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# Differential chemokine expression in tubular cells in response to urinary proteins from patients with nephrotic syndrome

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**Abstract**

*Background and aim:* Comparison of urine proteins in idiopathic minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) patients has been previously conducted, but the relationship between the severity of tubular injury and the composition of urine proteins in various kidney diseases is unknown. This study aimed to investigate the chemokine expression in human tubular cells in response to urine proteins from patients with nephrotic syndrome. *Methods:* Urine proteins collected from patients with MCD or FSGS were extracted by ultrafiltration and coincubated with HK-2 cells. The expression of the *RANTES* and *MIF* genes and the activation of p38 and extracellular regulated kinase were determined. *Results:* The urine proteins from both MCD and FSGS patients contained a primary band of proteins with Mr of ~62 kDa. The major cytokines present in urine proteins from MCD and FSGS patients were IL-6 and IL-8, while IL-1β, IL-10, IL-12p70 and TNF-α were only detectable. We observed time- and dose-dependent increases in RANTES and MIF expression with urine protein treatment in HK-2 cells. The urine proteins from FSGS patients induced a higher expression of these two chemokines in HK-2 cells compared to the urine proteins from MCD patients. ERK and p38 were activated by urine proteins from either MCD or FSGS patients. Pretreatment with SB203580 or PD98059 abolished the increase in RANTES and MIF expression induced by urine proteins from FSGS patients, while only SB203580 suppressed the high expression induced by urine proteins from MCD patients. *Conclusion:* These findings indicate that the urine proteins from MCD and FSGS patients induce a differential expression of RANTES and MIF in tubular cells through distinct activation of MAPK-related signaling pathways.


*Keywords:* Nephrotic syndrome; Human serum albumin; Proteinuria; Chemokine; MAPK

## 1. Introduction

Progressive nephropathies are characterized by a highly enhanced glomerular permeability to proteins, in turn leading to proteinuria and concomitant tubulointerstitial damage [1–3]. How proteinuria results in such precipitating interstitial changes is not fully understood, but it is certainly multifactorial and involves numerous pathways of cellular damage [2–4]. Earlier studies in vitro have already confirmed that certain serum protein components (e.g. albumin and transferrin) can stimulate proximal tubular cells to secrete a large number of chemokines, such as regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5, chemokine (C–C motif) ligand 5) [2] and macrophage immigration inhibitory factor (MIF) [5] into the basolateral medium, and the polarized secretion of these chemoattractants in vivo is considered to promote monocyte and lymphocyte recruitment into the renal interstitium [6,7]. Additionally, it is well established that activation of the extracellular signal regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signal transduction pathway plays an important role in the inflammatory response [8]. p38 and ERK as important signaling molecular have been proved to mediate release of MIF and RANTES in tubular cells exposed to a single component such as albumin or IgG, suggesting

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a possible link between proteinuria and inflammatory damage of tubular cells involving MAPK pathway [9,10]. However, these studies have a significant limitation. They have generally used a single serum component (e.g. albumin) at very high and physiologically irrelevant concentrations to study the effect of protein overload on proximal tubular epithelial cells (PTECs) [11,12]. However, the glomerular ultrafiltrate often contains a mixture of proteins and a single component might not be responsible for the damage of tubular cells in renal disease development [13].

Focal segmental glomerulosclerosis (FSGS) is one of the most prevalent histopathological lesions in idiopathic nephrotic syndrome. It is distinguished clinically from minimal change disease (MCD) by non-selective proteinuria, steroid resistance and progressive renal insufficiency. Furthermore, it has been shown that FSGS patients presented more often with progressive tubular degeneration and interstitial fibrosis compared to MCD patients even though both diseases had a similar degree of proteinuria [14–17]. Thus in an effort to find therapeutic targets in proteinuria, comparison of urine proteins in MCD and FSGS patients has been previously conducted [18–20], but the relationship between the severity of tubular injury and the composition of urine proteins in various kidney diseases is unknown.

To seek further evidence, we have hypothesized that the use of a wide range of proteins extracted from proteinuria might closely resemble the glomerular ultrafiltrate in tubular lumen and serve as a model for further study in the mechanism of tubular damage induced by proteinuria in kidney diseases. This study was undertaken to assess and compare the expression of two important chemokines, RANTES and MIF, in cultured proximal tubular cells treated with urine proteins extracted from MCD or FSGS patients and to explore the possible role of ERK and p38-MAPK signaling transduction pathways in differential chemokine production.

## 2. Materials and methods

### 2.1. Reagents and drugs

HK-2 cells, a primary human proximal tubular cell line, was purchased from American Type Culture Collection (Manassas, VA); Dulbecco's Modified Eagle's medium (DMEM)/F-12, fetal bovine serum (FBS) and trypsin/ethylenediamine tetraacetic acid (EDTA) solution were purchased from Gibco (Langley, OK); TRIzol and reverse transcriptase were from Invitrogen (Carlsbad, CA); PCR buffer, Taq polymerase and dNTPs were from Takara Bio Inc. (Shiga, Japan), and ELISA Quantikine kit for RANTES was from R&D Systems (Minneapolis, MN). The specific inhibitors, SB203580 and PD98059, were from CalBiochem (San Diego, CA). Primary antibodies of p38 and ERK were from Cell Signaling Technology (Danvers, MA). Purified human serum albumin (HSA, A5843), Triton X-100, and dimethyl sulfoxide (DMSO) were from Sigma–Aldrich (St. Louis, MO). Polymyxin B-immobilized columns (Detoxi-GelTM) were from Pierce Chemical (Rockford, IL). The ultrafilter (Labscale TTF system, PXC 100C 50) was purchased from Millipore Co. (Billerica, MA). The human inflammation Cytometric Bead Array kit for determining interleukins (IL-8, IL-1b, IL-6, IL-10 and IL-12p70), and tumor necrosis factor-α (TNF-α) and BD LSR II flow cytometer were from BD Pharmingen (San Jose, CA).

### 2.2. Patients and urine collection

Informed consent was obtained from all patients participating in this study, after local Ethics Committee approval. Diagnosis was established based on the criteria of ICD-11 for 10 patients with MCD and 12 patients with FSGS through biopsy using light and electron microscopy. Only patients who have never been treated with steroid or any other immunosuppressive agents were enrolled in this study. The spontaneous urine was collected in a clean container on ice (~4 °C) and stored at −80 °C until analysis. Some key clinical data of the patients studied are summarized in Table 1. The mean urine protein concentration in MCD and FSGS patients was 3.5 ± 1.4 mg/ml and 3.7 ± 2.0 mg/ml, respectively. Thus, a concentration range of 0.1–10 mg/ml for the urine proteins was used for our in vitro experiments.

