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J. Zwirner, who produced the 20/70-antibody is his laboratory, is retracting his authorship from this paper. The reasons for the retraction are (1) he was not informed about the manuscript before it was published and (2) he could not observe, under the experimental conditions used in his laboratory, the immunoreactivity described in Fig. 10.

All authors accept this decision, which is the result of an agreement mediated by the Ombudsman of the German Research Council.

Urokinase-induced activation of the gp130/Tyk2/Stat3 pathway mediates a pro-inflammatory effect in human mesangial cells via expression of the anaphylatoxin C5a receptor

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Summary

Glomerular mesangial cells (MCs) are central to the pathogenesis of progressive glomeruli-associated renal diseases. However, molecular mechanisms underlying changes in MC functions still remain poorly understood. Here, we show that in MCs, the urokinase-type plasminogen activator (uPA) induces, via its specific receptor (uPAR, CD87), upregulated expression of the complement anaphylatoxin C5a receptor (C5aR, CD88), and modulates C5a-dependent functional responses. This effect is mediated via the interaction of the uPA-specific receptor (uPAR, CD87) and gp130, a signal transducing subunit of the receptor complexes for the IL-6 cytokine family. The Janus kinase Tyk2 and the transcription factor Stat3 serve as downstream components in the signaling cascade resulting in upregulation of C5aR expression. In vivo, expression of C5aR and uPAR was increased in the mesangium of wild-type mice in a lipopolysaccharide (LPS)-induced model of inflammation, whereas in *uPAR*$^{-/-}$ animals C5aR expression remained unchanged. This is the first demonstration in vitro and in vivo that uPA acts in MCs as a modulator of immune responses via control of immune-competent receptors. The data suggest a novel role for uPA/uPAR in glomeruli-associated renal failure via a signaling cross-talk between the fibrinolytic and immune systems.

Key words: Urokinase, uPA receptor, C5a receptor, Mesangial cells, Inflammation

Introduction

The early stages of inflammatory processes are accompanied by activation of the complement system. One of the biological consequences of this activation is the release of potent inflammatory molecules, C3a and C5a anaphylatoxins. Anaphylatoxins act through specific receptors that are members of the rhodopsin family of seven transmembrane-spanning G protein-linked receptors (Gerard and Gerard, 1994). Expression of these receptors, initially thought to be restricted to peripheral blood leukocytes, appears to occur in several tissues. Recent reports provide evidence for the expression of anaphylatoxin C5a receptor (C5aR, CD88) on human mesangial cells (MCs), which play a pivotal role in renal physiology (Braun and Davis III, 1998; Wilmer et al., 1998). Moreover, C5aR activation in MCs induced proliferation, selective production of cytokines and growth factors, as well as upregulation of certain transcription factors and early response genes (Wilmer et al., 1998). All these parameters might determine the degree of mesangial injury, which in turn dictates the final outcome of the inflammatory process. These data indicate a role for the MC-expressed C5aR in mediating glomerular injury and pathogenesis of progressive glomeruli-associated renal diseases. This implication is strengthened by the demonstration of enhanced expression of C5aR in human diseased kidney (Abe et al., 2001) and by the recent studies on C5aR-mediated renal dysfunction (de Vries et al., 2003; Aramugam et al., 2003). However, molecular mechanisms of MC stimulation and upregulated C5aR expression in MCs remain unexplored. Most probably important candidates might be complement proteins, platelet products and components of the fibrinolytic system, in particular the urokinase-type plasminogen activator (uPA) and its specific receptor (uPAR, CD87).

uPA is a multifunctional molecule that serves either as a proteolytic enzyme or as a signal-inducing ligand. The urokinase receptor uPAR was originally identified as a proteinase receptor for uPA, directing pericellular proteolysis. However, uPAR also mediates intracellular signaling via surface proteins such as integrins, growth factors receptors and G-protein-coupled membrane proteins. These dual properties enable the uPA/uPAR system to control pericellular fibrinolytic and proteolytic activities, as well as cell adhesion, migration, proliferation and differentiation (Blasi and Carmeliet, 2002).

Moreover, recent findings in vitro and in vivo indicate that the uPA/uPAR system is an active participant in the majority of infection and inflammatory diseases and might serve as a modulator of immunological responses (Gyetko et al., 1996; Gyetko et al., 2000; May et al., 1998; Mondino and Blasi, 2004). Remarkably, modulating effects of the uPA/uPAR system on immunological responses may involve not only migration of different types of leukocytes to the site of inflammation but also contribute to generating pro- and anti-inflammatory signals, such as TNF-α neo-synthesis of lipopolysaccharide (LPS)-stimulated mononuclear phagocytes in vitro (Sitrin et al., 1996) and IFN-γ and IL-12 in vivo in response to pulmonary infection (Gyetko et al., 2002). Moreover, in the murine model of endotoxemia-induced lung injury, uPA increases LPS-induced activation of neutrophils via activation of a uPAR-directed intracellular signaling pathways including Akt and c-Jun N-terminal kinase, nuclear translocation of NF-κB and enhanced expression of IL-1β, TNF-α and MIP-2 (Abraham et al., 2003). These data suggest the interplay of the uPA/uPAR system and transcription factors that may represent an important cell-specific mechanism that upregulates the inflammatory response and facilitates MC proliferation upon progressive glomerular diseases.

In this study we show that through Tyk2/Stat3 activation, uPAR occupancy by uPA and its association with gp130 protein, a signal transducing subunit of the IL-6 receptor complexes, upregulates expression of anaphylatoxin C5aR on human MCs and modulates C5aR-dependent functional cell responses. These findings identify components of a novel pathway that couples fibrinolytic and immune systems in kidney inflammatory diseases.

Materials and Methods

Materials

Chemicals of high quality commercial grade were purchased from Sigma, Amersham Bioscience Inc., Merck, Serva (Heidelberg, Germany), Carl Roth GmbH (Karlsruhe, Germany) and Bio-Rad Laboratories (Hercules, CA, USA). Chemiluminescent signal enhancer was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). Mounting medium was purchased from Polysciences, Inc. (Warrington, PA, USA). uPA was from Loxo (Dossenheim, Germany).

Antibodies

Anti-human CD88, anti-human gp130 and horseradish peroxidase-conjugated secondary polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-STAT3 and anti-Tyk2 monoclonal antibodies were from Transduction Laboratories (Lexington, KY, USA), anti-phospho-STAT3 (Tyr705) and anti-phospho-Tyk2 (Tyr1054/1055) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA, USA). Monoclonal anti-human uPAR clone R3 antibody was from Monozyme (Copenhagen, Denmark). Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated donkey anti-goat antibodies were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Normal rabbit IgG and normal mouse IgG were from Upstate Biotechnology, Inc. (Lake Plasid, NY, USA). Monoclonal anti-mouse C5aR antibodies (clone 2/70, rat anti-mouse IgG) were generated as described previously (Werfel et al., 1996).

Cell culture

Normal human mesangial cells were obtained from Clonetics (San Diego, CA, USA). The cells were cultured in MsGM medium (Clonetics) supplemented with 5% fetal bovine serum (Clonetics) and were used in passage 8. For the uPA stimulation experiments, cells were starved for 24 hours in serum-free medium. For inhibition experiments cells were pretreated before uPA stimulation for 1 hour with the appropriate antibody at 5 μg/ml medium.

Cell infection, Tyk2 and Stat3 expression

Adenoviral Tyk2 constructs were generated as previously described (Kusch et al., 2000). Additionally, the deletion mutant Ad5Tyk2ΔC was generated by removing the kinase domain after amino acid 776. As a control, an adenovirus expressing β-galactosidase (Ad5βGal) was used. Adenoviral Stat3F construct with a point mutation in the tyrosine phosphorylation site at residue 705 (Tyr to Phe) was generated as previously described (Yahata et al., 2003). Cells were grown up to 80% confluency and infected for 1 hour with recombinant adenovirus stock at a multiplicity of infection of 500 plaque-forming units/cell in a total volume of 1 ml/well in six-well plates. The efficiency of infection was assessed by western blotting using anti-Tyk2 or anti-Stat3 antibody, respectively. Cells were serum-starved overnight after 24 hours of infection and used for experiments on the second day after infection. Total RNA was prepared using an RNeasy Kit (QIAGEN GmbH, Germany).

