnology or polyclonal rabbit anti-TNF-R1 from Santa Cruz Biotechnology) were performed for 2 h at RT. Incubations with Alexa 488-conjugated secondary Abs (Molecular Probes) were performed for 1 h. After staining, cells were embedded in Poly-Mount mounting medium (Polysciences). The fluorescence cell images were captured using a Leica TCS-SP2 AOBS confocal microscope (Leica Microsystems). All the images were taken with oil-immersed × 63 objective, NA = 1.4.

ICAM-1 cell ELISA
Cell surface expression of ICAM-1 on MAEC was measured by cell ELISA as described previously (34). In brief, MAEC were grown in 96-well plate until 80% confluent, starved for 4 h and then stimulated for 2 h with 100 ng/ml murine C5a (mC5a; Sigma-Aldrich) or with 5 ng/ml recombinant murine TNF-α (mTNFα; R&D Systems). The cell monolayers were harvested by trypsinization. Then, cell pellets were washed 2 times with PBS and incubated with goat anti-mouse ICAM-1 Ab (Santa Cruz Biotechnology) for 1 h. After washing, cells were incubated with 1/2000 diluted goat anti-rabbit IgG (Santa Cruz Biotechnology) followed by addition of
tetramethylbenzidine substrate solution (R&D Systems), stopping the reaction with 0.5 M H2SO4 and measuring the OD at 450 nm. The substrate was then washed away with deionized water, the plate allowed to dry and 0.5% trypan blue was added to stain for the number of cells/well. Excess of trypan blue was washed away and 1% SDS was added to solubilize the trypan blue stained cells. Each well was then read at 595 nm. The OD of ICAM-1 staining was divided by the OD of trypan blue staining to yield ICAM-1 expression for each well.

Peripheral blood mononuclear cell (PBMC) adhesion assay ex vivo

The adhesion of WT2 PBMC isolated by Ficoll-Paque separation to the endothelial surface of aortas obtained from WT2 or uPAR⁻/⁻ mice was determined by the counting of adherent cells fluorescently labeled with the acetyloxymethyl ester of calcein (Calbiochem). A total of 1–2 mm pieces of aortas cleaned carefully of periadventitial fat and connective tissue and opened longitudinally were placed adventitia-side down on collagen I coated 96-well plates containing 10 μl of endothelial cell basal medium 2 (Clonetics/Cambrex) supplemented with 5% FCS to allow adherence of the aortic pieces to the substratum. When the pieces were well-attached (after 4 h), 200 μl of EBM-2 medium containing 50 ng/ml mrC5a, or 50 ng/ml mrTNFα was added. These concentrations were chosen in preliminary experiments ranging from 0.5 ng/ml to 200 ng/ml for each stimulus. For inhibitory experiments the pieces were preincubated for 2 h with 100 ng/ml pertussis toxin or with 20 μg/ml neutralizing hamster anti-mouse-TNFR1 mAb. Aorta pieces incubated in medium alone served as a control. After 16 h, the aorta pieces were washed twice and 100 μl fluorescently labeled WT2 PBMC in 200 μl medium were added. The cells were allowed to adhere for 45 min at 37°C. Unbound cells were removed by washing three times. Photographs of aorta pieces were then made using the Leica imaging microscope with the digital image-processing program. The bound leukocytes were counted without knowledge of the group assignment in four different view-fields per aorta piece.

Statistical analysis

Data are shown as mean ± SEM. Normal distribution was analyzed by Klomogorov-Smirnov test and statistical significance was calculated by Student’s t test for independent groups. SPSS 12.01 software was used.