### 2.3. Urine protein ultrafiltration and preparation

Following initial centrifugation (4000*g* for 15 min at 4 °C) the supernatant of urine sample was collected and filtered by a 0.45-μm filtration membrane to remove non-soluble deposits and cellular particles. The samples were then applied to an ultrafilter with polyethersulfone membrane (5 kDa molecule weight cut-off and low protein binding membrane). After lyophilization using a freeze dryer to remove extra water, the urine protein extract was stored at −80 °C. In all in vitro experiment, dried and powdered urine proteins were dissolved in sterile phosphate-buffered saline (PBS) and then added to cells. Endotoxin in urine proteins was removed by polymyxin B-immobilized columns and cleared reagents contained <0.1 EU/ml of endotoxin as determined by the limulus amebocyte lysate assay. Urine proteins were eluted in 0.5 mM NaCl and 50 mM Tris at pH 7.5. Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to verify that there was no gross degradation of the proteins prepared using this protocol.

Table 1
Clinical data of patients

| Patient characteristic | MCD | FSGS |
|---|---|---|
| Number | 10 | 12 |
| Sex (male/female) | 9/1 | 9/3 |
| Mean age (years) | 21 ± 10 | 35 ± 17 |
| Proteinuria (g/day) | 3.5 ± 1.4 | 3.7 ± 2.0 |
| Serum creatinine (μmol/L) | 73.0 ± 8.8 | 92.0 ± 86.7 |

*Abbreviations:* FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease.

### 2.4. Quantification of cytokines using cytometric bead array (CBA)

Six cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α) in patient urine proteins were quantified simultaneously using a human inflammation CBA kit according to the manufacture's instructions (BD Pharmingen, San Jose, CA). The BD LSR II flow cytometer was calibrated with setup beads and 3000 events were acquired for each sample. Six standard curves (standard ranging from 0 to 5000 pg/ml) were obtained from one set of calibrators and data on the six cytokines were simultaneously obtained for each test sample. Individual cytokine concentration ratios were indicated by their fluorescent intensities. The urine proteins from each patient were dissolved in sterile PBS to a concentration of 60 mg/ml and assayed. The limits of detection for IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were 7.2, 2.5, 3.6, 3.3, 1.9 and 3.7 pg/ml, respectively.

### 2.5. Cell culture

HK-2 cells were grown in DMEM/F-12 plus 10% FCS. The cells were grown in an atmosphere of 5% $CO_2$ and 95% air at 37 °C and given fresh medium every 3–5 days. Experiments were performed on cells within 10 passages. Viable cells were counted using the trypan blue exclusion method.

### 2.6. MTT assay and lactate dehydrogenase (LDH) leakage assay

The effect of urine proteins on HK-2 cell proliferation was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). In brief, cells were grown in 96-well culture plates and treated by urine proteins from MCD or FSGS patients or HSA for 72 h. The culture volume was 100 μl. Ninety minutes before the end of the appropriate treatment periods, 10 μl of MTT (5 mg/ml in PBS) was added to each culture well. After incubation for a further 4 h at 37 °C, the culture medium was removed and the purple crystals formed were dissolved in 150 μl of 0.1 M HCl in isopropanol. The absorbance was measured using a microplate reader at 570 nm with a background correction at 690 nm.

The LDH leakage assay was used to assess cytosolic membrane integrity. HK-2 cells were seeded in 24-well plates at a cell density of $3 \times 10^5$ cells in 1000 μl/well and treated by urine proteins for 72 h. Thereafter, 600 μl incubation medium was collected from the culture wells and intracellular LDH was harvested by solubilizing cells with 600 μl 0.1% Triton X-100. Enzyme leakage was determined in the incubation medium and compared with total LDH activity (extracellular plus intracellular LDH) detected with an automatic biochemical autoanalyzer (Hitachi 7170A, Tokyo, Japan). The experiments were performed at least three times, using 4 wells for each test compound at different concentrations.

### 2.7. Treatment of HK-2 cells with ultrafiltered urine proteins

Cells were preincubated with serum-free DMEM/F-12 for 24 h. HK-2 cells were exposed to 5 mg/ml HSA, or urine proteins from MCD or FSGS patients at concentrations of 0.1–10 mg/ml over 48 h to detect RANTES and MIF expression. p38 or ERK protein expression were evaluated by Western blot analysis in cell lysates from HK-2 cells treated with HSA or urine proteins at 1 mg/ml for 20 min. To evaluate the effect of ERK and p38 inhibitor on chemokine expression, cells were pretreated with 10 mM SB203580 and 50 mM PD98059 for 1 h before and during incubation with urine proteins. Both inhibitors were prepared in DMSO with a final concentration of 0.1% (v/v) of the solvent in the test sample.

### 2.8. Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Tri-Reagent. Extracted RNA was quantified by absorbance at 260 nm using a spectrometer. Two micrograms of total RNA was reverse transcribed to cDNA in a 20 μl reaction mixture containing 50 ng random primer, 0.5 mmol/L dNTPs and 20 U of RNase inhibitor. Specific primer sequences were as followed: GAPDH, sense 5′-AGT CCA CTG GCG TCT TCA C-3′, antisense 5′-GCTTGACAAAGTGGTCGT TGA G-3′, 639 bp; RANTES/CCL5, sense 5′-GTTTA CGGGAAATCT TCGCACC-3′, antisense 5′-GTCGGA GCGGCTGTAGTC-3′, 300 bp; and MIF, sense 5′-CCT GGTCCTTCT GCCATCAT-3′, antisense 5′-CTTGCTG TAGGAGCGGTTCT-3′, 252 bp. PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Images of the gel were captured using the Gel Doc 1000 gel Documentation System from Bio-Rad (Hercules, CA). Gene expression was then analyzed by the IP Lab gel software from Signal Analytics (Vienna, VA). Data were expressed as the ratio of specific *MIF* or *RANTES*/*CCL5* mRNA normalized to *GAPDH* mRNA.

### 2.9. SDS–PAGE and immunoblotting

To demonstrate activation of p38 and ERK, Western blot analysis of protein extracts from HK-2 cells treated with HSA or urine proteins was performed. Twenty micrograms of protein were subjected to SDS–PAGE, using a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4 °C with PBS containing 5% BSA. The blots were incubated for 1 h with the following primary antibodies: rabbit polyclonal antibody p-p38 and p38; mouse monoclonal antibody p-ERK; or rabbit polyclonal antibody ERK. After incubation with the secondary antibodies (horseradish peroxidase-conjugated rabbit anti-mouse IgG or rabbit anti-rabbit IgG) for 1 h, protein bands were detected by Super-Signal chemiluminescent substrate.

### 2.10. Determination of RANTES levels by ELISA

The culture media of proximal tubular cells on a 24-well plate were collected and stored at −80 °C until assay. Concentrations of RANTES in the culture supernatants of HK-2 cells were measured by commercial ELISA kits according to the manufacture's instructions. All samples were assayed in triplicate and the data were presented as means ± SD. The optical density (OD) was measured at 450 nm and RANTES concentrations were extrapolated from a standard curve using recombinant human RANTES. RANTES measurement was also performed on the solutions used to stimulate the cells to exclude the effects of contaminant RANTES in urine protein extracts.