Design and cloning of lentiviral siRNA vectors

The human *uPAR* cDNA sequence (NM 002659) was searched for suitable siRNA target sequences starting with aag followed by 18 nucleotides. The 21 nucleotides sense and antisense sequences were subjected to BLAST searches, eliminating sequences with more than 16 bp homologies in the human genome. uPAR siRNA oligonucleotides (uPARsi): sense, 5′-GATCCCC**AAGCTGTACCC-ACTCAGAG**TTCAAGAGA**CTCTGAGTGGGTACAGCTT**TTTT-TGGAAA; antisense, 5′-AGCTTTTCCAAAA**AAGCTGTACC-CACTCAGAG**TCTCTTGAA**CTCTGAGTGGGTACAGCTT**GGG. Sense and antisense siRNA sequences are shown in bold and loop sequences are underlined. Hybridized oligonucleotides were ligated into the pSuper.retro vector (purchased from Oligo Engine, Seattle, WA, USA), into the *Bgl*II-*Hin*dIII-site, downstream of the H1 promoter. The H1-hairpin-precursor cassette was excised from pSuper.retro with *Eco*RI and *Cla*I (Fermentas GmbH, Germany) and further cloned into *Eco*RI-*Cla*I site of the pLV-TH plasmid.

Lentiviral vector production and cell infection

Lentiviral vectors were produced by transient transfection of 293T cells according to standard protocols. Briefly, 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and, when subconfluent, transfected with 15 μg of pCMV-ΔR8.91, 6 μg of pMD2G and 20 μg of pLV-TH-uPARsi, or pLV-TH as control. The transfection was performed by calcium-phosphate precipitation. Medium was changed to DMEM, supplemented with 2% FCS, after 6 to 8 hours. Vector supernatants, containing viral particles, were harvested approximately 48 hours later and concentrated by ultracentrifugation (1.5 hours at 25,000 ***g*** at 4°C). The virus obtained was assigned as LV-uPARsi. The viral batches were titered on 293 cells. MCs were infected in the presence of 8 μg/ml polybrene with viruses at 10^8 TU/ml and used for experiments day 3 after infection. 95% of cells have been shown to be infected under these conditions.

Quantitative RT-PCR analysis of C5aR in human MCs

The total RNA was isolated from human MCs and real-time quantitative RT-PCR for C5aR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA,

USA). *GAPDH* was used as a reference gene. The following oligonucleotide primers and probes were used: GAPDH, 5′-GAAGGTGAAGGTCGGAGTC-3′ (sense), 5′-GAAGATGGTGATGGGATTC-3′ (antisense), 6-FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA (probe); C5aR, 5′-GTGGTCCGGGAGGAGTACTTT-3′ (sense), 5′-GCCGTTTGTCGTGGCTGTA-3′ (antisense), 6-FAM-CACCAAAGGTGTTGTGTGGCGTGG-TAMRA (probe).

Immunoprecipitation and western blotting

Subconfluent, serum-starved MCs were treated with 10 nM uPA for 5-30 minutes at 37°C, lysed in RIPA buffer containing 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin. The cell lysates were clarified by centrifugation and 1.3-1.7 mg of total protein was incubated overnight at 4°C with appropriate antibodies and protein A/G PLUS-agarose. The immunoprecipitates were subjected to 7.5% SDS-PAGE, and proteins were transferred to PVDF western blotting membrane (Roche Diagnostics GmbH, Mannheim, Germany). Membranes were probed with appropriate antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The immune complexes were visualized by an enhanced chemiluminescence detection system.

FACS analysis

MCs unstimulated or stimulated with 20 nM uPA for 20 hours were detached from culture plates with 10 mM EDTA, washed with ice-cold PBS, and stained with a murine monoclonal FITC-conjugated anti-C5a receptor antibody that recognized the extracellular peptide corresponding to residues 1-31 (clone W17/1; Serotec Ltd, Oxford, UK), or a FITC-conjugated isotype matched control antibody (Serotec Ltd, Oxford, UK). FACS analysis was performed with a FACScan instrument (Becton-Dickinson).

Immunofluorescence microscopy

Phospho-Stat3 was detected using polyclonal anti-phospho-Stat3 (Tyr705) antibody (Cell Signaling Technology (Beverly, MA, dilution 1:1000) and Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, Inc., dilution 1:150). For C5aR immunofluorescent staining, cells were incubated for 2 hours at room temperature with polyclonal anti-human C5aR antibody (Santa Cruz Biotechnology, dilution 1:40) and Alexa Fluor 488-conjugated donkey anti-goat secondary antibody (Molecular Probes, Inc., dilution 1:150). Confocal microscopy studies were performed as previously described (Dumler et al., 1998).

Animals

Male *uPAR*$^{-/-}$ mice on a mixed C57BL/6J (75%) × 129 (25%) background and their wild-type littermate controls were kindly provided by P. Carmeliet and M. Dewerchin (Leuven, Belgium) and were further bred under the same pathogen-free conditions in the animal facility of Hanover Medical School. All mice, weighing 25-30 g, were used at 8-12 weeks of age. Experiments were conducted in accordance to the regulations of the local authorities.

LPS-induced nephritis

Mice were injected intraperitoneally with either 50 μg LPS (2.0 mg/kg; *Escherichia coli* serotype O111:B4; Sigma Chemical Co, St Louis, MO, USA) in 200 μl of saline or with saline vehicle alone. At 8 hours after injection, the mice were killed and immediately perfused for 10 minutes through the left ventricle into the opened vena cava at an approximate rate of 1 ml/minute of cold PBS. Perfusion quality was optically controlled by observing the organ color change. Large perfusion volumes were used to remove contaminating C5aR-expressing leukocytes from the blood vessels. The kidneys were then harvested, sectioned, the medulla was removed and pieces of kidney cortex containing predominantly glomeruli were snap frozen for RT-PCR experiments and immunohistochemical staining.

Quantitative RT-PCR analysis of uPA, uPAR, C5aR and MCP-1 expression in mouse kidney cortex

Total RNA was isolated from kidney cortex using the RNeasy Mini Kit (QIAGEN GmbH, Germany) and real-time quantitative RT-PCR for uPA, uPAR, C5aR, and MCP-1 serving as a marker of LPS-induced inflammation was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). β-tubulin was used as a reference gene. The following oligonucleotide primers and probes were used: β-tubulin, 5′-CACCATGAGCGGCGTCA-3′ (sense), 5′-TTCGAAGGTCAGCATTAAGCTG-3′ (antisense), 6-FAM-ACCTGCCTCCGTTTCCCGGG-TAMRA (probe); MCP-1, 5′-CCAACTCTCACTGAAGCCAGC-3′ (sense), 5′-CAGGCCCAGAAGCATGACA-3′ (antisense) 6-FAM-CTCTCTTCCTCCACCACCATGCAGGT-TAMRA (probe); uPA, 5′-CGATTCTGGAGGACCGCTTA-3′ (sense), 5′-CCAGCTCACAATCCCACTCA-3′ (antisense), 6-FAM-CTGTAACATCGAAGGCCGCCCAACT-TAMRA (probe); uPAR, 5′-CCACAGCGAAAAGACCAACA-3′ (sense), 5′-CGGTCTCTGTCAGGCTGATGA-3′ (antisense), 6-FAM-ATGAGTTACCGCATGGGCTCCA-TAMRA (probe); C5aR, 5′-TGTGGGTGACAGCCTTCGA-3′ (sense), 5′-CCGCCAGATTCAGAAACCAG-3′ (antisense), 6-FAM-CCAGACGGGCCGTCAAACGC-TAMRA (probe).

