Urinary Excretion of Monocyte Chemoattractant Protein-1 in Autosomal Dominant Polycystic Kidney Disease

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Abstract. Autosomal dominant polycystic kidney disease (ADPKD) progresses to renal insufficiency in >50% of patients and is characterized by interstitial inflammation and fibrosis in the end stage. In a rat model of ADPKD, monocytes accumulate within the renal interstitium in association with increased levels of monocyte chemoattractant protein-1 (MCP-1) in cyst mural cells and increased excretion of this chemokine into the urine. For determining the extent to which this chemokine is abnormally expressed in patients with ADPKD, a cross-section study was performed of MCP-1 in urine, serum, and cyst fluid and MCP-1 production by mural epithelial cells cultured from the cysts of human patients with ADPKD. Upper boundaries for urinary MCP-1 excretion (>263 pg/mg creatinine) and serum creatinine concentration (>1.5 mg/dl) determined in 19 normal individuals were used to sort 55 ADPKD patients into three groups. In group 1 ($n = 13$), urine MCP-1 excretion (136 ± 14 pg/mg creatinine) was not different from normal volunteers (152 ± 16 pg/mg); serum creatinine levels and urine total protein excretion were normal as well. In group 2 ($n = 27$), urine MCP-1 excretion was increased (525 ± 39 pg/mg creatinine), but serum creatinine levels and urine protein excretion were not different from normal. In group 3 ($n = 15$), urine MCP-1 excretion increased further (1221 ± 171 pg/mg), serum creatinine levels increased to 4.3 ± 0.8 mg/dl, and urine protein excretion rose to 0.64 ± 0.28 mg/mg creatinine. Serum MCP-1 levels of ADPKD patients (84 ± 9.9 pg/ml; $n = 15$) did not differ from normal. Levels of MCP-1 much higher than in serum or urine were found in cyst fluids obtained from nephrectomy specimens (range, 767 to 40,860 pg/ml; mean, 6434 ± 841 pg/ml; $n = 73$). Polarized, confluent cultures of ADPKD cyst epithelial cells secreted MCP-1 into the apical fluid to levels eightfold greater than in the basolateral medium. Similar results were obtained with tubule epithelial cells cultured from normal human renal cortex. On the basis of these results, it is concluded that urinary excretion of MCP-1 is increased in the majority of adult patients with ADPKD and that the source of some of this chemokine may be the mural epithelium of cysts. Furthermore, it seemed that urinary MCP-1 excretion may have increased in these ADPKD patients before appreciable increases in serum creatinine concentration or urine protein excretion were detected. It is reasonable to include urine MCP-1 excretion among candidate surrogate markers in controlled, longitudinal studies of ADPKD.

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited disorder in which renal tubules develop in fluid-filled cysts in childhood that progressively enlarge for decades (1,2). In approximately one half of the patients, the progressive enlargement of the kidneys leads to the loss of renal function manifested in the early stages by hypertension and later by proteinuria and progressive azotemia. How to account for the fact that only a portion of the patients with ADPKD progress to end-stage renal failure is a mystery that has not been solved.

Morphologic studies of cystic kidneys in humans and animals indicate that cysts develop in a relatively small fraction of renal tubules (3,4), possibly the consequence of a "second-hit" process (5,6). Most of these early cysts exhibit abnormalities of the tubule basement membranes and infiltration of the adjacent interstitium with macrophages and fibroblasts, suggesting a tissue response to the presence and the enlargement of the cysts. A rat model of ADPKD (Han:SPRD Cy) mimics the renal pathology and the clinical course in humans to a remarkable extent (4). Cysts develop in a minority of renal tubules shortly after birth and progressively enlarge, leading to death of males from renal insufficiency in approximately 1 yr. Remarkably, females are protected from renal insufficiency for approximately 2 yr, although renal cysts develop and enlarge as in males. The major difference between the male and female kidneys is the inflammatory infiltrate and fibrosis found relatively early in the Han:SPRD Cy males. In a recent study, Cowley *et al.* (7) found increased expression of monocyte chemoattractant protein-1 (MCP-1) and osteopontin, chemotactic cytokines, within epithelial cells lining the cysts in association with an increased number of interstitial macrophages. This raised the possibility that chemotactic cytokines expressed abnormally in PKD might have a role in determining the variable course of ESRD. In the current study, we determined the extent to which patients with ADPKD may elaborate increased amounts of MCP-1 in the urinary tract.

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Materials and Methods

ADPKD Patients

Aliquots of discarded urine from 55 ambulatory ADPKD patients (22 men, 33 women; age 22 to 79 yr) in the Polycystic Kidney Clinic at the Kansas University Medical Center during 1998 to 2001 were evaluated in the study. Fresh samples were collected and frozen at −20°C. ADPKD was diagnosed by characteristic ultrasound or computed tomography presentation of the kidneys and confirmed by the presence of liver cysts or positive family history. None of the patients had evidence of clinical renal infection when urine samples were obtained. None had been treated with dialysis or renal transplantation. Serum creatinine levels ranged between 0.5 and 11.9 mg/dl (median, 1.2). In 17 patients, additional urine samples (two to seven) were obtained over a maximum interval of 21 mo. The initial urine sample was used to date entry of an individual patient into the study. Aliquots of serum were obtained from 15 patients after informed, written consent. Unfortunately, in this retrospective study, serum samples were not available in all patients from whom urine was obtained.