### 2.11. Immunofluorescent staining and confocal laser scanning microscopy

Human HK-2 cell cultures were fixed in ice acetone for 10 min, washed and blocked with 5% BSA. Cells were incubated overnight with anti-MIF or anti-RANTES antibody and were then incubated for 45 min with FITC-labeled donkey anti-goat or mouse secondary antibody. Primary antibody was replaced with isotype IgG as a negative control. The cells were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI).

### 2.12. Quantification of cellular MIF and RANTES expression by flow cytometry

RANTES and MIF expression on HK-2 cells was evaluated by flow cytometric analysis. Following treatment as described above, cells were collected and incubated with either FITC-labeled mouse anti-human RANETS and MIF monoclonal antibody or an appropriate isotype-matched antibody for 45 min at 4 °C and then were analyzed by FACS (Coulter Corp., Hialeah, FA) with WinMDI software (TSRI Cytometry, San Diego, CA). The mean specific fluorescence intensity was measured in each channel (10,000 cells/sample). Mean values from three measurements and standard deviations were determined.

### 2.13. Statistical analysis

Data are expressed as means ± standard deviation. Data were analyzed using one-way ANOVA followed by post hoc least significant difference test. Analyses were performed using SPSS software 12.0. A *P*-value of less than 0.05 was regarded as statistically significant.

## 3. Result

### 3.1. Analysis patterns of ultrafiltered urinary proteins from MCD and FSGS patients in gel electrophoresis

The composition of ultrafiltered urine proteins was analyzed by gel electrophoresis. The results are shown in Fig. 1. All proteins with Mr of < 5 kDa were removed by ultrafiltration. Human HSA shows one primary band only, with a molecular weight of approximately 66 kDa. The gel revealed two similar protein band patterns but with some differences in the protein band range analyzed. The urine proteins from both MCD and FSGS patients contained a primary band of proteins with Mr of approximately 62 kDa, followed by bands with Mr of approximately 83, 130 and 200 kDa. Both urine protein samples also contained some protein bands with Mr < 50 kDa. However, the urine proteins from FSGS patients contained several remarkable bands with Mr > 250 kDa, but they were not remarkable for urine samples from MCD patients.

### 3.2. Detection of cytokines in urine proteins from MCD or FSGS patients

Experiments were performed to evaluate six cytokine levels by CBA in the urine proteins of MCD and FSGS patients. There was a large variability in the levels of six cytokines in urine proteins from MCD or FSGS patients (Fig. 2). For both MCD and FSGS patients, the major cytokines present in urine proteins were IL-6 and IL-8. In MCD patients, there was a 15-fold variability in the urinary levels of IL-6 and IL-8. The variability was 14- and 39-folds in FSGS patients. The mean levels of urinary IL-6 in MCD and FSGS patients were 76.1 ± 85.9 and 73.0 ± 71.2 pg/ml, respectively. The mean value for IL-8 in MCD and FSGS patients was 92.4 ± 92.5 and 134.4 ± 213.8 pg/ml, respectively. In most MCD and FSGS patients, IL-1β, IL-10, IL-12p70 and TNF-α were only detectable by our CBA-based assays (Fig. 2). No significant difference existed between the urinary levels of all cytokines tested in FSGS and MCD patients.

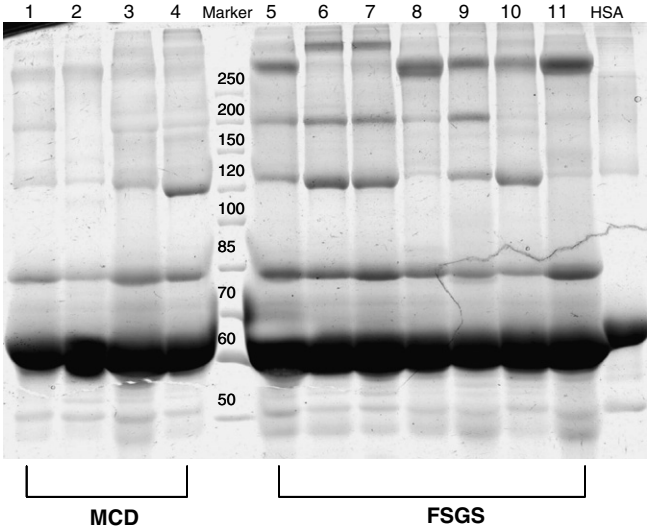


Fig. 1. Representative band patterns of urine proteins from MCD and FSGS patients by SDS–PAGE electrophoresis. Lanes 1–4, urine proteins from MCD patients and lanes 5–11, urine proteins from several FSGS patients. Human HSA shows one primary band only, with a molecular weight of approximately 66 kDa.

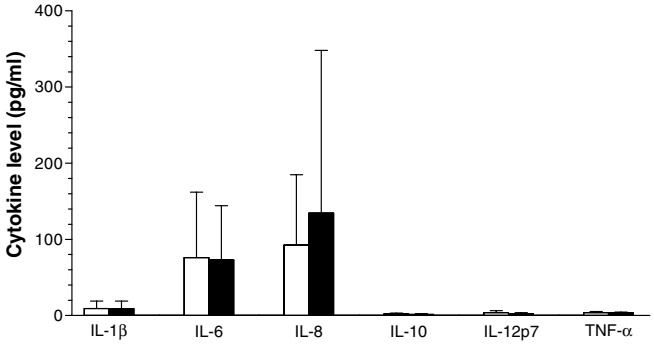


Fig. 2. Concentrations of six cytokines in urine proteins from MCD and FSGS patients by the CBA method. IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α protein levels were measured from urinary samples of MCD or FSGS patients. The samples with 60 mg/ml urine proteins of all patients were assayed and cytokine quantity is depicted as pg/ml. $^{*}P < 0.05$, FSGS vs. MCD group. Data are means ± SD of 10–12 patients.

### 3.3. Cytotoxicity of urine proteins from MCD or FSGS patients in HK-2 cells

The urine proteins from MCD or FSGS patients at concentrations ⩽5.0 mg/ml did not elicit significant cytotoxicity after incubation with HK-2 cells for 72 h (data not shown). When the urine protein concentrations increased to 10 mg/ml, a marked cytotoxicity was observed. The urine proteins from MCD or FSGS patients at 10 mg/ml caused a 52.3% and 56.6% ($P < 0.05$) reduction of cell survival in HK-2 cells, respectively (Fig. 3). HSA at 5 and 10 mg/ml only slightly reduced the cell proliferation in HK-2 cells.

We further examined the effects of urine proteins from MCD and FSGS patients on the lactate dehydrogenase leakage in HK-2 cells. After 72 h incubation, the urine proteins from MCD patients at 1, 5 and 10 mg/ml induced a 12.3%, 94.7% and 247.3% increase in LDH leakage, respectively, compared to the control (Fig. 3). The LCD leakage in HK-2 cells cotreated with urine proteins from FSGS patients at 1, 5 and 10 mg/ml for 72 h was increased by 76.8%, 137.85% and 249.1%, respectively, compared to the control. The urine proteins from FSGS patients induced a greater leakage of LDH than those from MCD patients.