Immunohistochemical studies

The cryosections (6 μm) were fixed with ice-cold acetone and air-dried. Non-specific binding sites were blocked with 10% normal donkey serum (Jackson Immuno Research Lab, West Grove, USA) for 30 minutes. Then sections were incubated with the monoclonal rat anti-mouse C5aR antibody for 1 hour. For fluorescent visualization of bound primary antibodies, sections were further incubated with Cy3-conjugated secondary donkey anti-rat antibody (Jackson Immune Research Lab, West Grove, USA) for 1 hour. Specimens were analyzed using a Zeiss Axioplan-2 imaging microscope with the digital image-processing program AxioVision 3.0 (Zeiss, Jena, Germany). C5aR expression in the glomeruli was evaluated in a blind fashion in arbitrary units (0-5+) based on the staining intensity and positivity of the mesangium using the following criteria: 5+: >90% positive glomeruli with strong immunoreactivity; 4+: >75% positive glomeruli with strong immunoreactivity; 3+: >75% positive glomeruli with weak immunoreactivity; 2+: >25% positive glomeruli with weak immunoreactivity; 1+: >10% positive glomeruli with weak immunoreactivity. Fifteen different cortical areas of each kidney (n=6 for each group) were analyzed.

Statistical analysis

All values are expressed as the mean ± s.e.m. To analyze differences in mean values the two-sided unpaired Student's t-test was used; $P<0.05$ was considered significant, and $P<0.01$ was considered highly significant.

Results

uPA upregulates expression of C5aR in MCs

We began by examining the ability of uPA to regulate expression of C5aR in normal human mesangial cells. The changes in C5aR expression were monitored at the mRNA and protein levels using TaqMan analysis and immunoblotting, respectively. Treatment of MCs with physiological

concentrations of uPA resulted in dramatic increase (up to sevenfold) in C5aR mRNA. This activation was time-dependent and reversible, starting as early as 4 hours, peaking at 16 hours, and returned to basal level at 48 hours (Fig. 1A). Consistent with these data, C5aR protein expression was also upregulated, as shown by immunoblotting using specific anti-C5aR antibody (Fig. 1B). Additionally, we performed FACS analysis and immunocytochemical studies to visualize and quantify C5aR on the cell surface. Indeed, treatment of MCs with uPA led to substantial expression of C5aR on the surface of stimulated cells (Fig. 1C,D). To verify whether uPA exerts its effect on C5aR activation via uPAR, specific uPAR-blocking antibody was used for cell pretreatment. Neither C5aR mRNA nor C5aR protein was upregulated in response to uPA after uPAR blockage (Fig. 2). These data suggest a requirement for uPAR in the chain of signaling events mediating uPA-induced C5aR expression.

uPA potentiates C5aR-related responses in MCs

Since C5aR activation in MCs was shown to induce proliferation and selective production of cytokines and growth factors, we tested whether these functional C5aR-directed responses might be affected by uPA. MCs were stimulated with uPA and C5a separately and simultaneously, and then cell proliferation and production of MCP-1 were analyzed (Fig. 3). In both cases stimuli worked synergistically when added together. These data provide evidence that uPA functions as a modulator of the C5aR-dependent processes in human MCs. To verify the role of uPAR in the observed effects, MCs were pretreated with uPAR blocking antibody. This antibody, in contrast to non-relevant IgG, abolished synergistic effects of uPA and C5a for both MC proliferation and MCP-1 release (Fig. 3).

uPA induces activation of Stat3 and Tyk2 in MCs

Next, we investigated the molecular mechanisms underlying the revealed uPA-induced expression of C5aR. As no data on signaling pathways mediating C5aR expression are available, we asked whether the signal transducer and activator of transcription Stat3 might be one likely candidate. This transcription factor was recently shown to regulate expression of another G-protein-coupled receptor (Senga et al., 2003). To test this hypothesis, we first checked whether uPA could activate Stat3 in MCs. Indeed, stimulation with uPA caused a significant increase in Stat3 tyrosine phosphorylation, as shown in immunoblotting experiments using an antibody that specifically recognized Stat3 phosphorylation on Tyr 705 (Fig. 4A). Stat3 activation was time-dependent and reversible, peaking after 20 minutes of uPA stimulation. This activation was specific for Stat3, as no changes in phosphorylation state of other Stat proteins, namely Stat1, Stat2, Stat4 and Stat5 were observed (data not shown). To determine whether uPA-induced activation of Stat3 results in its translocation to the cell nucleus as required for the functional effect

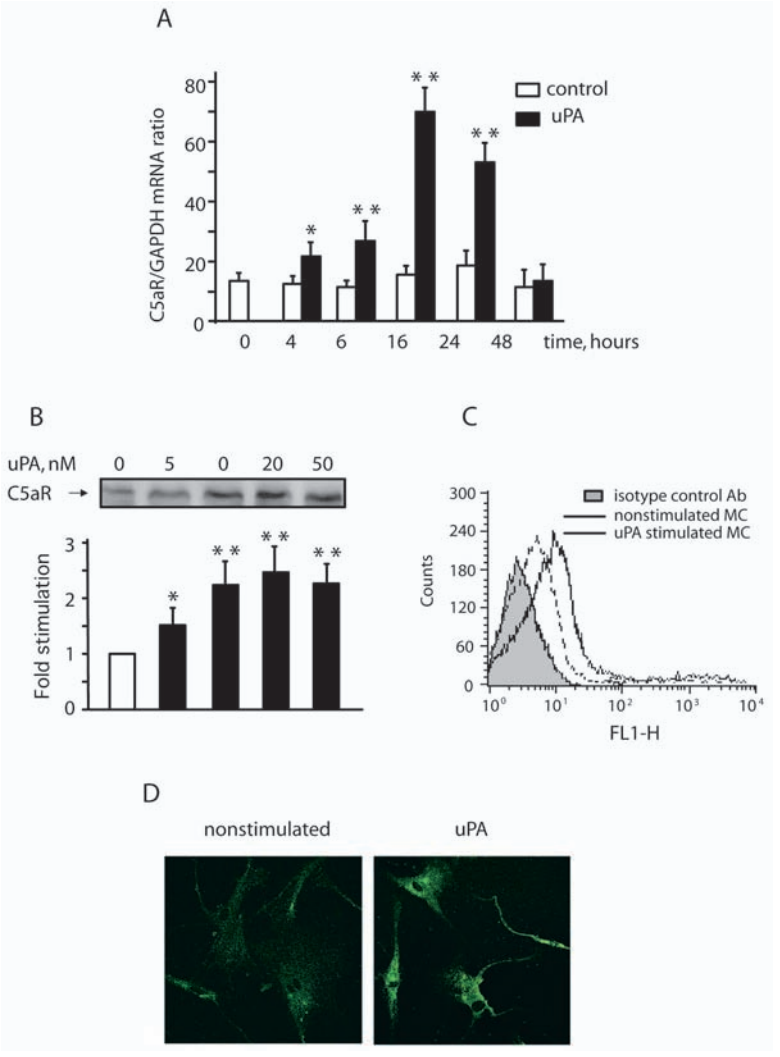

Fig. 1. uPA upregulates expression of C5aR in MCs. (A) RT-PCR analysis for C5aR mRNA was performed using the TaqMan method. RNA was isolated from quiescent MCs incubated for the indicated times with 20 nM uPA or in medium without uPA (control). *GAPDH* served as a house keeping gene. The results are presented as mean ± s.e.m. of four separate and independent experiments. (B) The quiescent MCs were stimulated for 20 hours at 37°C with the indicated concentrations of uPA, and C5aR protein was visualized in the membrane fractions by immunoblotting with anti-C5aR antibodies. MCs incubated in medium without uPA served as a control. The data on the upper panel are representative of four separate and independent experiments. Quantification of the results of these experiments by densitometry presented as mean ± s.e.m. is shown in the lower panel. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (*P<0.05; **P<0.01). (C) FACS analysis of MCs stimulated (solid line) or not (dashed line) for 20 hours at 37°C with 20 nM uPA using a FITS-labeled murine monoclonal anti-C5aR antibody. FITS-labeled isotype-matched antibody was used as a negative control (grey area). The results are representative of two separate and independent experiments. (D) The quiescent MCs were stimulated for 20 hours with 20 nM uPA or in medium without uPA (control). The cells were then fixed and stained with primary anti-C5aR antibodies and Alexa Fluor 488-conjugated secondary antibodies. Results are representative of three separate experiments.