Samples of cyst fluid were obtained from 73 different individuals with ADPKD (7 to 73 yr old) who were nephrectomized in preparation for renal transplantation. Sealed kidneys were placed in wet ice and transported to the laboratory, usually by overnight, expedited delivery. Samples of clear cyst fluid of widely varying amounts were obtained from one to approximately 50 cysts on the surface of each pair of kidneys. Fluids were generally pooled together, although a few individual cyst fluid samples were also obtained from two patients.

Normal Subjects

Nineteen healthy volunteers (9 men, 10 women; 25 to 65 yr old) had serum creatinine values ranging from 0.6 to 1.1 mg/dl. Early morning midstream urine samples were obtained, in women in the middle of the menstrual cycle. The protocol was approved by the Kansas University Medical Center Institutional Review Board, and each volunteer gave informed consent.

Enzyme-Linked Immunosorbent Assay

Samples of urine, serum, and cyst fluid were thawed, and aliquots were taken for the determination of MCP-1, MCP-3, IL-8, and TNF-α by enzyme-linked immunosorbent assay (ELISA) using methods recommended by the manufacturer (Biosource International, Amarillo, CA). These assays used a multiple sandwich, solid-phase enzyme immunoassay that included monoclonal antibodies raised against the respective cytokines. Specifically, the MCP-1 kit used a hamster monoclonal MCP-1 capture antibody and a rabbit polyclonal MCP-1 antibody for detection. Standard buffer in the kit was used to dilute samples with high levels of MCP-1. The enzymatic reactions were quantified in an automatic microplate photometer (Dynatech Laboratories, Chantilly, VA). Cytokine concentrations of unknown samples were determined by interpolation using a standard curve based on recombinant MCP-1 supplied by the manufacturer. Recovery of MCP-1 added to urine was 110 ± 7% (mean ± SEM, $n = 5$). Urine samples were generally diluted 1:2 to 1:20, cyst fluids 1:30 to 1:50, and serum 1:2. The sensitivity of the ELISA for MCP-1 was 20 pg/ml. Samples could be thawed and frozen at least three times without causing a change in MCP-1 concentration (data not shown). Urine cytokine samples (pg/ml) were related to creatinine (mg/ml) concentration and expressed as pg/mg. Urine creatinine was determined with a kit method based on an alkaline picrate colorimetric assay (Sigma Diagnostics, St. Louis, MO) and protein by a kit method (Quantimetrix Corporation, Redondo Beach, CA).

Immunoprecipitation

Samples of urine and cyst fluid were immunoprecipitated to increase sensitivity of Western blots of MCP-1. Protein A Sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) were washed with Triton lysis buffer (TLB; 20 mM Tris [pH 7.4], 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM Pyrophosphate, 1 mM phenymethylsulphonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM benzamidine, and 0.5 mM dithiothreitol) three times, then incubated with polyclonal rabbit anti-human MCP-1 (Research Diagnostics Inc., Flanders, NJ) for 2 h. The antibody-laden beads were washed with TLB three times, and aliquots were incubated with urine (2.5 ml) or cyst fluid (150 to 500 μl) overnight at 4°C, each aliquot sufficient for a single PAGE lane (*vida infra*). MCP-1 bound to the beads was washed with TLB three times then released by heating to 95°C for 5 min in TLB and 2× sample buffer (132 mM Tris-HCl [pH 6.8], 4.2% SDS, 21% glycerol, 0.72 mM β-mercaptoethanol, 20 mM dithiothreitol, and 0.005% bromophenol blue).

Western Blotting of MCP-1

Samples of urine or cyst fluid concentrated by immunoprecipitation were resolved by 15% SDS-PAGE. MCP-1 standards and polyclonal rabbit anti–MCP-1 were obtained from Research Diagnostics Inc., and anti-rabbit IgG–horseradish peroxidase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The transfer of proteins from gels to nitrocellulose membranes (Midwest Scientific, St. Louis, MO) was carried out at 4°C. Kaleidoscope prestained standards or prestained polypeptide markers (Bio-Rad Laboratories, Inc., Hercules, CA) were transferred from gel to membranes, and their position in relation to the experimental samples was aligned carefully. Blocking was carried out with 5% dry milk in Tris-buffered saline Tween (TBS-T; 20 mmol/L Tris-HCl [pH 8.0], 137 mmol/L NaCl, and 0.05% Tween 20). Blocked membranes were cut into left and right portions through the lane of visible markers, then incubated in separate systems. One portion of the divided membrane was incubated with 1:1000 dilution of rabbit polyclonal anti-human MCP-1 and the other with anti–MCP-1 antibody that had been incubated for 8 h in a 5:1 excess (wt:wt) of immunizing peptide (MCP-1). Both portions were incubated in 5% dry milk in TBS-T at 4°C for 20 h, followed by washing three times with TBS-T and incubation with a 1:2500 dilution of anti-rabbit IgG–horseradish peroxidase (Santa Cruz biotechnology) in 5% dry milk in TBS-T for 2 h. The membranes were washed three times in TB-T and visualized with an enhanced chemiluminescence system (Amersham Life Sciences, Arlington Heights, IL).