### 3.4. Effects of ultrafiltered urine proteins from MCD or FSGS patients on RANTES and MIF expression in HK-2 cells

We first examined the effect of urine protein treatment on the *RANTES* and *MIF* mRNA expression in HK-2 cells when incubated over 48 h. With exposure of the HK-2 cells to ultrafiltered urine proteins from MCD or FSGS patients at 5.0 mg/ml over 48 h, the increase of both *RANTES* and *MIF* mRNA levels was time-dependent, with increasing induction of both genes when the incubation time increased over 48 h (Fig. 4). As shown in Fig. 4, treatment of HK-2

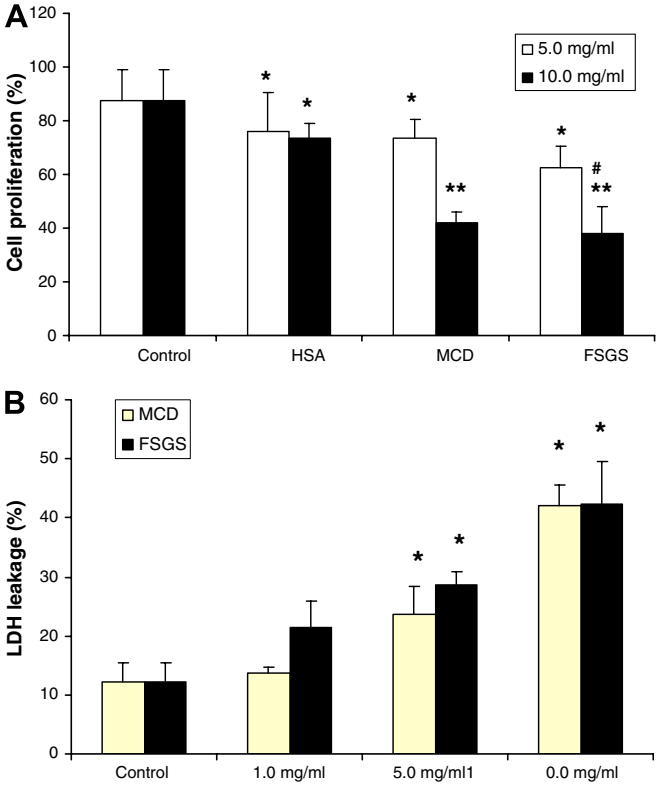


Fig. 3. Effect of urine proteins from MCD and FSGS on cellular proliferation (A) and lactate dehydrogenase (LDH) leakage (B) in HK-2 cells. For MTT assay, HK-2 cells were treated with 5 or 10 mg/ml urine proteins and incubated for 72 h. For LDH leakage assay, HK-2 cells were treated with 1, 5 or 10 mg/ml urine proteins and incubated for 72 h. The LDH activity was determined using an automatic biochemical autoanalyzer. Data are means ± SD of four independent determinations.

cells with 5.0 mg/ml urine proteins from MCD patients for 3, 6, 12, 24 and 48 h significantly increased the expression of *RANTES* and *MIF* 5.3-, 6.8-, 14.1-, 17.9- and 23.8-fold; and 1.7-, 2.0-, 2.0-, 2.8- and 3.5-fold, respectively, compared to the control (zero time incubation) ($P < 0.05$, Fig. 4). When the cells were treated with ultrafiltered urine proteins from FSGS patients for 3, 6, 12, 24 and 48 h, the expression of *RANTES* mRNA was significantly increased 8.3-, 14.4-, 17.5-, 22.5- and 24.1-fold, and the *MIF* mRNA levels were also significantly increased 2.0-, 3.2-, 4.1-, 4.3- and 4.4-fold, respectively, compared to the control (zero time incubation) ($P < 0.05$, Fig. 4). The urine proteins from FSGS patients induced a greater magnitude of both *RANTES* and *MIF* mRNA expression compared to those from MCD patients after 6 h incubation up to 48 h ($P < 0.05$).

With exposure of the HK-2 cells to ultrafiltered urine proteins from MCD or FSGS patients at concentrations in the range of 0.1–10.0 mg/ml for 24 h, the increase of *RANTES* and *MIF* mRNA levels was dose-dependent, with a maximal induction at 5–10 mg/ml (Fig. 5). The induction of *RANTES* and *MIF* by urine proteins from MCD and FSGS patients appeared saturable, when the concentrations of urine proteins were greater than 0.1 and 0.5 mg/ml, respectively. As shown in Fig. 5, treatment

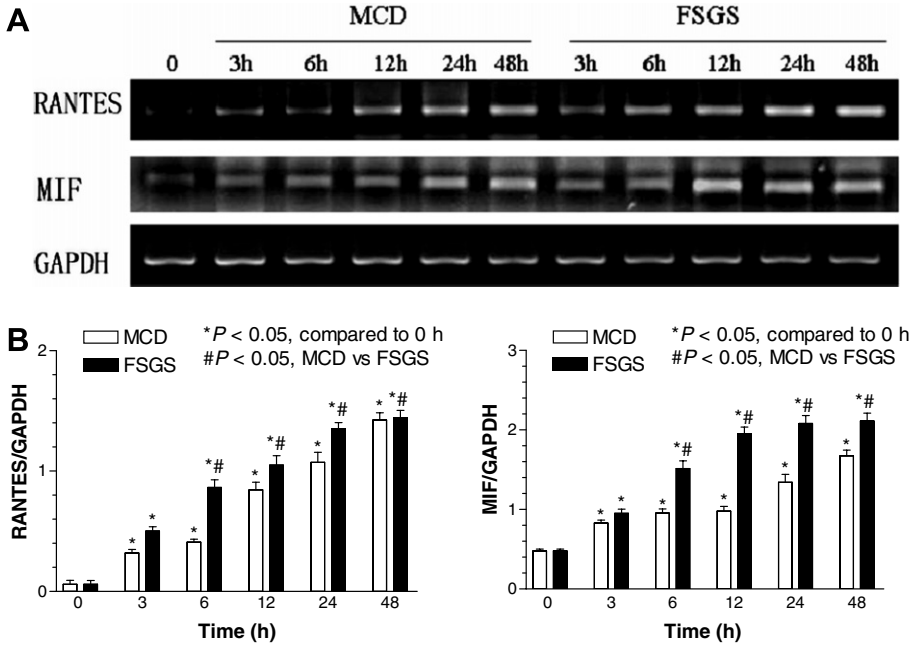


Fig. 4. Effects of treatment of HK-2 cells with urine proteins from MCD and FSGS patients at 5 mg/ml on *RANTES* and *MIF* mRNA expression over 48 h. HK-2 cells were treated with urine proteins at 5 mg/ml from MCD or FSGS patients over 48 h. The mRNA was extracted and its level was determined by RT-PCR. The pictures shown are representative of experiments performed on urine proteins of all MCD and FSGS patients. Data are means ± SD of at least three independent determinations. $^{*}P < 0.05$, vs. zero time; $^{\#}P < 0.05$, FSGS vs. MCD patients.