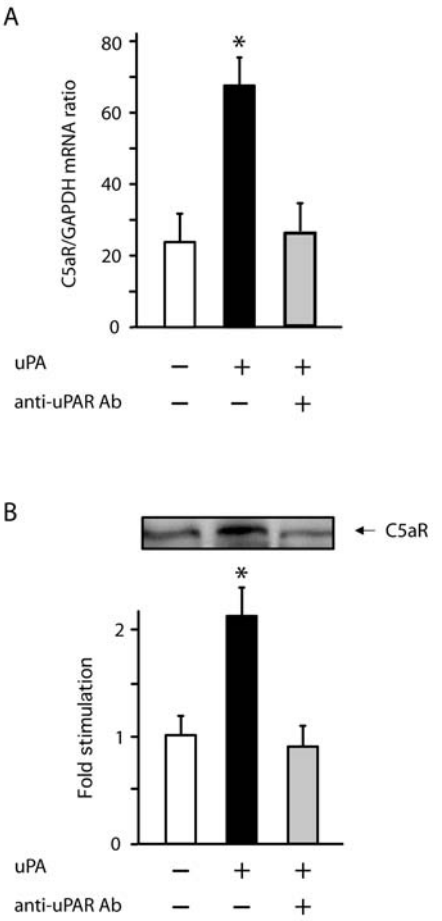

Fig. 2. uPAR mediates uPA-dependent upregulation of C5aR expression. The quiescent MCs were stimulated for 20 hours with 20 nM uPA in the presence or absence of 5 μg/ml anti-uPAR monoclonal antibody, and C5aR expression was investigated at the mRNA (A) and protein (B) levels using RT-PCR TaqMan analysis and western blotting, respectively. MCs incubated in medium without uPA and anti-uPAR antibody served as a control. The results in A are presented as mean ± s.e.m. for three separate and independent experiments. The data on the upper panel in B are representative of three separate and independent experiments. Quantification of the results of these experiments by densitometry presented as mean ± s.e.m. is shown in the lower panel. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05).

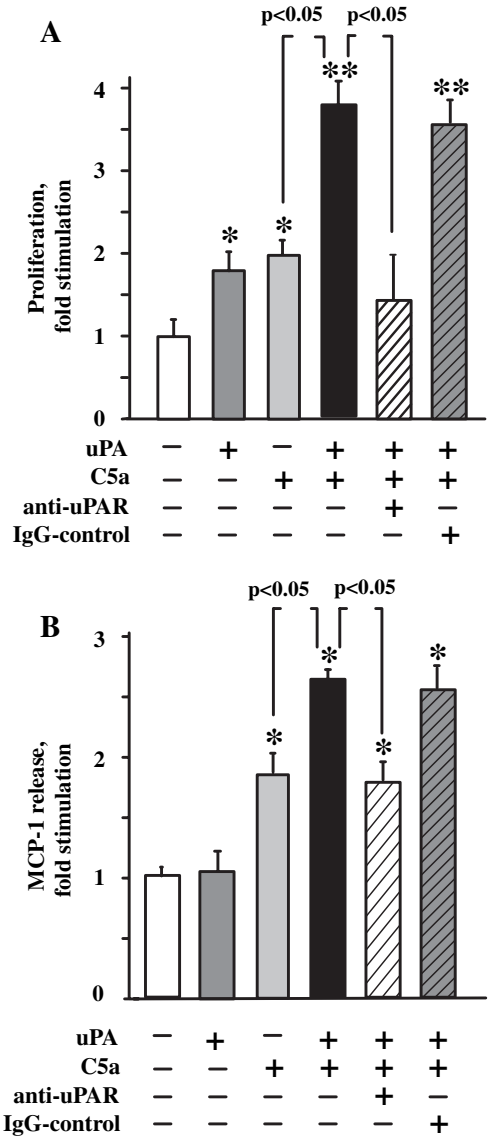

Fig. 3. uPA potentiates C5aR-related responses in MCs. (A) Quiescent MCs were incubated in 96-well microtiter plates with 20 nM uPA or 20 nM C5a alone or in combination with both stimuli in the presence or absence of 5 μg/ml anti-uPAR monoclonal antibody or irrelevant mouse IgG for 24 hours. The cells incubated in medium without stimuli served as a control. After 16 hours labeling with BrdU DNA synthesis was used as measure of the proliferation rate. Results are given as mean ± s.e.m. of four independent experiments performed in six parallel wells for each condition. (B) MCP-1 release was evaluated in supernatants from MC monolayers that were incubated as described in A for 48 hours. MCs incubated in medium without stimuli and producing 868±95 pg/ml MCP-1 served as a control. The data are given as mean ± s.e.m. of four separate and independent experiment performed in triplicate for each condition. Significance between control unstimulated and stimulated cells, as well as between the cells stimulated in the presence or not of 5 μg/ml anti-uPAR monoclonal antibody was determined by Student's *t*-test (**P*<0.05; ***P*<0.01).

of Stats on gene transcription, immunocytochemical studies were performed. As shown in Fig. 4B, Stat3 translocated efficiently into the nuclei of MCs stimulated with uPA.

Phosphorylation of signal transducers and activators of transcription of the Stat family is mediated via the Janus kinases (Jaks) (Chatterjee-Kishore et al., 2000; Darnell et al., 1994). This interplay is highly specific, thus contributing to the specificity of cell functional responses to cytokines, growth factors and polypeptide hormones utilizing the Jak/Stat pathway for signal generation. We therefore examined activation of Jaks in MCs stimulated with uPA. Treatment with uPA rapidly increased specific tyrosine phosphorylation of the Janus kinase Tyk2 (Fig. 5A), whereas other Jaks, namely Jak1, Jak2 and Jak3 were not affected (data not shown). The kinetics of this activation correlates with those for Stat3 phosphorylation. To provide more direct evidence that the uPA-induced Stat3 phosphorylation was really mediated by Tyk2, a dominant negative form of Tyk2, devoid of kinase activity, was