Culture of ADPKD Cyst Epithelial Cells and Human Kidney Cortex Cells

Epithelial cells from the renal cysts of ADPKD patients and cells from humans with normal renal cortex were harvested and grown as primary cultures using methods described in detail previously (8,9). Briefly, primary epithelial cell cultures from renal cysts of ADPKD patients or normal renal cortex were used for up to four passages. Cells were maintained in DMEM and Ham's F12 (DMEM/F12; JRH Biosciences, Lenexa, KS) supplemented with 5% FBS (Hyclone, UT), 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (ITS; Collaborative Biomedical Products, Bedford, MA). Monolayers of ADPKD and human kidney cortex (HKC) cells were grown on Snapwell supports (0.4-μm pore size; Corning Costar Corp., Cambridge, MA) until they were confluent. The FBS content was reduced to 1% FBS DME/F12 supplemented medium for 48 h, then the FBS and ITS were removed for 24 h to reduce cytokine stimulation. Fresh DMEM/F12 lacking FBS and ITS was added to both apical (0.4 ml)

and basolateral (3 or 5 ml) compartments for 24 h. Samples of apical and basolateral media were taken for the determination of MCP-1 by ELISA. For recovering sufficient MCP-1 for Western blotting, DMEM/F12 supplemented with 1% FBS and ITS was added to the chambers for 72 h after which apical samples were taken.

Statistical Analyses

Data are presented as means ± SEM, and unpaired two-tailed *t* test was used for comparisons between two groups; $P < 0.05$ was considered significant.

Results

Determination of MCP-1 in Urine and Cyst Fluid by Western Blot

The amounts of MCP-1 in urine and cyst fluid were too low to detect in unmodified samples of urine or cyst fluid by conventional Western blotting. Consequently, urine and cyst fluid samples were immunoprecipitated using anti–MCP-1–coated Protein-A Sepharose beads. Western blots were performed on three urine samples from normal and ADPKD patients and on three samples of cyst fluid obtained from surgically removed ADPKD specimens. MCP-1 standards run as a control for the Western blots showed a distinct band at approximately 9 kD (Figure 1A). In two urine samples (70 and 79) selected on the basis of representative levels of MCP-1 detected by ELISA (*vida infra*), two adjacent bands were detected, the lower of which matched the mobility of the monomeric MCP-1 standard. The heavier band is consistent with a posttranslational glycosylated MCP-1 product (10). Both of these bands as well as the standard band were eliminated after preincubation of the antibody with an excess of immunizing MCP-1 peptide (Figure 1A).

In immunoprecipitated samples of cyst fluid, two bands were found, one aligned with the MCP-1 standard and the other at a slightly higher molecular weight (Figure 1B). Both of these bands were eliminated by an excess of competing antigen. These findings indicate that proteins with a molecular weight and immunoreactivity characteristic of MCP-1 are present in ADPKD urine and cyst fluids.

MCP-1 in Urine, Serum, and Cyst Fluid

MCP-1 levels in urine and serum are summarized in Table 1. Urinary MCP-1 was referenced to creatinine, thereby providing an indication of cytokine excretion in relation to GFR (Tables 1 and 2, Figure 2). The concentration of urine MCP-1 referenced to creatinine of 55 ADPKD patients ranged from 43 to 2835 pg/mg. By contrast, the range of values in 19 normal subjects, 36 to 262 pg/mg, was much narrower. The greater urinary excretion of MCP-1 in ADPKD was reflected in a higher mean level (623 ± 73 *versus* 151 ± 16 pg/mg; $P < 0.001$). There did not seem to be a gender difference in the urinary excretion of MCP-1 in either ADPKD patients or normal subjects (Tables 1 and 2). The range and mean values for serum concentrations of MCP-1 did not seem to be different between ADPKD patients and normal subjects, and there was no a gender effect (Table 1).