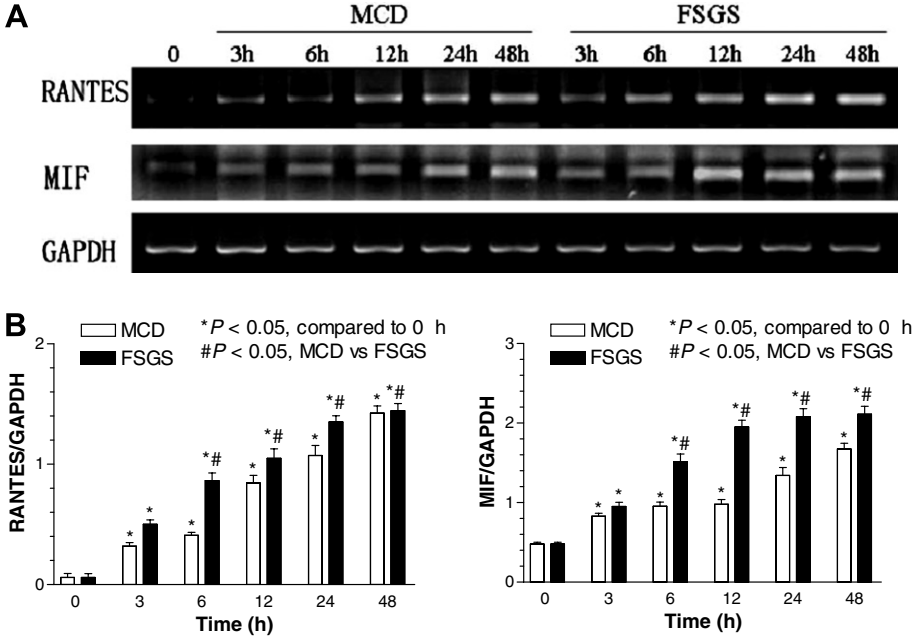


Fig. 5. Effects of urine protein concentrations (0.1–10) mg/ml on *RANTES* and *MIF* mRNA expression in HK-2 cells over 24 h. HK-2 cells were treated with urine proteins at 0.1–10 mg/ml from MCD or FSGS patients for 24 h. The mRNA was extracted and its level was determined by RT-PCR. The pictures shown are representative of experiments performed on urine proteins of all MCD and FSGS patients. Data are means ± SD of at least three independent determinations. $^{*}P < 0.05$, vs. control vehicle; $^{\#}P < 0.05$, FSGS vs. MCD patients.

of HK-2 cells with ultrafiltered urine proteins from MCD patients at 0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 mg/ml for 24 h significantly increased the expression of *RANTES* 2.3-, 3.3-, 3.6-, 4.2-, 4.2- and 4.2-fold; and 1.1-, 1.1-, 1.7-, 1.7-, 1.9- and 2.2-fold for *MIF* expression, respectively, compared to the control (zero time incubation) ($P < 0.05$, Fig. 5). When the cells were treated with urine proteins at above concentrations from FSGS patients for 24 h, the expression of *RANTES* mRNA was significantly increased 3.4-, 3.5-, 4.4-, 4.7-, 4.8- and 6.3-fold, and the *MIF* mRNA

levels were significantly increased 1.3-, 2.5-, 2.6-, 3.0-, 2.8- and 3.0-fold, respectively, compared to the control (zero time incubation) ($P < 0.05$, Fig. 5). The urine proteins from FSGS patients at 0.1 and 10 mg/ml induced a greater magnitude of *RANTES* mRNA expression compared to those from MCD patients ($P < 0.05$), and the urine proteins from FSGS patients at 0.5–10 mg/ml induced a greater magnitude of *MIF* mRNA expression compared to those from MCD patients ($P < 0.05$).

We compared the effects of HSA and urine proteins from MCD or FSGS patients on the expression of *RANTES* and *MIF* mRNA in HK-2 cells. Fig. 6 shows a significantly elevated *RANTES* mRNA expression by 122.5% and 228.3% in HK-2 cells when treated with 5 mg/ml urine proteins from either FSGS or MCD patients, respectively; and *MIF* mRNA levels in HK-2 cells treated with 5 mg/ml urine proteins from either FSGS or MCD patients were significantly increased by 197.3% and 273.3%, respectively. In contrast, HSA at 5 mg/ml only slightly (11.1–17.9%, $P > 0.05$) increased the *MIF* and *RANTES* RNA levels in HK-2 cells.

### 3.5. Effects of ultrafiltered urine proteins from MCD or FSGS patients on RANTES and MIF protein expression in HK-2 cells

The protein level of RANTES was monitored during our studies by a validated ELISA assay. Consistent with the increased mRNA level, the release of RANTES protein by HK-2 cells treated with urine proteins from MCD or FSGS patients was also significantly increased in a time-dependent manner from 12 to 48 h (Fig. 7). Treatment of HK-2 cells with 5 mg/ml urine proteins from MCD or FSGS patients increased RANTES production 52.2-, 80.2- and 138.2-; and 135.1-, 259.8- and 351.5-fold, respectively, compared to the control treatment. The urine proteins from FSGS patients induced a significantly higher production of RANTES in HK-2 cells compared to those from MCD patients.

The marked increase in RANTES and MIF protein levels in HK-2 cells with exposure to urine proteins from MCD or FSGS patients was further confirmed by flow cytometric analysis (Fig. 8). The basic levels of RANTES and MIF proteins in HK-2 cells were low as determined by flow cytometry. HSA treatment induced RANTES and MIF production by 230% ($P < 0.05$) and 39.4% ($P > 0.05$). The urine proteins from MCD and FSGS patients significantly increased the production of RANTES and MIF 17.0- and 49.0-fold and 3.1- and 4.4-fold, respectively. Apparently, the urine proteins from FSGS patients induced a significantly higher production of both RANTES and MIF proteins.

Our immunofluorescence analysis demonstrated a low (background) level of MIF and RANTES proteins in untreated HK-2 cells. The intensity of these two proteins in HK-2 cytoplasmic regions was markedly increased following treatment with urine proteins from MCD or FSGS patients. Representative photomicrographs were exhibited in Fig. 8A and C. The protein levels of RANTES and MIF in HK-2 cells treated by urine proteins from FSGS