Results

uPA/uPAR deficiency in IR injury

The uPA/uPAR system is involved in a variety of signaling cascades which mediate IR injury. We first tested whether uPA or uPAR are mediators or effectors of renal IR injury. We performed
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FIGURE 4. Allograft uPAR-deficiency strongly protected kidneys from transplantation-induced apoptosis. TUNEL assay was performed in WT2 and uPAR−/− allografts at 4 (upper row) and 24 h (middle row) after transplantation. WT2 allografts (a and c) had many TUNEL-positive nuclei at 4 h (a) with further increase at 24 h (c) after transplantation. In contrast, uPAR-deficient allografts had almost no TUNEL positive cells at (b) and 24 h (d) after transplantation. (TUNEL assay, original magnification ×200). The quantification results are presented as mean ± SEM in the right-hand panel (**, p < 0.01; *** p < 0.001 vs WT2, six mice were investigated for each group). The immunohistochemistry for cleaved caspase-3 showed in WT2 allografts an intense up-regulation of cleaved caspase-3 in the tubuli of the outer stripe of the outer medulla, the area most sensitive to hypoxic damage (e). uPAR-deficient allografts in contrast showed only few cleaved caspase-3 positive tubuli (f).

bilateral renal pedical clamping in uPA−/− and uPAR−/− mice and their corresponding wild-type controls (WT1 and WT2, respectively). Both WT1 and WT2 controls and uPA−/− mice demonstrated severe loss of renal function within 24 h postischemia, as reflected in elevation of serum creatinine levels. In contrast, uPAR-deficient animals were protected from IR injury and demonstrated statistically significant attenuated loss of renal function (Fig. 1A). Because apoptosis is a hallmark of IR injury, we performed a TUNEL assay in normal kidney tissue and in kidneys 24 h after ischemia (Fig. 1B). No TUNEL-positive cells were detected in normal kidneys in any of the four groups of animals (data not shown). We detected increased numbers of TUNEL-positive nuclei in WT1, WT2 and uPA−/− kidneys. Because WT1 and WT2 mice showed almost identical results, only the data from WT2 mice are shown. However, in uPAR−/− kidneys the amount of TUNEL-positive cells was significantly reduced. These results suggest that uPAR contributes to hypoxia-induced apoptosis independently from uPA.

uPA/uPAR expression is up-regulated in rejecting kidney allografts

Because activation of uPA/uPAR system has been shown to correlate with allograft rejection in a human biopsy study (25), we tested whether or not a similar phenomenon may be observed in kidney allografts in mice. We performed allogenic kidney transplantation using WT2 mice (H2b) as donors, and BALB/c mice (H2d) as recipients. The expression of uPA and uPAR was then investigated in normal WT2 kidneys and in WT2 kidney allografts at 4 and 24 h after transplantation. We detected strong up-regulation of uPAR (Fig. 2A) mRNA 4 h after transplantation with further increase after 24 h. The up-regulation of uPAR mRNA was less pronounced but also could be observed at 24 h after transplantation (Fig. 2B). At protein level (Fig. 2C), the weak expression of uPAR seen in normal WT2 kidney was strongly up-regulated within 24 h after transplantation and further increased at day 6. As expected, no positive signal was detected in uPAR−/− allografts (data not shown). These data demonstrate that transplantation results in local activation of the uPA/uPAR system in rejecting allografts from WT mice.

uPAR but not uPA deficiency improved kidney allograft survival and attenuated loss of renal function

Further, we analyzed an influence of uPA or uPAR deficiency of the allograft on recipient survival (Fig. 3A). We performed allogenic kidney transplantation using uPA- and uPAR-deficient mice (H2b) and their corresponding WT controls as donors, and BALB/c mice (H2d) as recipients. All recipients of WT as well as uPA−/− and uPAR−/− allografts died shortly after allogenic kidney transplantation. In contrast, recipients of uPAR-deficient allografts showed superior survival for more than 20 wk after transplantation.