If renal MCP-1 has either an active or a passive role in the

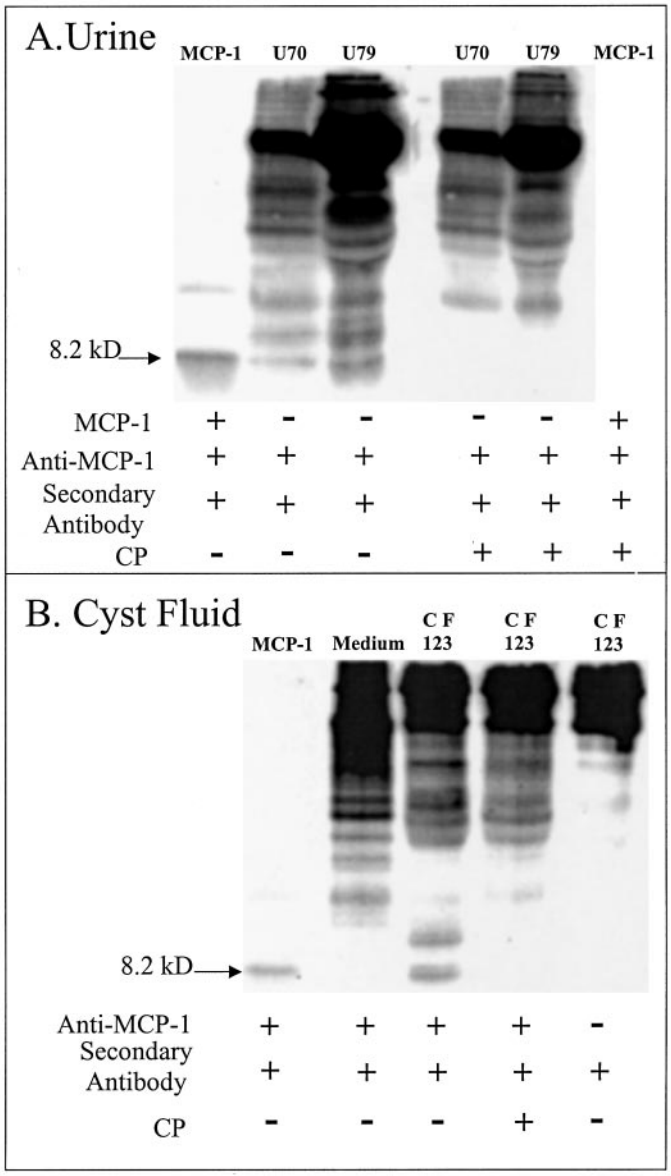

Figure 1. Monocyte chemoattractant protein-1 (MCP-1) protein in autosomal dominant polycystic kidney disease (ADPKD) urine and cyst fluid. Western blots of immunoprecipitated MCP-1 in urine (A) and cyst fluid (B). (A) MCP-1 alone (10 ng) and urine samples from two ADPKD patients (70 and 79) examined with and without an excess of competing peptide (CP). MCP-1 standard in lanes 1 and 6 were not subjected to immunoprecipitation. Molecular weight of monomeric MCP-1 (indicated by arrow at 8.2 kD noting kaleidoscope marker) was approximately 9 kD. The faint band at approximately 18 kD in lane 1 is dimeric MCP-1. The band immediately above MCP-1 in urine (lanes 2 and 3) is most likely glycosylated MCP-1. Heavy staining of the upper portions of the gels, unaffected by incubating anti–MCP-1 with an excess of CP (lanes 4 and 5) was due to secondary antibody binding to nonspecific proteins added during the immunoprecipitation procedure. This staining was absent in the first and last lanes containing MCP-1 standards in medium. (B) Cyst fluid 123 examined with and without anti–MCP-1 incubated with an excess of CP. As in urine, MCP-1 of molecular weight approximately 9 kD was detected as well as a heavier band, both of which were eliminated by incubating anti–MCP-1 with an excess of CP. Lane 5 contains cyst fluid to which secondary antibody was added alone.

Table 1. Components of urine and serum[a]

Group	*n*	Age (Years)	MCP-1 Urine (pg/ml)	MCP-1 Urine (pg/mg Creatinine)	MCP-1 Serum[b] (pg/ml)
Normal	19	39 ± 3	194 ± 44	152 ± 16	73 ± 11 (9)
range			10 – 550	36–262	29–148
female	10	41 ± 4	187 ± 43	148 ± 22	67 ± 6 (6)
male	9	36 ± 4	202 ± 80	155 ± 29	85 ± 35 (3)
ADPKD	55	43 ± 2	511 ± 65	623 ± 73	84 ± 9.9 (15)
range			26 – 1941	43–2835	21–162
female	33	44 ± 2	443 ± 80	664 ± 90	71 ± 19 (5)
male	22	42 ± 2	613 ± 107	562 ± 126	91 ± 12 (10)

[a] MCP-1, monocyte chemoattractant protein-1; ADPKD, autosomal dominant polycystic kidney disease.
[b] Number of serum samples in parentheses.

Table 2. MCP-1 excretion in urine

Group	*n*	Age (Years)	MCP-1 Urine (pg/mg)	Creatinine Serum (mg/dl)	Protein Urine (mg/mg)
Normal	19	39 ± 3	152 ± 16	0.88 ± 0.05	0.059 ± 0.017
female	10	41 ± 4	148 ± 18	0.73 ± 0.02	0.075 ± 0.020
male	9	36 ± 4	155 ± 29	1.05 ± 0.04	0.041 ± 0.028
Group 1	13	43 ± 3	136 ± 14	1.00 ± 0.06	0.017 ± 0.008
female	7	44 ± 3	139 ± 23	1.01 ± 0.10	0.019 ± 0.015
male	6	42 ± 5	134 ± 16	0.98 ± 0.08	0.015 ± 0.008
Group 2	27	39 ± 2	525 ± 39[c,f]	0.97 ± 0.05	0.094 ± 0.031
female	18	40 ± 3	565 ± 55[c,f]	0.87 ± 0.06	0.092 ± 0.031
male	9	37 ± 3	444 ± 26[c,f]	1.18 ± 0.06	0.097 ± 0.070
Group 3	15	50 ± 3[a]	1221 ± 171[c,f,i]	4.3 ± 0.8[c,f,i]	0.64 ± 0.28[b,d,h]
female	8	53 ± 5[g]	1345 ± 174[c,f,i]	4.1 ± 0.7[c,f,i]	0.30 ± 0.18[b,d,h]
male	7	48 ± 3[a,g]	1080 ± 315[a,b,d]	4.6 ± 1.5[a,d,g]	0.92 ± 0.47[a]

Versus normal: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.
Versus group 1: [d] $P < 0.05$, [f] $P < 0.001$.
Versus group 2: [g] $P < 0.05$, [h] $P < 0.01$, [i] $P < 0.001$.

pathogenesis of disease progression as suggested by the earlier study of the Han:SPRD rat (7), then one would expect to see an inverse relationship between the urinary excretion of MCP-1 and the decrease in renal function reflected in the GFR or increased levels of proteinuria, traditional markers of disease progression. Because of the nature of this study, we could evaluate only two classical markers of renal dysfunction, serum creatinine concentration and urine protein excretion. There seemed to be a direct relation between urine MCP-1 excretion and serum creatinine concentration, but it was not linear (Figure 2). Urine MCP-1 excretion and serum creatinine concentration were best fit to a parabolic curve inscribed by a two-parameter equation (curve and equation not shown, $r^2 = 0.605$). The relation between urine MCP-1 and serum creatinine concentration suggests but does not prove a possible linkage between the magnitude of chemokine excretion and the degree to which creatinine clearance may be decreased.