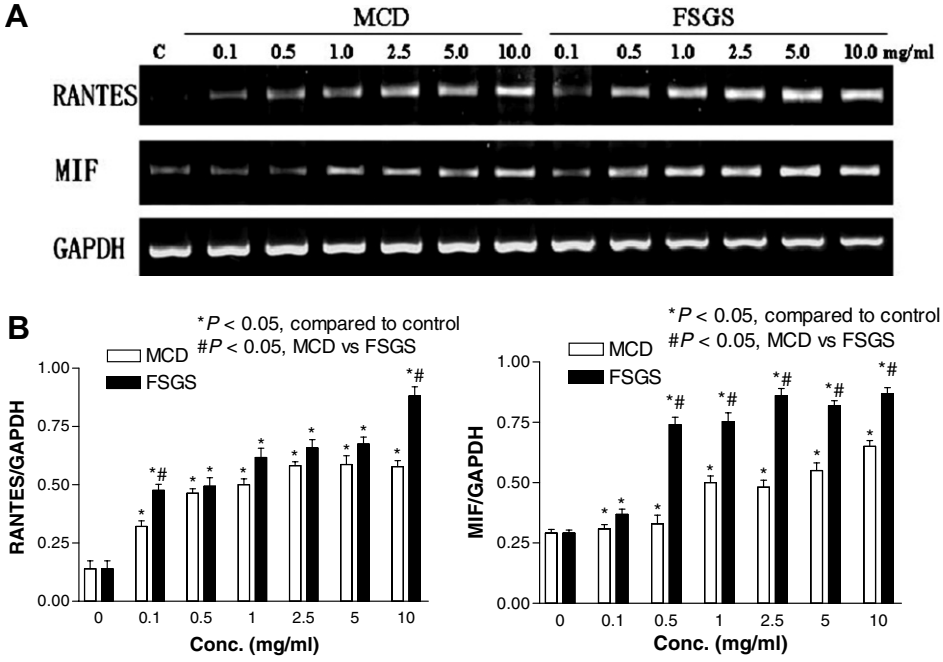


Fig. 6. *MIF* and *RANTES* mRNA expression in HK-2 cells treated with 5 mg/ml HSA or urine proteins from MCD and FSGS patients. HK-2 cells were treated with 5 mg/ml urine proteins or HSA for 24 h. (A) M1–M4: MCD patients; and F1–F6: FSGS patients. The pictures shown are representative of experiments performed on urine protein of all patients. (B) Data are expressed as RANTES/GAPDH or MIF/GAPDH ratio and are given as means ± SD from three experiments. $^{*}P < 0.05$, urine proteins vs. HSA; $^{\#}P < 0.05$, FSGS vs. MCD patients.

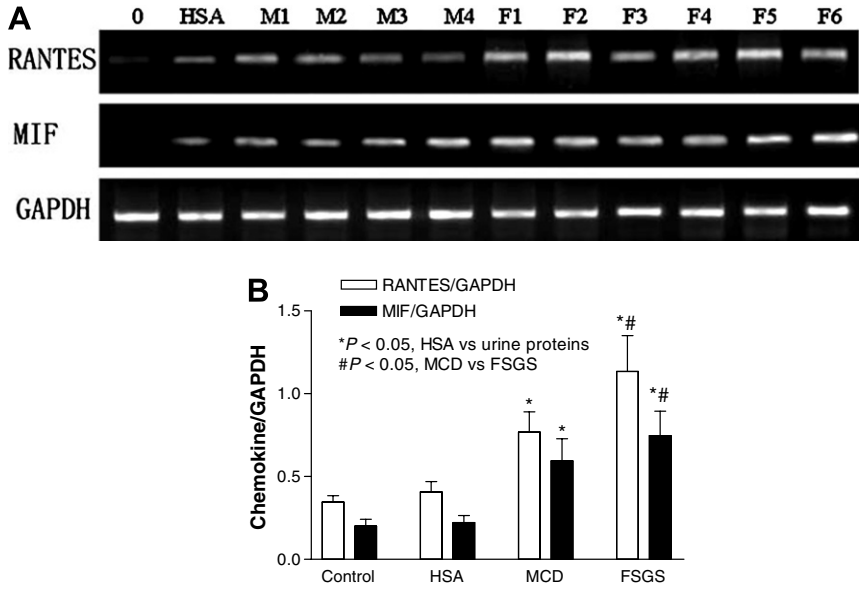


Fig. 7. Release of RANTES in HK-2 cells treated with 5 mg/ml urine proteins from MCD or FSGS patients. HK-2 cells were incubated with urine proteins at 5 mg/ml over 48 h. The release of RANTES protein in culture medium at 12, 24 and 48 h after urine protein treatment was analyzed by ELISA. Data are mean of at least three independent determinations. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$; FSGS vs. MCD patients.

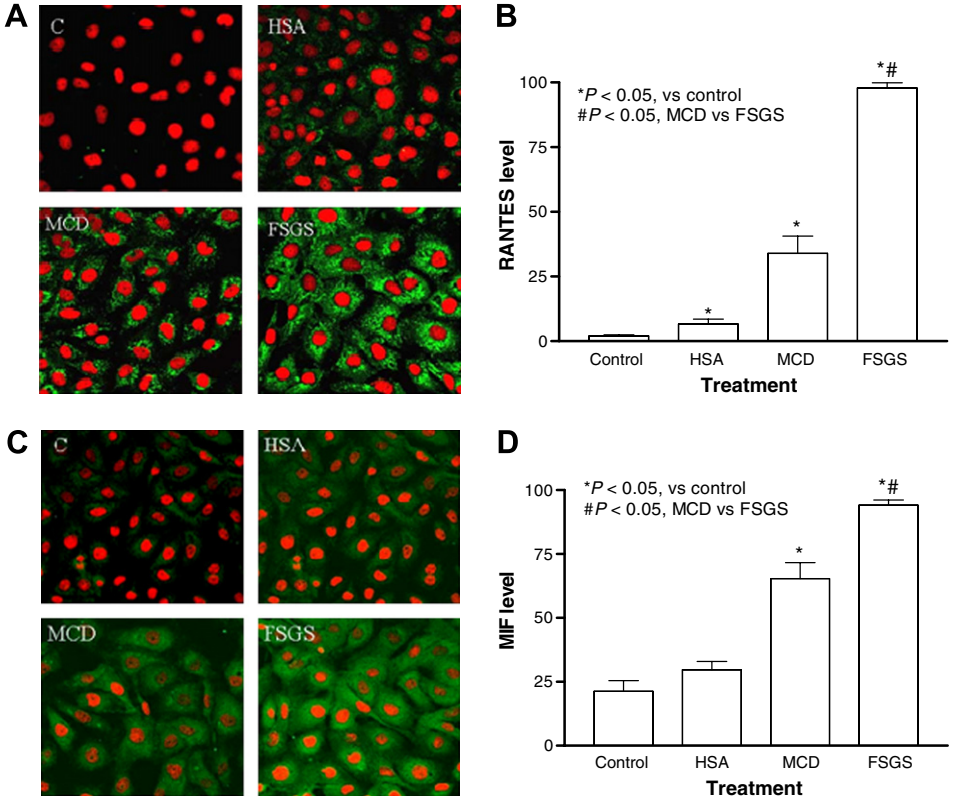


Fig. 8. Expression of RANTES and MIF proteins in HK-2 cells treated with 5 mg/ml HSA or urine proteins from MCD and FSGS patients by confocal immunofluorescence microscope and flow cytometric analysis (FACS). The expression of RANTES (A) and MIF (C) were observed by confocal microscopic image stained immunofluorescently. The images are magnified at 100×. The cells were analyzed by FACS, and RANTES (B) and MIF (D) expression was measured by mean fluorescence intensity. Data are mean values from three measurements. $^{*}P < 0.05$, urine proteins vs. HSA; $^{\#}P < 0.05$, FSGS vs. MCD group.

patients was significantly greater than those of cells treated by urine proteins from MCD patients. However, HSA at the same concentration failed to significantly elevate RANTES and MIF protein expression in HK-2 cells.