Next, we studied the effect of uPA/uPAR deficiency on renal function after transplantation by measuring serum creatinine levels (Fig. 3B). To monitor the effects of rejection on renal function we removed both recipient kidneys and transplanted the donor kidney in the same operation. An increase in serum creatinine was detected in all groups 24 h after transplantation; however this effect...
was strongly attenuated in mice which received uPAR−/− allografts. Six days after transplantation, recipients of uPA-deficient allografts and WT allografts developed deleterious loss of renal function as reflected in severe creatinine elevation. In contrast, the renal function of recipients of uPAR-deficient allografts remained stable without any significant increase in serum creatinine. Histological analysis revealed severe diffuse acute tubular necrosis (ATN) in WT and uPA−/− but not in uPAR−/− allografts at d1 after transplantation (Fig. 3C). These results indicate that the uPAR but not uPA of allograft origin contributes significantly to the loss of renal function and allograft rejection in this model of allogenic kidney transplantation. Because uPA deficiency had no protective effects in either the IR model or in kidney transplantation, the following experiments were performed using only uPAR-deficient animals and their respective WT2 controls.

**Apoptosis was markedly reduced in uPAR-deficient allografts**

Because hypoxia-induced apoptosis is the pathophysiological correlate for ATN, we performed a TUNEL assay to investigate the cell death rate in uPAR−/− and WT2 kidney allografts (Fig. 4). WT2 allografts ubiquitously developed TUNEL positive nuclei as early as 4 h (a) and more pronouncedly 24 h (c) after transplantation. In contrast, uPAR−/− allografts demonstrated almost no signs of apoptosis at these time points (b and d). To elucidate whether the differences in cell death between WT2 and uPAR−/− allografts were due to necrosis or to apoptosis we performed immunohistochemistry for cleaved caspase-3. Especially in the outer stripe of the outer medulla, the area which is most sensitive to hypoxic damage, and in the medium of the vessels, cleaved caspase-3 level was increased in WT2 allografts at 24 h after transplantation (data not shown) and even more so at day 6 after kidney transplantation (e). In comparison, uPAR−/− allografts (f) showed markedly lower levels of cleaved caspase-3. These results were in line with our previous finding in the IR injury model and clearly demonstrate that local uPAR expression is also pivotal in ischemia triggered apoptosis in the allogenic kidney transplantation model.

**Generation of ROS was reduced in uPAR-deficient allografts**

Hypoxia leads to tissue damage via generation of ROS contributing significantly to the mitochondrial apoptotic pathway (35). Scavengers for ROS, H2O2, and superoxide have been shown to inhibit apoptosis induced by ischemia-reperfusion (36). Therefore, we tested the hypothesis that strongly reduced apoptosis in uPAR−/− allografts might be due to decreased production of ROS. DHE staining (Fig. 5) performed in normal WT2 (a) and uPAR−/− (b) kidneys revealed similar basal levels of ROS. However, in contrast to WT allografts demonstrating severe up-regulation of ROS at 4 (c) and 24 h (e) after transplantation, ROS generation was markedly reduced in uPAR-deficient allografts (d and f). This result suggests that uPAR contributes to the initial apoptosis triggering step - the generation of ROS.

**uPAR deficiency of the allograft and inflammatory cell infiltration**

The inflammatory cell infiltration of the allograft is a hallmark of allograft rejection (37, 38). Therefore, we performed immunohistochemistry for different cell subsets to elucidate the composition of the cell infiltrates. No differences were observed between the groups in CD8 and CD4 infiltrates. Moreover, the CD25 expression on CD4 T cells was comparable in WT2 and uPAR−/− allografts (Fig. 6a). In line with this observation the MLR assay did not reveal any differences between splenocytes obtained from WT2 and uPAR−/− allograft recipients at day 6 after transplantation (Fig. 6B). The splenocytes from WT2 and uPAR−/− allograft recipients showed a similar increase of proliferative response to WT2 stimulator cells compared with splenocytes from naive BALB/c mice. The frequency of alloantigen-specific IFN-γ-producing T cells tested by ELISPOT assay was also comparable in WT2 and uPAR−/− allograft recipients (Fig. 6C). This result shows that uPAR deficiency did not decrease the level of donor-reactive T cell priming in this model.