When the data for normal subjects were added to this plot, the narrow ranges compared with that of the ADPKD cohort suggested another way to examine the data. In this snapshot study of ADPKD including patients with the disease ranging from relatively mild to severe, more than likely some of the 40 patients with serum creatinine levels in the normal range would ultimately progress to the point at which the serum creatinine level would become clearly abnormal. Furthermore, it would be interesting to know whether urine MCP-1 or protein excretion might indicate which of those patients might be at higher risk of progressing to ESRD.

To facilitate the evaluation, we selected upper limits for urine MCP-1 (263 pg/mg) and serum creatinine (1.5 mg/dl) on the basis of the normal subjects in the study. These limits are represented by dashed lines in Figure 2. Examined in this light, the ADPKD patients were sorted into three groups (Table 2). Thirteen patients fell within the normal limits (group 1), and in these the urine protein excretion rate was normal as well.

Twenty-seven of the ADPKD patients had urinary MCP-1 excretion levels higher than normal, yet their serum creatinine concentrations were within normal limits (group 2). In these group 2 patients with urine MCP-1 excretion levels that were clearly elevated, there was no detectable increase in urine

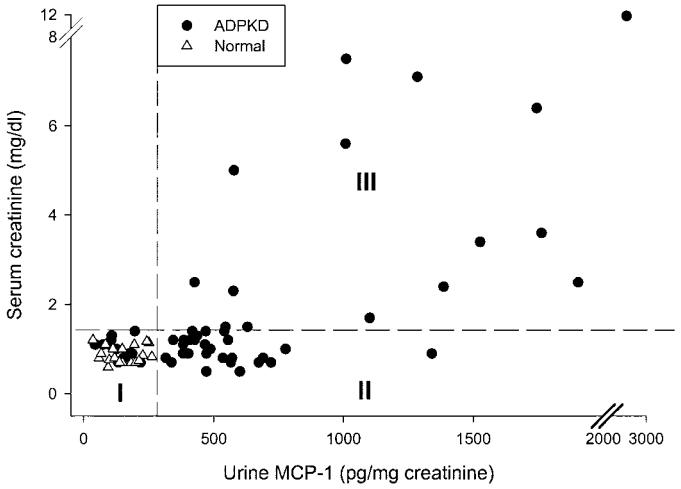

Figure 2. Relation between urine MCP-1 excretion and serum creatinine concentration. Normal values for urine MCP-1 fell below 263 mg/mg (vertical dashed line) and serum MCP-1 below 1.5 mg/dl (horizontal dashed line). ADPKD patients were divided into groups 1 to 3 determined by urine MCP-1 excretion and serum creatinine levels. The ADPKD data could be fit best to a two-parameter exponential equation that inscribed a parabolic curve ($r^2 = 0.605$).

protein excretion (Table 2). Fifteen of the ADPKD patients had elevated urine MCP-1 and serum creatinine levels (group 3); in these patients, urine protein excretion was also significantly increased.

In 17 ADPKD patients, urine MCP-1 measurements were made on more than one occasion (Figure 3). In most of the patients, MCP-1 excretion was relatively stable for as long as 21 mo.

In cyst fluids removed from ADPKD kidneys in a different cohort of patients, MCP-1 concentrations ranged from 767 to 40,860 pg/ml (mean, 6434 ± 841 pg/ml; $n = 73$). This mean level greatly exceeded the average MCP-1 concentrations in urine or serum from both ADPKD patients and normal subjects (Tables 1 and 2).

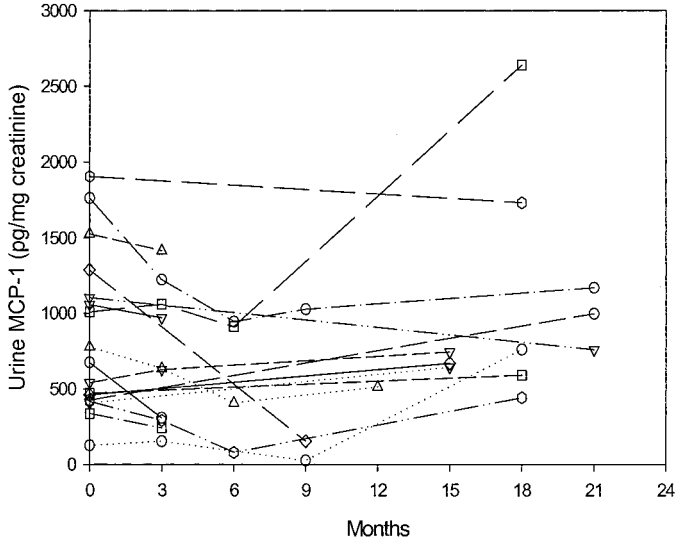

Figure 3. Time course of urine MCP-1 excretion in ADPKD patients. Seventeen ADPKD patients had sequential measurements of MCP-1 for as long as 21 mo.

Secretion of MCP-1 by ADPKD Cyst Epithelial Cells in Culture

We cultured ADPKD cyst cells on polarized supports to determine whether the mural epithelium might contribute to the high levels of MCP-1 found in cyst fluid. After the cultures reached confluence, they were washed thoroughly with fresh medium and placed in a nutrient medium containing minimal growth factors and no known cytokine agonists. Primary cultures from normal human renal cortex were treated identically. After 24 h, the conditioned media bathing the apical and basolateral surfaces of the cultured cells were collected and MCP-1 was determined by ELISA. The concentrations of MCP-1 achieved in the fluids bathing the apical surfaces of both ADPKD and HKC cells were seven to eightfold higher than in the basolateral fluids (Figure 4). This indicates that the MCP-1 was released by the cells across the apical plasma membranes. Taking into account the different volumes of the apical (0.4 ml) and basolateral compartments (3 or 5 ml), the total amounts of MCP-1 produced *in vitro* were not different between ADPKD and HKC cells (Figure 4).