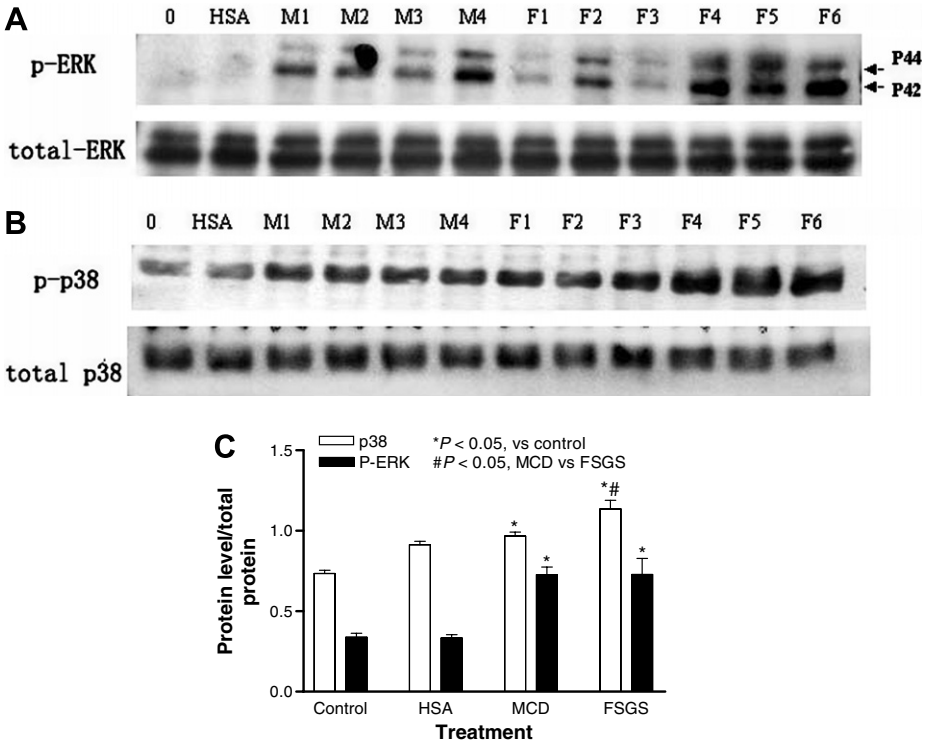


Fig. 9. ERK1/2 and p38 protein expression in HK-2 cells treated with 5 mg/ml urine proteins from MCD and FSGS patients. HK-2 cells were incubated with urine proteins for 20 min and ERK1/2 and p38 levels were determined by Western blotting analysis. M1–M4: MCD patients; F1–F6: FSGS patients.

### 3.6. Effect of urine proteins on activation of MAPK pathway

Fig. 9 shows a significantly elevated phospho-ERK1/2 and p38 levels by 114.9% and 31.7%, respectively, as determined by immunoblotting analysis, in HK-2 cells treated with 1.0 mg/ml urine proteins from MCD patients for 20 min. The urine proteins from FSGS also significantly increased phospho-ERK1/2 and p38 levels by 115.1% and 54.85%, respectively. However, treatment of HSA at 1.0 mg/ml insignificantly increased ERK1/2 and p38 expression by 0–24.5% ($P > 0.05$).

### 3.7. Effects of SB203580 and PD98059 on RANTES and MIF mRNA expression

Because some controversy exists regarding the specific form of MAPK that is necessary and sufficient for production of chemoattractants in response to damage caused by urine proteins, we evaluated the effects of two specific MAPK inhibitors, PD98059 and SB20358, on urine protein mediated chemotaxis of human PTECs. PD98059 at 50 mM, a specific inhibitor of ERK1/2 pathway, almost completely inhibited urine protein-mediated activation of ERK1/2. Pretreatment of PD98059 at 50 mM led to significant reduction of *RANTES* and *MIF* mRNA expression in cells exposed to urine proteins from FSGS patients (Fig. 10). However, it had no significant inhibitory effect on elevated *RANTES* and *MIF* expression by urine proteins from MCD patients.

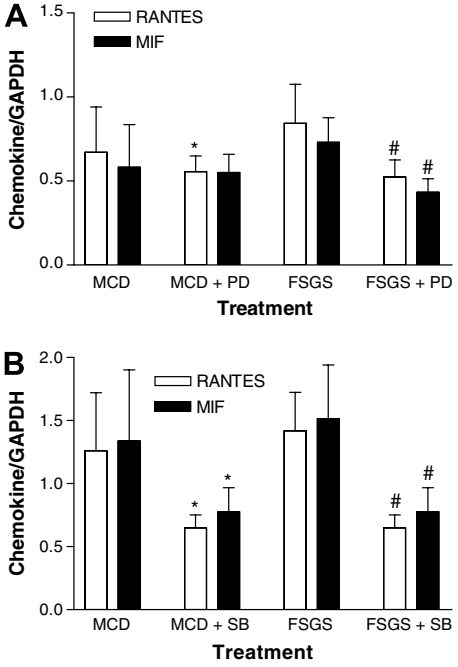


Fig. 10. Effects of PD98059 and SB203580 on *RANTES* and *MIF* mRNA expression induced by urine proteins from MCD and FSGS patients in HK-2 cells. HK-2 cells were incubated with urine proteins at 1 mg/ml for 20 min in the presence or absence of SB203580 or PD98059. The mRNA expression of *RANTES* and *MIF* was detected by RT-PCR. (A) PD: PD98059; (B) SB: SB203580. Data are expressed as RANTES/GAPDH or MIF/GAPDH ratio and are given as means ± SD from three independent experiments. $^{*}P < 0.05$, MCD+SB/PD vs. MCD; $^{\#}P < 0.05$, FSGS+SB/PD vs. FSGS.

On the other hand, SB20358 at 10 mM, a specific inhibitor of p38 pathway, almost abolished the activation of p38 mediated by urine proteins in our preliminary experiment. Increase of *RANTES* and *MIF* mRNA expression induced by urine proteins from MCD or FSGS patients was significantly suppressed by pretreatment with SB20358 at 10 mM. Incubating HK-2 cells with the control vehicle alone (0.1% DMSO, v/v) resulted in no significant inhibition of both ERK1/2 and p38 expression.

## 4. Discussion

In our study, we extracted the urine protein mixture using ultrafiltration techniques and treated PTECs with such extract to imitate the overall damage of proximal tubule in renal disease in vivo. Ultrafiltration techniques have a number of advantages over other chemical purification techniques, being a less time-consuming process and excluding any chemical and heating processing to maintain activity and integrity of proteins to a maximum extent. The urine proteins from both MCD and FSGS patients were carefully processed during lyophilization and ultrafiltration to retain the activity of protein molecules and to avoid contamination and addition of external substances. The resultant ultrafiltrates are totally different from those from plasma in terms of the presence of cytokines and other potentially toxic molecules that are secreted by tubular cells.