Furthermore, we analyzed infiltration of neutrophils (Fig. 7A, upper panel) and monocytes/macrophages (Fig. 7A, middle panel). We found that uPAR deficiency of the allograft decreased the number of infiltrating neutrophils and monocytes/macrophages as compared with WT2 control allografts. These results underline the
up-regulation was even significantly higher in uPAR
MCP-1 mRNA up-regulation was also similar in WT and
MIP-2 is necessary for an adequate neutrophil infiltration (39).
Therefore, we compared the transplantation-induced changes of
ICAM-1 after stimulation of cells with TNF-
important role of local renal uPAR for monocyte/macrophage and
neutrophil infiltration.
Adhesive interaction between up-regulated adhesion molecules
on activated endothelium with blood leukocytes is a crucial step
for leukocyte infiltration into the site of inflammation. Therefore,
we analyzed the expression of ICAM-1 in WT2 and uPAR
allografts after kidney transplantation. Baseline ICAM-1 expres-
sion was comparable (data not shown). However, a strong ICAM-1 up-regulation seen in WT2 allografts after transplantation was significantly impaired in
uPAR-deficient allografts (Fig. 7A, lower panel).
Members of the chemokine family play a central role in inflam-
atory cell infiltration into extravascular sites by attracting and
stimulating specific subsets of leukocytes (39). MCP-1 is an
important mediator for monocyte recruitment (40) whereas
MIP-2 is necessary for an adequate neutrophil infiltration (39).
Therefore, we compared the transplantation-induced changes of
MCP-1 and MIP-2 mRNA expression in WT2 and uPAR
allografts. As expected, we observed a strong up-regulation of
these proinflammatory mediators in WT2 allografts. Surpris-
ingly, uPAR
allografts demonstrated a practically identical pattern of the transient overexpression of MIP-2 mRNA, with
a maximum at 24 h after transplantation. The kinetics of
MCP-1 mRNA up-regulation was also similar in WT and
uPAR
allografts, and moreover, the grade of MCP-1 mRNA
up-regulation was even significantly higher in uPAR
kidney (Fig. 7B). These results suggest that attenuated leukocyte infiltration into uPAR-deficient allografts may be due to impaired up-regulation of ICAM-1 but not to altered chemokine expression.

FIGURE 7. uPAR-deficiency of the allograft protects from host leukocyte infiltration after transplantation. A. At day six after transplantation, WT2 allografts (a) showed more tubulo-interstitial infiltrates of Gr-1 positive neutrophils as compared with uPAR-deficient allografts (b). Dense infiltration of F4/80 positive monocytes/macrophages seen in the tubulo-interstitium of WT2 allografts (c) was strongly reduced in uPAR-deficient allografts (d). ICAM-1 expression was studied by immunohistochemistry. Twenty-four hours after transplantation ICAM-1 was heavily up-regulated in the glomeruli of WT2 allografts (e) whereas uPAR
allografts (f) showed almost no ICAM-1 up-regulation. The results of the semiquantification analysis are presented as mean ± SEM in the right-hand panel (**, p < 0.01; ***, p < 0.001 vs WT2, original magnification ×200, six mice were investigated for each group).
B. The expression of MCP-1 (upper panel) and MIP-2 (lower panel) mRNA was analyzed by TaqMan RT-PCR in normal kidneys (control) and in WT2 and uPAR
allografts obtained at 24 h and day 6 after transplantation. Data are expressed as the mean ± SEM (**, p < 0.01 vs WT2, six mice were investigated for each group).