To determine the nature of the proteins secreted by ADPKD and HKC cells in culture, we performed Western blots on unmodified conditioned medium collected over a 72-h interval. As was found in ADPKD urine and cyst fluid (Figure 1), two adjacent bands were detected in conditioned medium obtained from both ADPKD and HKC cells. Both bands were eliminated by incubation of the antibody used in the Western blot with an excess of immunizing peptide (Figure 4).

We could not quantify MCP-1 in urine and cyst fluid by Western blotting because the samples were concentrated by immunoprecipitation. The concentrations of MCP-1 in conditioned medium were high enough and that of other proteins sufficiently low that Western blots could be obtained after direct application of sample to the gels. The levels of MCP-1 in conditioned media determined by ELISA and those determined by quantitative Western blot analysis fell within the same order of magnitude (data not shown).

Other Cytokines Excreted in Urine and Cyst Fluid

MCP-1, MCP-3, IL-8, and TNF-α levels were determined in the urine selected from five ADPKD and three normal individuals and in cyst fluids from seven polycystic kidneys (Table 3). Because the intent of this experiment was to determine the extent to which the different cytokines were expressed in concert with MCP-1, the ADPKD urine and cyst fluids were selected to give a broad range of MCP-1 concentrations. Because we did not anticipate any correlation among the cytokines in the normal urine, we selected three samples from those with the higher levels of MCP-1 to gain a small glimpse of the level of cytokine excretion in normal individuals. In ADPKD patients, the excretion rates of MCP-3, IL-8, and TNF-α were significantly less than MCP-1, as were the levels of these

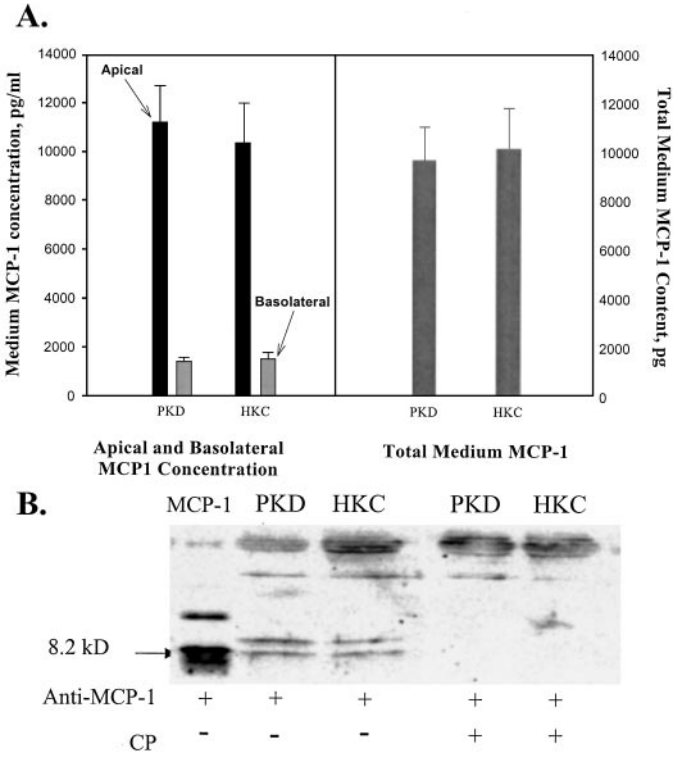

Figure 4. Appearance of MCP-1 in medium used to culture renal epithelial cells. (A) MCP-1 concentrations determined in cyst epithelial cultures from four ADPKD specimens and four human kidneys without ADPKD (human kidney cortex [HKC]). Vertical bars indicate MCP-1 concentrations (mean ± SEM) in apical and basolateral fluids. The total amounts of MCP-1 accumulated in both apical and basolateral fluids of the cultures are shown by the two vertical bars on the right. (B) Western blot of conditioned medium (72 h) from ADPKD and HKC cultures with and without incubation with anti–MCP-1 antibody containing an excess of CP. In the first lane, MCP-1 (10 ng) was added to medium alone. The bands represent monomeric MCP-1 of approximately 9 kD and dimeric MCP-1 of approximately 18 kD. Two bands were detected in the conditioned medium of PKD and HKC cells, one consistent with MCP-1 and the other a glycosylated derivative.

cytokines in cyst fluid (Table 3). The urinary excretion of MCP-1 correlated directly with the excretion of IL-8, although the urine levels of IL-8 did not seem to rise to the same extent as MCP-1. There was no correlation between urinary MCP-1 and MCP-3 or TNF-α. In cyst fluid, MCP-1 levels seemed to correlate with IL-8 and to a lesser extent with MCP-3 but not with TNF-α.

Discussion

ADPKD is the product of mutated genes, *PKD*1 and *PKD*2, that causes a relatively small fraction of the renal tubules to form cysts (1). The initiation of focal cyst formation is dependent on the malfunction of the gene products, polycystin-1 and polycystin-2, leading to altered intracellular calcium levels and impaired ciliary function that provoke tubular epithelial cells to proliferate abnormally (11). Thickening and fragmentation of tubule basement membranes and expansion of the interstitium with infiltration of macrophages appears early in the evolution of the cysts (12–15). The early changes in the interstitium are thought to be important in the formation and enlargement of cysts and in their more long-term effects to reduce renal function.