Electrophoretic analysis of the urine protein extracts showed similar band profiles between FSGS and MCD patients, with a primary band of proteins with Mr of approximately 62 kDa (Fig. 1.). However, the urine extracts from FSGS patients contained several marked bands with Mr > 250 kDa, but these bands were not apparent for urine samples from MCD patients. It appeared that FSGS patients excreted more proteins with a large molecular weight from their urine compared to MCD patients.

Some large molecular proteins such as immunoglobin and complement were well known frequently detected in non-selective proteinuria. The urine protein extracts appear largely mimic the composition of glomerular ultrafiltrates in tubular lumen in nephrotic syndrome and thus provide a useful in vitro model for mechanistic study of tubular damage induced by proteinuria.

To examine the concentrations of six proinflammatory cytokines in urine proteins, the CBA technique was applied. Our study showed that there was a large variability in the levels of six cytokines in urine proteins from MCD or FSGS patients (Fig. 2). The major cytokines present in urine proteins from both MCD and FSGS patients were IL-6 and IL-8, while IL-1β, IL-10, IL-12p70 and TNF-α were only detectable. The urinary cytokines at low concentrations in MCD and FSGS patients may act as a less important foster to activate tubular cells in proteinuria. Recent evidence suggested that urinary cytokines may be secreted by activated and proliferative glomerular cells and correlated with unfavorable clinical outcomes [21,22]. High urinary levels of cytokines have been more frequently demonstrated in the patients with proliferative glomerulonephritis, but not in the patients with non-proliferative glomerulonephritis such as MCD and FSGS [6]. Urinary cytokines, especially TNF-α and IL-6, can be secreted by activated and proliferative glomerular cells [21,22].

Our studies have demonstrated that the urine proteins from both MCD and FSGS patients at higher concentrations (>5 mg/ml) significantly inhibited the proliferation of HK-2 cells (Fig. 3), which was accompanied by increased leakage of LDH from the cells. Such cytotoxicity was more apparent when the cells were treated with urine proteins from FSGS patients compared to those from MCD patients. These results are in agreement with previous studies where exposure to protein overload induces a decrease in proliferation and significant release of LDH of tubular cells in vivo and in vitro and protein fractions containing high molecular weight proteins overload are more cytotoxic [23–25]. The mechanism for the cytotoxicity of urine proteins may be related to apoptosis induction and cell necrosis initiated by toxic substances such as TNF-α and other cytokines in the urine.

Through tubular cells with exposure to urine proteins, we found that urine protein induced significantly elevation in expression of *RANTES*/*CCL5* and *MIF* at both mRNA and protein levels (Figs. 4–6), which is consistent of previous studies in the stimulation of single component (e.g. albumin or transferrin) overload in vitro [26,27]. Therefore, our study has further confirmed that proteinuria may induce tubulointerstitial damage by activating the synthesis of inflammatory mediators and induce intrinsic renal toxicity.

To check on the protein production of RANTES and MIF in HK-2 cells coincubated with urine proteins from MCD and FSGS patients, the following techniques were applied: ELISA, immunofluorescence microscope and flow cytometric analysis. Consistent with increased *RANTES* and *MIF* mRNA levels, urine proteins from MCD and FSGS significantly increased the production of RANTES and MIF in HK-2 cells but the latter elicited a greater effect compared to the urine proteins from MCD (Figs. 7 and 8). The distinct effect was considered due to different composition of proteins in the urine samples from MCD and FSGS patients. Similar to the lack of effect on *RANTES* and *MIF* mRNA expression, HSA treatment did not significantly alter the production of RANTES and MIF by HK-2 cells.

We observed that the proinflammatory potential of urine proteins from FSGS patients appeared to be greater than that of urine proteins from MCD patients because of the more significantly elevated levels of RANTES and MIF in PTECs induced by urine proteins from FSGS patients. The differential effects might be attributed to the difference in the components of urine proteins from FSGS and MCD patients. Despite of unknown composition as yet, presence of macromolecular proteins in FSGS non-selective proteinuria perhaps serve as one of the major mechanism. High molecular weight proteins such as immu-

noglobin and complement have already been proved to induce the higher expression of chemokine and more significant inflammatory activation compared with albumin in renal tubular cells [28,29]. Such evidence perhaps account for the discrepancy over the effect of urine protein between FSGS and MCD nephropathy.

To investigate the mechanism behind the urine protein induced inflammatory activation, we examined the role of ERK and p38-MAPK pathways in HK-2 cells. Our study showed that treatment of urine proteins from MCD or FSGS patients mediated a significantly activation of p38-MAPK and ERK (Fig. 9). The result has been supported by previous finding that the stimulation of plasma component (e.g. albumin or transferrin) overload activated MAPK pathway in cultured tubular cells [30].

To explore the molecular mechanism for the differential chemokine induced turnover in tubular cells, we proceeded to apply two widely used specific inhibitors, PD98059 and SB203580, in our inhibition studies. Our data shown that the elevated expression of *RANTES* and *MIF* induced by urine proteins from MCD patients was mediated by p38-MAPK pathway but not ERK pathway, while both ERK and p38-MAPK pathways were involved in the increased MIF and RANTES secretion induced by urine proteins from FSGS patients. The discrepancies of the intracellular signaling mechanisms may be due to the differential regulation of some specific components in urine proteins on chemokine secretion. Earlier studies had reported that serum IgG, other than albumin or transferrin, could induce and increase IL-6 secretion via early activated of ERK in PTECs [31].

Previous studies have shown that exposure to albumin induced increased expression and secretion of various chemokines in tubular cells via activated MAPK pathway [31–35]. However, in our study, purified albumin (5 mg/ml) alone had no or minor effect on up regulation of *RANTES* and *MIF* expression. Moreover, HSA at 1 mg/ml failed to effectively activate ERK and p38-MAPK pathways [10]. Discrepancies over the effect of albumin in response to protein overload can be attributed to differences in experimental design. The concentration of albumin (>10 mg/ml) that is required to induce inflammatory responses in previous studies exceeded the concentration of albumin in proximal tubular fluid even in severe nephrotic syndromes [36–38]. Thus, our findings further confirmed that the stimulation of serum albumin should not be considered as the major factor responsible for tubular cell damage in the progression of tubulointerstitial fibrosis though it is the most abundant component in proteinuric conditions.

Our study has important clinical implications. The differential production of RANTES and MIF and involvement of p38 and ERK pathways in tubular cells in response to urine proteins from MCD and FSGS patients suggest that these cytokines and signaling pathways may represent potential novel therapeutic targets but the therapeutic outcome might be very different. The differential chemokine production and p38 and ERK activation in vitro by urine proteins from MCD and FSGS patients may be used to predict disease status and progression in vivo, although further clinical studies are needed.

In conclusion, we have demonstrated that urine proteins from FSGS induced the significantly higher expression of chemokines in tubular epithelial cells compared to urine proteins from MCD patients through distinct MAPK-associated signaling pathways. Though a comprehensive analysis of components in urine proteins should be required in our further study, it has provided initial evidence that the quality of proteinuria plays an important role in eventually determining the severity and progression of tubular inflammatory injury associated with different kidney diseases.

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