Members of the chemokine family play a central role in inflam-
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stimulating specific subsets of leukocytes (39). MCP-1 is an
important mediator for monocyte recruitment (40) whereas
MIP-2 is necessary for an adequate neutrophil infiltration (39).
Therefore, we compared the transplantation-induced changes of
MCP-1 and MIP-2 mRNA expression in WT2 and uPAR
allografts. As expected, we observed a strong up-regulation of
these proinflammatory mediators in WT2 allografts. Surpris-
ingly, uPAR
allografts demonstrated a practically identical pattern of the transient overexpression of MIP-2 mRNA, with
a maximum at 24 h after transplantation. The kinetics of
MCP-1 mRNA up-regulation was also similar in WT and
uPAR
allografts, and moreover, the grade of MCP-1 mRNA
up-regulation was even significantly higher in uPAR
kidney (Fig. 7B). These results suggest that attenuated leukocyte infiltration into uPAR-deficient allografts may be due to impaired up-regulation of ICAM-1 but not to altered chemokine expression.

uPAR deficiency of blood vessel decreases TNFα/C5a-induced ICAM-1 up-regulation and reduces WT leukocyte adhesion in vitro

Recently, the interaction of complement activation product C5a with its receptor (C5aR) has been shown to induce a strong in-
crease in gene expression for cell adhesion molecules in endothelial cells similar to those induced by TNF-α (41). Because both
TNF-α and activated complement are important mediators of IR
injury and transplant rejection (42, 43), we hypothesized that
TNF-α and/or C5a-dependent up-regulation of endothelial adhe-
sion molecules may be impaired in uPAR
allografts and may be an explanation for the reduced monocyte/macrophage and gran-
ylocyte infiltration. To test this hypothesis, we performed addi-
tional in vitro experiments with the primary culture of MAEC from
WT2 and uPAR
mice. After demonstration of similar cell surface
expression of both TNFR1 and C5aR on WT2 and uPAR
MAEC by immunocytochemistry (Fig. 8A) we investigated the
expression of ICAM-1 after stimulation of cells with TNF-α and
C5a for 16 h by cell ELISA. The treatment with 5 ng/ml mrTNF
resulted in a strong up-regulation of ICAM-1 expression in both
WT2 and uPAR
MAEC, however, this effect was significantly
decreased in uPAR
MAEC compared with WT2. The pretreat-
ment with TNFR1 blocking Ab strongly decreased the TNF-α-
duced up-regulation of ICAM-1 suggesting the involvement of
TNFR1 (Fig. 8B). The treatment of MAEC with 100 ng/ml mrC5a
resulted in a moderate but significant up-regulation of ICAM-1 in
WT2 MAEC; however, this effect was completely abrogated in
uPAR
MAEC. The pretreatment with the C5aR blocking per-
tussis toxin prevented the C5a-induced up-regulation of ICAM-1 in
WT2 MAEC verifying the role of C5aR (Fig. 8C).
These results were confirmed by a PBMC adhesion assay ex vivo. In these experiments, adhesion of normal WT2 PBMC to the endothelial surface of aortas obtained from WT2 or uPAR/−/− mice and stimulated in vitro with mrC5a or mrTNFα was investigated. As demonstrated in Fig. 8D, 16-h stimulation of WT2 aorta with TNF-α or C5a induced significant increase in adhesion of WT2 PBMC. These effects could be strongly reduced or completely abolished when aortas were pretreated with TNFR1 blocking Ab and pertussis toxin, respectively. In contrast, the TNF-α and C5a-dependent up-regulation of endothelial adhesion was completely abolished in uPAR/−/− aortas. These results clearly demonstrate that uPAR expression on endothelial cells is necessary for adequate TNF-α and C5a signaling leading to up-regulation of adhesion molecules and mediating leukocyte adhesion.

Discussion

Our data provide the first evidence that local renal uPAR but not uPA expression plays a pivotal role in the pathogenesis of IR injury and allogenic transplant rejection. We ruled out that uPAR deficiency protected kidney tissue from generation of ROS and consecutively from severe apoptosis in IR injury and acute kidney allograft rejection. In the transplantation model, the resistance of the allograft against hypoxia-induced apoptosis due to uPAR deficiency was linked to better renal function and increased allograft survival. The inadequate up-regulation of ICAM-1 in the blood vessels of uPAR/−/− allografts resulted in attenuated monocyte/macrophage and neutrophil infiltration.