In the Han:SPRD Cy rat with ADPKD, tubulointerstitial abnormalities appear coincident with the formation of cysts (13–15). In this model, the mural epithelial cells in the cysts express increased levels of chemokines such as MCP-1 and osteopontin (7). These findings raised the possibility that early events in cystogenesis, *e.g.*, stress or injury in response to the altered tubule growth, development, and maturation, may provoke the expression of cytokines by the cells. It seemed appropriate, therefore, to determine whether MCP-1 may have a potential pathogenetic role in ADPKD patients.

We used immunoblotting to demonstrate that urine and cyst fluid harbored proteins with molecular weight and immunoreactivity properties consistent with MCP-1 (Figure 1). On the whole, urine MCP-1 concentrations were higher in ADPKD patients than in a cohort of normal individuals without renal disease (Tables 1 and 2); MCP-1 reached extraordinarily high levels in cyst fluids removed from patients with ADPKD. However, MCP-1 concentrations in ADPKD sera were not greater than in normal individuals (Table 1). These findings suggested that increased amounts of the chemokine in urine were most likely dependent on diminution of tubular reabsorption or possibly net tubular secretion.

The amount of MCP-1 excreted in the urine seemed to be related directly to the serum creatinine concentration in ADPKD patients (Figure 2). The upper boundaries for urine MCP-1 referenced to creatinine ($<$263 pg/mg) and for serum creatinine concentration ($<$1.5 mg/dl) in normal individuals defined three groups of ADPKD patients: group 1 had no increase in urine MCP-1 excretion or serum creatinine concentration, group 2 had increased levels of urine MCP-1 excretion and normal serum creatinine concentration, and group 3 had increased levels of both urine MCP-1 excretion and serum creatinine concentration. The excretion of total urinary protein was not different from normal in group 1 and in group 2 but was significantly elevated in group 3 (Table 2). The findings in group 2 suggest that MCP-1 excretion may rise to a detectable level before either the serum creatinine concentration or the excretion of total protein rise to an appreciable degree.

The relation between urine MCP-1 excretion and serum creatinine concentration in the 55 ADPKD patients shown in Figure 2 could be fit to a linear equation ($r^2 = 0.544$). However, the correlation coefficient was even higher ($r^2 = 0.605$) when it was described by a two-parameter exponential equation (not shown), which yielded a parabolic curve (not shown). This suggests that urine MCP-1 and the rise in serum creatinine concentration may be reflecting the impact of similar mechanisms, but because of the kidney's capacity to compensate for large-scale losses of functioning glomerular units by hyperfiltration in unaffected glomeruli, serum concentrations of creatinine will increase above normal limits later than pathologic substances that are secreted into the urine. In view of these considerations, we suggest that the severity of renal injury and

Table 3. Cytokines in urine and cyst fluid[a]

	MCP-1	MCP-3	IL-8	TNF-α
ADPKD urine	(pg/mg)	(pg/mg)	(pg/mg)	(pg/mg)
60	43.5	15.8	3.2	7.3
59	99.1	23	42.2	7.8
4	1763	0	56.2	0
31	1905	19.9	77.6	1.4
79	2835	25.1	162.1	29.4
mean	1329	16.8	68.3	9.2
Correlation with MCP-1 (r^2)		0	0.79	0.20
Cyst fluid	(pg/ml)	(pg/ml)	(pg/ml)	(pg/ml)
121	1023	0	21.2	20.6
103	1041	0	52.3	7.1
126	1873	0	120.5	14.3
64	14494	439	823	0
153	21457	75.4	1689	7.1
120	34526	914	7167	7.1
123	40858	840	7341	15.2
mean	16467	324	2459	10.2
Correlation with MCP-1 (r^2)		0.81	0.90	−0.18
Normal urine	(pg/mg)	(pg/mg)	(pg/mg)	(pg/mg)
8	182	8.1	7.8	80.9
10	213	4.8	1.2	18.7
9	227	7.3	61.7	21.5
mean	207	6.7	23.6	40.4

[a] Urine samples referenced to urine creatinine concentration. Normal urine selected on the basis of MCP-1 levels in upper range.

dysfunction in ADPKD may be reflected in an increased rate of urinary MCP-1 excretion before there are clear-cut changes in serum creatinine concentration or in the rate of urinary protein excretion. Longitudinal, controlled studies will be needed to determine whether MCP-1 may be a useful surrogate marker for detecting progression of ADPKD relatively early in the course of the disease.

The observation that MCP-1 levels in ADPKD cysts were much greater than in serum is consistent with accumulation of filtered chemokine in a blind sac or with net secretion of the protein by the abnormal mural epithelium. More than likely, the relatively large cysts aspirated in this study were not in communication with afferent tubule segments of MCP-1 reaching the cyst cavity via glomerular filtration (3). The vast majority of macroscopic cysts have no tubule connections and function as isolated sacs that fill with liquid by transepithelial secretion (16,17). It is most economical to suppose that the MCP-1 build up within the fluid was secondary to the transepithelial secretion of interstitial MCP-1 or by direct synthesis of the chemokine in the cyst epithelial cells with secretion into the lumen.