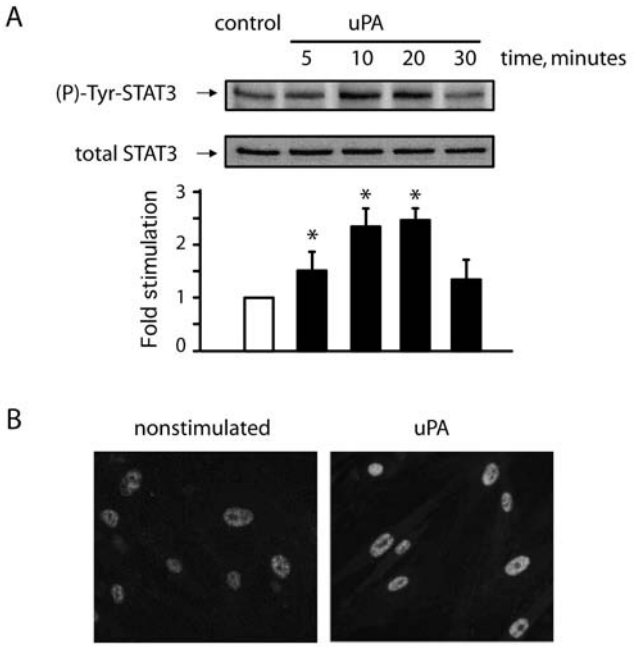

Fig. 4. uPA induces activation and nuclear translocation of Stat3 in MCs. (A) Quiescent MCs were treated with 10 nM uPA for the indicated times and phosphorylated Stat3 protein was visualized in cell lysates by immunoblotting with specific anti-(P)-Tyr-Stat3 antibodies. MCs incubated in medium without uPA served as a control. The middle panel demonstrates the amount of total Stat3 loaded on the gel for each sample. Results are representative of three independent experiments. Quantification (mean ± s.e.m.) of the results of these experiments by densitometry is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05). (B) A subconfluent MC monolayer was treated with 10 nM uPA at 37°C or left untreated, fixed and stained using primary anti-(P)-Tyr-Stat3 antibody and the corresponding Alexa Fluor-488 secondary antibody. The data are representative of three separate and independent experiment performed in duplicate for each condition.

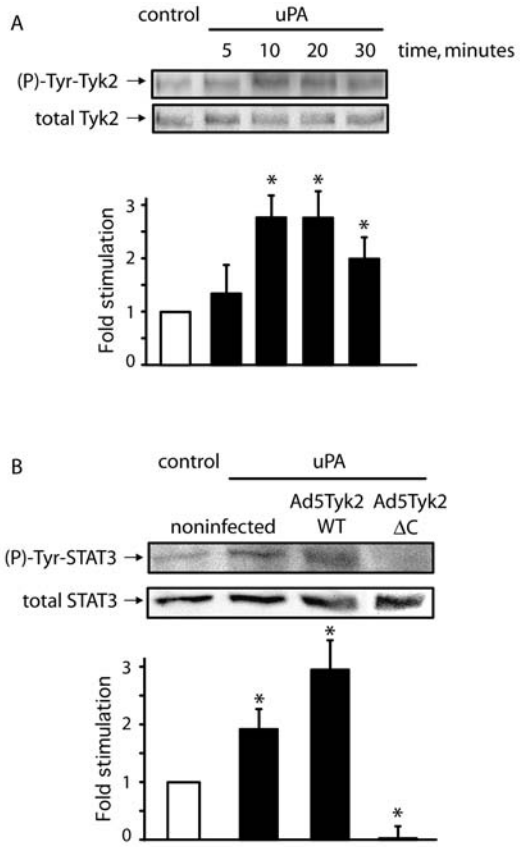

Fig. 5. uPA induces activation of Tyk2 in MCs. (A) Quiescent MCs were treated with 20 nM uPA for the indicated times and phosphorylated Tyk2 protein was visualized in cell lysates by immunoblotting with specific anti-phospho-Tyk2 antibodies (upper panel). MCs incubated in medium without uPA served as a control. The middle panel demonstrates the amount of total Tyk2 loaded on the gel for each sample. Results are representative of three independent experiments. Quantification (mean ± s.e.m.) of the results for these experiments by densitometry is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05). (B) MCs were left uninfected or were infected with wild-type Ad5Tyk2 or with the mutant form, Ad5Tyk2ΔC, and then stimulated for 20 minutes with 20 nM uPA. Phosphorylated Stat3 protein was visualized in cell lysates by immunoblotting with specific anti-(P)-Tyr-Stat3 antibodies. Noninfected MCs incubated in medium without uPA served as a control. The middle panel demonstrates the equal amount of total Stat3 loaded on the gel for each sample. Results are representative of three independent experiments. Quantification (mean ± s.e.m.) of the results by densitometry is shown for three experiments is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05).

expressed in MCs. As shown in Fig. 5B, uPA-induced Stat3 activation was completely abrogated in Ad5Tyk2ΔC-expressing MCs, as compared to non-infected and wild-type Ad5Tyk2-expressing cells. We conclude from these results that Tyk2 is required for the activation of Stat3 by uPA in MCs.

To examine the specific role of uPAR in propagation of the revealed Tyk2/Stat3 signaling, MCs with downregulated uPAR expression were used. Stable and specific inhibition of uPAR expression was achieved using a lentiviral RNA interference vector LV-uPARsi (Fig. 6A). Neither Tyk2 nor Stat3 phosphorylation was observed in LV-uPARsi-MCs, in contrast to cells infected with control vector (Fig. 6B,C).

Tyk2/Stat3 pathway mediates C5aR expression in MCs in response to uPA

To evaluate the role of the Tyk2/Stat3 pathway in the uPA-directed control of C5aR expression, several experiments were performed. MCs transiently infected with wild-type Ad5Tyk2 and two mutant forms, Ad5Tyk2KE and Ad5Tyk2ΔC, were stimulated with uPA, and C5aR expression was monitored by TaqMan analysis. Whereas there was a strong increase in C5aR mRNA in non-infected and in Ad5Tyk2-infected cells, Ad5Tyk2KE- and Ad5Tyk2ΔC-expressing cells did not respond to uPA stimulation. Consistent with these data, no uPA-induced stimulation in C5aR expression was observed in MCs expressing the Stat3F mutant form of Stat3 (Fig. 7). These results assign a direct role to the Tyk2/Stat3 pathway in controlling immune competent receptors response to uPA.

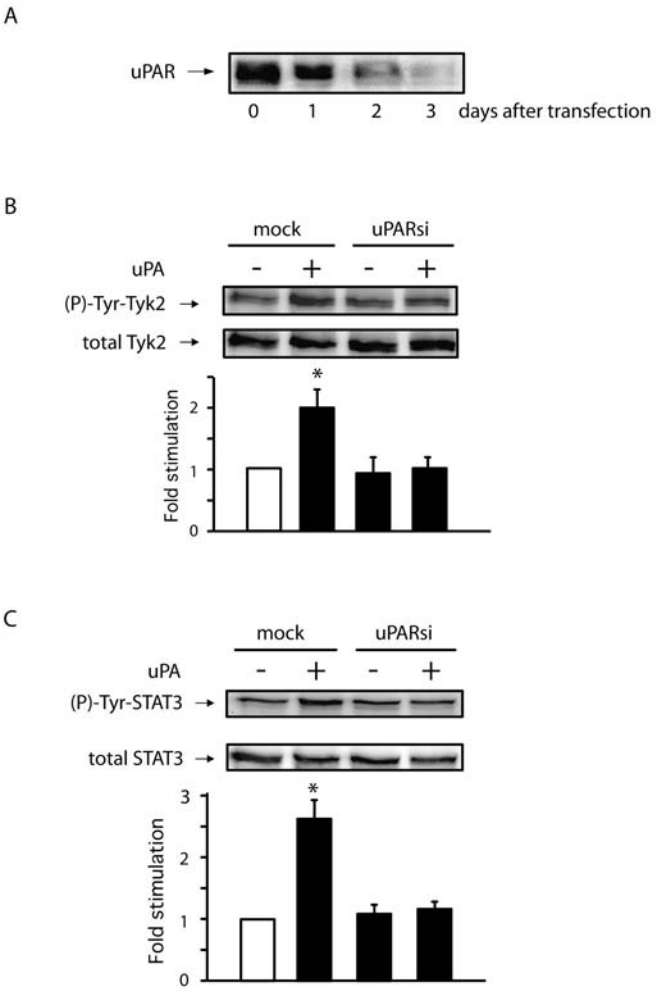

Fig. 6. uPAR mediates uPA-dependent Tyk2/Stat3 pathway activation in MCs. A. Quiescent MCs were infected with LV-uPARsi and time-dependent downregulation of uPAR expression was monitored by immunoblotting, using clone R3 monoclonal anti-uPAR antibody. MCs infected with LV-uPARsi or mock viruses were treated with 10 nM uPA for the indicated times at day 3 after infection and phosphorylated Tyk2 (B) or Stat3 (C) proteins were visualized in cell lysates by immunoblotting with specific anti-phospho-Tyk2 and anti-(P)-Tyr-Stat3 antibody, respectively. MCs incubated in medium without uPA served as a control. The upper panels is representative of three independent experiments. Quantification (mean ± s.e.m.) of the results by densitometry is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (*P<0.05).