Allogenic transplant rejection is triggered by several stimuli such as allograft hypoxia and subsequent apoptosis and inflammatory response. Tubular cell apoptosis is considered to be an important pathway leading to tubular atrophy in progressive renal disease. Recently, it has been shown that uPAR may modify the rate of apoptotic renal cell death (44). In vitro, human glioma cells exposed to uPAR antisense have been reported to undergo more apoptotic cell death (45). In contrast to these observations, the major finding of this study was that uPAR deficiency strongly protected renal cells from apoptosis via reduced ROS formation in both models, IR injury and kidney transplantation. One possible explanation for this discrepancy might be that uPAR exposes pro- or anti-apoptotic action and modulates the cell survival/apoptosis ratio depending on the cell type and/or the apoptosis-triggering pathways. This hypothesis is supported, for example, by demonstration of the
anti-apoptotic and uPA-independent action of uPAR in endothelial cells in which anti-uPAR Abs as well as soluble recombinant uPAR blocked the apoptotic effect of cleaved high molecular mass kininogen by preventing the binding of high molecular mass kininogen to these cells (46).

Recently, it has been shown that uPA stimulates ROS production in VSMC in vitro (47). Because hypoxia triggers ROS generation and mediates apoptosis, we investigated the possible role of uPA/uPAR interaction in ROS generation and subsequent ROS-dependent apoptosis using both uPA- and uPAR-deficient mice. Our results clearly demonstrate that in both models uPAR deficiency protected renal tissue from ROS generation independently from uPA. The molecular mechanisms underlying the attenuated ROS formation in uPAR−/− allografts remain to be investigated. uPAR can interact laterally with a wide variety of membrane proteins including integrins, endocytic receptors, caveolin, the gp130 cytokine receptor, the epidermal growth factor receptor, chemokine receptors (1) and platelet-derived growth factor receptor (2). This interaction might activate both pro- and anti-apoptotic downstream signaling pathways. For example, recently ROS-triggered apoptosis in polymorphonuclear leukocytes (PMNs) cells has been shown to be β2 integrin-dependent (48). Therefore, uPAR may stimulate ROS production via its lateral interaction with other molecules.

The early inflammatory response during reperfusion of allografts is initiated by the infiltration of PMNs into the graft (38) followed by infiltration of monocytes/macrophages (MO). Macrophages constitute 38 to 60% of infiltrating cells during acute allograft rejection and increased influx of MO has been strongly correlated to complement activation and acute rejection in a human protocol biopsy study (49). Furthermore, it has been shown that MO infiltration 3 mo after transplantation correlated inversely with graft survival (50). It is well known that uPAR deficiency on the surface of leukocytes strongly reduces their migratory capacity both in vivo and in vitro (15, 51, 52). However, it should be noted that in our transplant experiments, the host leukocytes expressed uPAR normally. Despite the normal expression of uPAR on host leukocytes, we found strongly reduced PMN/MO infiltration in kidney allografts from uPAR−/− mice as compared with those from WT2 mice. This finding stresses the importance of the local uPAR expression on resident renal cells in the allograft for adequate cell adhesion and subsequent PMN/MO accumulation after kidney transplantation, independently from the uPAR expression on infiltrating cells. Recently it has been shown that inhibition of PMN infiltration into cardiac allografts may have a significant downstream impact on the efficacy of recipient T cell responses to the allograft (38). In contrast, in our model of acute renal allograft rejection we did not find any differences in CD4 and CD8 positive cell infiltration between WT2 and uPAR−/− allografts after transplantation. Moreover, the expression of T cell activation Ag CD25 used as a marker for T cell activation (53) was also similar in WT2 and uPAR−/− allografts. Furthermore, by investigating of priming of alloreactive T cells by MLR and IFN-γ ELISPOT assays we did not found differences of T cell function of WT2 and uPAR−/− allografts recipients. These results suggest that superior survival of uPAR−/− deficient allografts is not mediated by T cell-dependent alloimmune response.

The migration of inflammatory cells into an extravascular site requires a series of coordinated signals including the generation of a chemotactic gradient by the cells of the extravascular compartment and up-regulation of adhesion molecules on activated endothelium. The strong up-regulation of MCP-1 and MIP-2 has been demonstrated in animal models during renal ischemia as well as in renal biopsies from patients with acute and chronic renal rejection (54). In line with this report, we observed a strong up-regulation of these proinflammatory mediators which was similar in WT2 and uPAR−/− allografts. Therefore, it could be concluded that the level of these chemotactants was not responsible for the diminished PMN/MO infiltration in uPAR−/− allografts.

Adhesion molecules are rapidly up-regulated early after transplantation (55) and after induction of IR injury (56). Therefore, we analyzed expression of ICAM-1 in WT2 and uPAR−/− allografts given the facts that, being the major counterreceptor for leukocyte β2-integrins, this adhesion molecule is rapidly up-regulated within the first 2–3 h after transplantation (57) and the blockade of ICAM-1 strongly attenuated PMN infiltration in some experimental models of renal ischemia (58–60). Indeed, we could demonstrate that the transplantation-induced up-regulation of ICAM-1 observed in WT2 allografts was strongly attenuated in uPAR−/− allografts. However, the reduced expression of ICAM-1 in uPAR−/− allografts was not sufficient to prevent T cell infiltration. This result coincides with observation of Zhang and coworkers (61) demonstrating that primed alloreactive T cells do not require allograft expression of ICAM-1 to infiltrate heart allografts. The interaction of integrin leukocyte function Ag-1 with its alternative ligand ICAM-2, or interaction of other T cell homing receptors with their ligands on endothelium, such as CD44/Selectin interaction, may be involved in lymphocyte adhesion to the vascular endothelium of the graft and its subsequent infiltration bypassing the requirement for ICAM-1 (62).

By performing in vitro experiments with primary aortic endothelial cells we could elucidate impaired TNF-α and C5a signaling in uPAR−/− cells leading to strongly decreased TNF-α-induced and completely abrogated C5a-induced up-regulation of ICAM-1. In line with a prior report that only the TNFR1 receptor is involved in TNF-α-induced ICAM-1 up-regulation (63) we could demonstrate that the up-regulation of ICAM-1 in MAEC after TNF-α stimulation was mediated predominantly via TNFR1 because the neutralizing anti-mouse TNFR1 Ab almost completely blocked this effect. These results coincide with strongly reduced adhesion of WT2 PBMC to isolated aortas from uPAR−/− mice compared with WT2 aortas after stimulation with TNF-α and C5a. This result is in line with a prior report that platelet uPAR is critical for the response to TNF-α (64) and coincides also with our previous observation that uPAR is necessary for C5a/C5aR-mediated responses in mouse alveolar macrophages (15) and in human mesangial cells (14). Collectively, these data suggest that uPAR plays a major role in mediating IR injury and subsequently influences early inflammation and allograft survival in renal transplantation rather than directly affecting T cell mediated alloimmune responses.

In summary, this study shows that the local expression of uPAR on resident renal cells of the allograft contributes to the development of acute allograft rejection via at least two different pathways, ischemia-induced apoptosis and the infiltration of host leukocytes. These results suggest that uPAR, whose role was presumed to be involved in the migratory behavior of infiltrating cells, has a broader critical function as an early regulator of ischemia-triggered initial generation of ROS, which, in turn, induces the apoptosis in intrinsic renal cells. uPAR is also necessary for adequate TNF-α and C5a signaling, leading to up-regulation of ICAM-1 on endothelial cells of the allograft which is a crucial step for adequate leukocyte recruitment to the inflamed tissue. These observations underscore a new role of uPAR in acute allograft rejection and highlights uPAR as a target for prevention of organ dysfunction and damage in IR injury.