uPAR utilizes gp130 adaptor protein to mediate C5aR expression in MCs

As uPAR is devoid of catalytic activity and linked to the outer membrane leaflet by a glycosylphosphatidylinositol (GPI) anchor, its signaling capacities and biological functions rely on interactions with other transmembrane molecules. Several proteins interacting with uPAR have been identified, such as integrins, tyrosine kinase receptors and G protein-coupled receptors. It was shown that in the human kidney epithelial tumor cell line TCL-598, uPAR associates with gp130 protein, a signal transducing subunit of the receptor complexes for the IL-6 cytokine family, to activate the Jak/Stat pathway

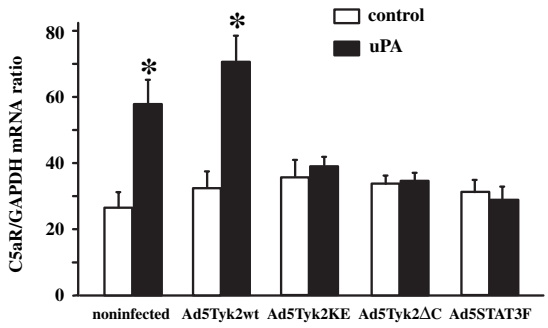

Fig. 7. TheTyk2/Stat3 pathway mediates uPA-induced upregulation of C5aR in MCs. Quiescent MCs that were uninfected or infected with wild-type Ad5Tyk2, the Tyk2 mutants, Ad5Tyk2ΔC and Ad5Tyk2KE, or with the Ad5STAT3F mutant form of Stat3 were stimulated with 20 nM uPA for 20 hours, and RT-PCR analysis for C5aR mRNA was performed using the TaqMan method. MCs incubated in medium without uPA served as a control. Results are presented as mean ± s.e.m. of three independent experiments.

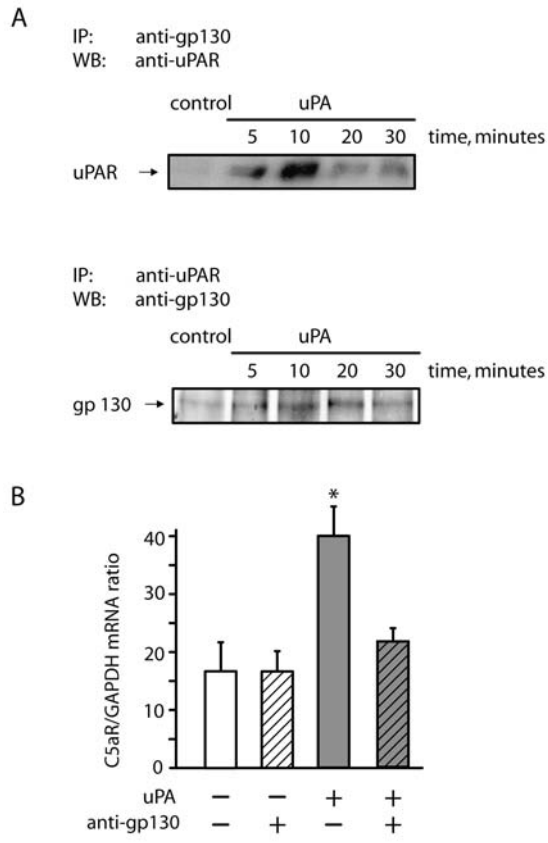

Fig. 8. uPAR utilizes gp130 adaptor protein to mediate C5aR expression in MCs. (A) Quiescent MCs were stimulated with 10 nM uPA for the indicated times or left unstimulated (control), and anti-gp130 (upper panel) or anti-uPAR (lower panel) antibody was used to coimmunoprecipitate gp130 and uPAR from the cell lysates. The immunoprecipitates were then analyzed with anti-uPAR (upper panel) or anti-gp130 antibody (lower panel). (B) RT-PCR analysis of C5aR mRNA was performed using the TaqMan method. RNA was isolated from quiescent MCs incubated for 6 hours with 20 nM uPA or in medium without uPA (control) in the presence or absence of 5 μg/ml of anti-gp130 antibody. Results are presented as mean ± s.e.m. of two independent experiments performed in duplicates for each condition.

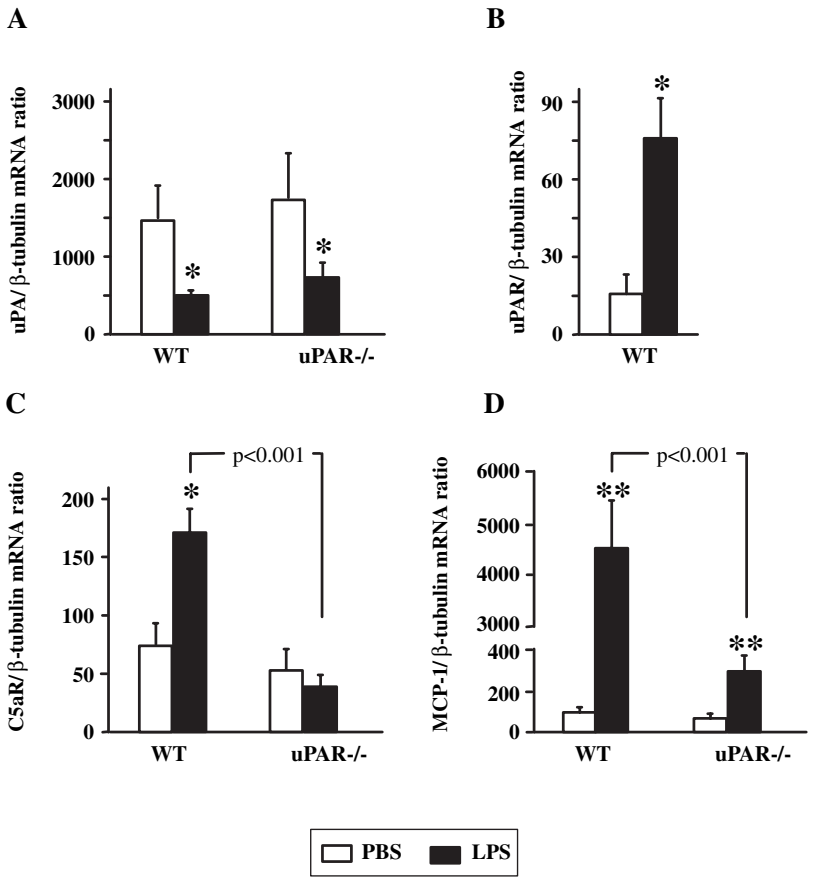

Fig. 9. uPAR is required for C5aR expression in mesangium upon LPS-induced renal inflammation in mice. LPS-induced nephritis was induced in wild-type and *uPAR*$^{-/-}$ mice by an intraperitoneal injection of 50 μg LPS in 200 μl PBS. Animals injected with PBS only, served as a control. 8 hours after challenge the mice were killed, RNA was isolated from kidney glomeruli and used for expression analysis by the TaqMan method. The mRNA levels were analyzed for uPA (A), uPAR (B), C5aR (C) and MCP-1 (D). β-tubulin served as a house-keeping gene. Results are expressed as mean ± s.e.m. (n=6 mice for each group). Significance between PBS controls and LPS-treated animals was determined by Student's t-test (*P<0.05; **P<0.01).

(Koshelnick et al., 1997). Numerous reports provide evidence that activation of Stat3 in response to various stimuli in several cell types is mediated by gp130. These observations point to the probable involvement of gp130 in the uPA-directed Stat3 activation and C5aR upregulation in MCs. To address this hypothesis, we performed immunoprecipitation using anti-gp130 antibody from MCs stimulated with uPA at different time points and determined uPAR in the immunoprecipitates using immunoblotting. Indeed, we observed a specific band for uPAR in the immunoprecipitates (Fig. 8A, upper panel). Specificity of this association was further confirmed in immunoprecipitation experiments using a reverse combination of antibodies, namely anti-uPAR antibody for precipitation and anti-gp130 antibody for western blotting (Fig. 8A, lower panel). The uPAR-gp130 association was uPA-dependent and displayed kinetics very similar to those observed by us for Stat3 and Tyk2 activation. These results suggest that in the uPA-stimulated MCs, uPAR may directly bind to the gp130 adaptor protein, which, in turn, provides a link to the Jak/Stat pathway.

To confirm that gp130 is required to propagate C5aR expression in MCs in response to uPA, cells were pretreated with anti-gp130 blocking antibody and the uPA-directed expression of C5aR was monitored by TaqMan RT-PCR. As expected, no increase in C5aR expression in response to uPA was observed after gp130 blockage (Fig. 8B).

uPAR is required for C5aR expression in mesangium upon LPS-induced renal inflammation in mice

To find out whether our findings might reflect a physiologically relevant process, in vivo studies were performed using a LPS-induced inflammation model. Kidney inflammation was induced by an intraperitoneal injection of LPS into wild-type and uPAR-deficient mice. For control experiments, phosphate-buffered saline (PBS) vehicle was injected. At 8 hours after injections kidney cortex, enriched in glomeruli, was isolated and used for expression analysis. uPA expression was decreased in both wild-type and *uPAR*$^{-/-}$ LPS-treated animals, although there was no difference between these two groups (Fig. 9A). In contrast, uPAR expression in glomeruli of wild-type mice after LPS-induced nephritis was strongly upregulated as compared to control PBS-treated animals (Fig. 9B). Interestingly, while C5aR and MCP-1 were upregulated in inflammed glomeruli of wild-type mice, these responses were abrogated in uPAR-deficient mice (Fig. 9C,D).

Finally, to exclude the possibility that the observed activation of C5aR in glomeruli of LPS-injected mice might be caused by infiltrating neutrophils and macrophages and not restricted to mesangial cells, immunohistochemical studies were performed. This examination revealed the upregulated expression of C5aR localized within mesangium of inflamed glomeruli of wild-type mice (Fig. 10A, upper panels). No infiltrating cells expressing C5aR were identified. The prominent immunohistochemical signal observed in the mesangium of LPS-treated animals was completely abolished in *uPAR*$^{-/-}$ mice with LPS-induced nephritis (Fig. 10A, lower panels). The difference in C5aR expression was further confirmed by a semi-quantification analysis of mesangial immunoreactivity (Fig. 10B).

Discussion

An important challenge in renal cell biology is to understand regulatory signaling pathways that control MC functional changes. Activated MCs play a direct role in the initiation and propagation of inflammatory events within the glomerulus. Recent studies have documented the expression of the complement anaphylatoxin receptors in the kidney (Fayyazi et al., 2000; Abe et al., 2001; de Vries et al., 2003), particularly on MCs (Braun and Davis III, 1998; Wilmer et al., 1998). These findings give a new insight into the mechanisms by which MCs may contribute to the pathogenesis of

glomerulonephritis and other glomeruli-associated kidney disorders. Thus, it is considered that the presence of complement receptors on MCs may mediate their specific interactions with activated complement products, which are decisive factors in tissue damage. However, there is still much that is not known about this process. One of the crucial, but still unexplored aspects, is the mechanism of regulated expression of the complement C5a anaphylatoxin receptor in MCs. This is of special importance, since uncontrolled or improperly activated C5aR expression may be crucial for the pathogenesis of complement-mediated tissue damage. In this study we provide convincing evidence that, in MCs, uPA, via its specific receptor uPAR and the association of uPAR with gp130 adaptor protein, regulates expression of C5aR and modulates C5a-dependent functional responses. The Janus kinase Tyk2 and transcription factor Stat3 serve as downstream components in the signaling cascade resulting in C5aR expression. Our data underscore a new role for uPA/uPAR-related signaling in the initiation and propagation of glomeruli-associated inflammation.

We suggest that at least two interrelated mechanisms are involved: activated expression on MCs of the complement anaphylatoxin C5a receptor and modulation of C5a-directed proinflammatory cytokines release and of cell proliferation. Thus, we show that uPA induces expression of C5aR on human MCs in a dose-dependent and transient fashion at both mRNA and protein levels. This process required functionally active uPAR, as proved by cell pretreatment with uPAR-blocking antibody. We provide evidence that uPA functions as a modulator of the C5aR-dependent processes in human MCs. Thus, we observed a synergistic, uPAR-mediated effect of uPA and C5a on MC proliferation and MCP-1 release, the events that could contribute to the progression of inflammatory renal injury.

We have extended these in vitro observations to an in vivo model of renal inflammation. We show that *uPAR*$^{-/-}$ mice are protected from LPS-induced glomerular inflammation. At the time point examined, no increase in C5aR expression in the mesangium of *uPAR*$^{-/-}$ mice was observed after LPS treatment, in contrast to the findings in wild-type animals. Consistent with these data, expression of MCP-1 was dramatically increased in wild-type animals, whereas only moderate increase was observed in uPAR-deficient mice. Our immunohistological studies demonstrate that under our experimental conditions, namely the low LPS concentration and short time of treatment, rapid upregulation of C5aR was restricted to mesangial cells and preceded LPS-induced leukocyte infiltration into glomeruli.

We investigated the molecular mechanism underlying the revealed uPA/uPAR-related upregulation of C5aR on MCs and delineated the gp130/Tyk2/Stat3 signaling pathway responsible for this effect. Although uPAR has been well documented as an important modulator and orchestrator of signaling events, the functional purpose of uPAR-directed signaling in the cell has only been elucidated in a few studies. One of the obvious limitations in this research field is the cellular specificity of uPA/uPAR-related signaling cascades. Thus, several studies have documented that uPAR-dependent tumor growth involves ERK/MAPK signaling (Liu et al., 2002), whereas FAK signaling mediated tumor dormancy (Ghiso, 2002). In human vascular smooth muscle cells the Tyk2/Stat1 pathway was shown to regulate cell migration and proliferation via a cross-talk with different downstream signaling cascades (Kusch et al., 2000; Kiian et al., 2003; Kunigal et al., 2003). By contrast, uPA-stimulated migration of rat smooth muscle cells requires activation of MEK and Erk kinases (Degryse et al., 2001). These and other data on the cell specificity of uPAR signaling emphasize the potential for different cell types to respond differently to a given signal. In

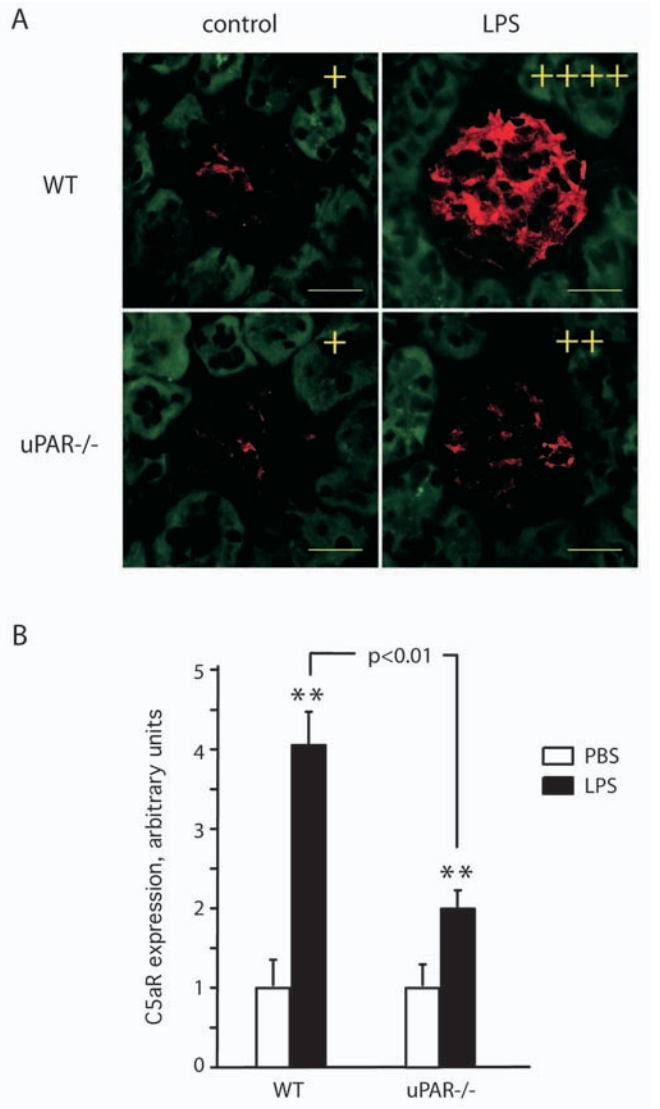

Fig. 10. uPAR is required for C5aR expression in mesangium upon LPS-induced renal inflammation in mice. (A) LPS-induced nephritis was induced in wild-type and *uPAR*$^{-/-}$ mice as described in Fig. 9. 8 hours after challenge the mice were killed and 6 µm cryosections of kidney cortex were stained for C5aR protein expression using anti-C5aR primary antibody and Cy3-conjugated secondary antibodies. Representative microphotographs are shown (6 mice for each group). Bar, 50 µm. (B) Statistical analysis of C5aR expression in mesangium of wild-type and *uPAR*$^{-/-}$ mice with LPS-induced nephritis was performed by evaluating the total number of positively stained glomeruli and the staining intensity (in arbitrary units) as described in the Materials and Methods. Results are expressed as mean ± s.e.m. (n=6 mice for each group). Significance between PBS controls and LPS-treated animals was determined by Student's t-test (*P<0.05; **P<0.01). +, arbitrary units (based on staining intensity using criteria described in Materials and Methods).

light of the multitude of biological functions played by the uPA/uPAR system it is of fundamental importance to determine different cascades of the uPAR-signaling machinery and then to assign particular pathways to particular cells and functions.

Signal transducers and activators of transcription of the Stat family play important roles in regulating immediate-early biological effects initiated by a variety of extracellular ligands including cytokines, chemokines and growth factors (Darnell et al., 1994; Schindler, 1999). Activation of latent, cytoplasmic, or membrane-associated Stat proteins requires their tyrosine phosphorylation, which in most cases depends on the activity of protein tyrosine kinases of the Janus family (Ziemiecki et al., 1994). After their phosphorylation, Stat proteins form homo- or heterodimers in order to translocate to the nucleus, where site-specific binding to enhancer elements leads to gene activation. uPA/uPAR has been documented to utilize Jak/Stat signaling in several cell types. Thus, in human kidney epithelial tumor cells uPAR is associated with the Jak1/Stat1 pathway, although cellular effects of this signaling remained unexplored (Koshelnick et al., 1997). In human vascular smooth muscle cells, the Tyk2/Stat1 pathway modulates, as mentioned above, uPA/uPAR-directed cell migration and proliferation.

In this study, we demonstrate that Stat3 is a key signaling molecule, which mediates upregulated expression of C5aR in human MCs in response to uPA. Our results show that uPA induces tyrosine phosphorylation of Stat3, but not other Stats, and its nuclear translocation. Obviously, these processes are required to induce uPA-related C5aR expression, since MCs expressing the Stat3 inactive mutant were unable to express C5aR when stimulated with uPA. Our studies on Janus kinases indicate that Tyk2 is responsible for the observed Stat3 tyrosine phosphorylation. Indeed, we observed fast and reversible Tyk2 activation in MCs in response to uPA, whereas Jak1, Jak2 and Jak3 were not affected. The kinetics of this activation correlates with those of the uPA-induced Stat3 phosphorylation, assuming that both processes are coordinated in time. Furthermore, MCs expressing kinase-dead Tyk2 mutants did not respond to uPA with Stat3 activation and C5aR expression, indicating a decisive role for Tyk2 kinase activity in these processes. Both Tyk2 and Stat3 activation by uPA required native cell surface uPAR, since no Tyk2/Stat3 phosphorylation was observed in uPARsi-MC where uPAR expression was downregulated. Our results confirm and extend the findings of others that expression of some G protein-coupled receptors is regulated by the Jak/Stat3 pathway (Senga et al., 2003).

Stat3 protein plays a central role in transmitting a multitude of different processes initiated by the IL-6 cytokine family (French et al., 2002). Moreover, IL-6 has been shown to mediate expression of C5aR in several cell types (Riedemann et al., 2003). Therefore, it was tempting to speculate that induction of the Tyk2/Stat3 pathway followed by the upregulation of C5aR might result from the uPA-triggered IL-6 activation, instead of reflecting a direct effect of uPA. To verify this hypothesis, separate experiments have been performed. Thus, we measured, using specific ELISA, IL-6 secretion from MCs stimulated dose-dependently with uPA for different times and examined expression of both α- and β-subunits of the IL-6 receptor in uPA-stimulated cells. No changes were observed (data not shown) thus indicating that C5aR expression in MCs is indeed attributed to the uPA/uPAR-induced signaling.

Although the revealed mechanism of uPA-mediated C5aR expression is IL-6 independent, uPAR utilizes gp130 protein, a signal transducing subunit of the receptor complexes of the IL-6 cytokine family, as a membrane interactor to mediate C5aR expression. In order to identify a transmembrane protein, which might serve as an adaptor for uPAR in mesangial cells and therefore couple uPAR to the pathway required for C5aR expression, several experiments have been performed aiming at analyzing uPAR-interacting proteins. Thus, we used the a325 peptide that dissociates uPAR-integrin complexes, FPR-family receptor inhibiting peptides, and pharmacological inhibitors of tyrosine kinase receptors. No effect on the uPA-mediated C5aR expression was observed in all these experiments (data not shown). Instead, we found that uPAR is associated in an uPA-dependent fashion with gp130. We also provide evidence that this association is required to mediate C5aR expression in MCs in response to uPA.

A relevant question regarding the functional role of uPAR signaling in C5aR regulation in MCs is the source of uPA in glomeruli. In our in vivo experiments we observed a decrease in uPA expression in mesangium of both wild-type and uPAR-deficient mice treated with LPS. These results correlate with the data of others on the reduction in urinary and renal uPA in LPS-injected mice, suggesting that an imbalanced fibrinolytic system might participate in kidney dysfunction upon inflammation (Yamamoto and Loskutoff, 1996). By contrast, we noticed strong upregulation of uPAR expression in inflammed mesangium of wild-type animals. Thus, it appears that the regulation of the C5aR expression pathway may result from the uPAR-mediated uptake of uPA via the increased number of uPAR available for its ligand on the surface of MCs. A paracrine loop of stimulation cannot be excluded, although the source of uPA in mesangium during inflammation remains to be elucidated.

In summary, the results of the present study demonstrate that uPA, via the uPAR-directed signaling machinery, regulates expression of the anaphylatoxin C5a receptor on MCs, and modulates C5a-dependent functional responses. These findings suggest the possibility that MCs may be therapeutic targets for inhibiting complement-mediated glomerular damage.

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