To test the hypothesis that mural epithelium in human cysts synthesize and secrete MCP-1, we cultured ADPKD mural cells on permeable supports. MCP-1 levels in fluid samples, taken 24 h after simplified medium lacking growth factors was added, were much higher in the apical than in the basolateral fluids (Figure 4). We also detected proteins in the apical fluids with molecular weights and immunoreactivities consistent with MCP-1 and its glycosylated derivative. Because the gradient for passive diffusion could not possibly generate an apical: basolateral transepithelial concentration difference of this magnitude, we conclude that MCP-1 is secreted by the cultured ADPKD cells to a large extent across the apical plasma membrane. Some of MCP-1 appearing in the basolateral fluid may have derived from direct movement across the basolateral plasma membrane or by passive diffusion of the chemokine from the apical compartment through paracellular junctions. The observation that ADPKD cells cultured *in vitro* generate steep transepithelial concentration gradients is consistent with the high levels of MCP-1 found in the cyst fluids removed from ADPKD kidneys and the high fractional excretion rates in ADPKD patients approaching renal insufficiency.

Cells cultured from human normal renal cortex (HKC) secreted MCP-1 to the same extent as ADPKD cells (Figure 4). Thus, the increased production of MCP-1 by renal cells in culture is not a unique reflection of one or more polycystin mutations. Recent studies have shown that cells from normal kidney cultured in this manner have the same lectin-staining profile as ADPKD cells, suggesting that they derive from similar if not identical cortex precursor cells (18). The synthesis of MCP-1 by HKC cells in a simplified medium lacking growth factors or other potential agonists indicates that renal epithelial cells in culture may generate this cytokine in response to mechanical or structural stresses. Serum proteins increase the production of MCP-1 by cultured renal epithelium and may be involved in the increased excretion of the chemokine in proteinuric states (19).

Increased amounts of protein in the urine would seem to be an unlikely explanation for the early appearance of MCP-1 in the urine of patients with ADPKD as there was no increase in urine protein excretion (Table 2).

Renal MCP-1 expression is increased in a host of progressive renal disorders, including obstructive nephropathy (20), lupus nephritis (21), glomerular disorders (22), and partial nephrectomy (22). MCP-1 has been demonstrated in renal tubule epithelial cells (20,23) and in glomerular capillaries (22). The levels of urinary MCP-1 observed in the current study (Tables 1 and 2) are in line with a previous report (22). The lack of correlation between serum and urine MCP-1 levels implicates an alteration in intrarenal MCP-1 handling rather than a simple overflow excretion of filtered chemokine.

On the basis of evidence presented here, we suggest a hypothesis to link the increased excretion of MCP-1 in ADPKD urine with the extent of disease progression. The current study is in keeping with the view that tubule cells undergoing cystogenesis express increased amounts of MCP-1 in response to a stress or injury stimulus that accompanies the transformation of normal renal epithelial cells to a cyst phenotype. We further suppose that MCP-1 generated within the tubule cells is secreted into the urine. As more tubules experience "second hits" and are converted into cysts, the amount of MCP-1 probably rises in the urine. Thus, at a relatively early stage of the disease, the level of urinary MCP-1 could be a reflection of the number of relatively small cysts that remain connected to the urinary collecting system. As the cysts enlarge, they lose their connections to the tubule of origin and function as isolated sacs of fluid (3). As a consequence, these relatively large cysts accumulate extraordinarily high concentrations of MCP-1 within them. Diffusion of the chemokine into the adjacent interstitium through paracellular pathways could lead to a local influx of macrophages that contribute to tubulointerstitial fibrosis. An examination of this hypothesis awaits further study.

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References

1. Calvet JP, Grantham JJ: The genetics and physiology of polycystic kidney disease. *Semin Nephrol* 21: 107–123, 2001
2. Grantham JJ: Polycystic kidney disease: From the bedside to the gene and back. *Curr Opin Nephrol Hypertens* 10: 533–542, 2001
3. Grantham JJ, Geiser JL, Evan AP: Cyst formation and growth in autosomal dominant polycystic kidney disease. *Kidney Int* 31: 1145–1152, 1987
4. Cowley BD Jr, Gudapaty S, Kraybill AL, Barash BD, Harding MA, Calvet JP, Gattone VHD: Autosomal-dominant polycystic kidney disease in the rat. *Kidney Int* 43: 522–534, 1993
5. Qian F, Watnick TJ: Somatic mutation as mechanism for cyst formation in autosomal dominant polycystic kidney disease. *Mol Genet Metab* 68: 237–242, 1999
6. Brasier JL, Henske EP: Loss of the polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss-of-function model for cyst pathogenesis. *J Clin Invest* 99: 194–199, 1997
7. Cowley BD Jr, Ricardo SD, Nagao S, Diamond JR: Increased renal expression of monocyte chemoattractant protein-1 and osteopontin in ADPKD in rats. *Kidney Int* 60: 2087–2096, 2001
8. Yamaguchi T, Pelling JC, Ramaswamy NT, Eppler JW, Wallace DP, Nagao S, Rome LA, Sullivan LP, Grantham JJ: cAMP stimulates the in vitro proliferation of renal cyst epithelial cells by activating the extracellular signal-regulated kinase pathway. *Kidney Int* 57: 1460–1471, 2000
9. Wallace DP, Grantham JJ, Sullivan LP: Chloride and fluid secretion by cultured human polycystic kidney cells. *Kidney Int* 50: 1327–1336, 1996
10. Jiang Y, Valente AJ, Williamson MJ, Zhang L, Graves DT: Post-translational modification of a monocyte-specific chemoattractant synthesized by glioma, osteosarcoma, and vascular smooth muscle cells. *J Biol Chem* 265: 18318–18321, 1990
11. Igarashi P, Somlo S: Genetics and pathogenesis of polycystic kidney disease. *J Am Soc Nephrol* 13: 2384–2398, 2002
12. Cuppage FE, Huseman RA, Chapman A, Grantham JJ: Ultrastructure and function of cysts from human adult polycystic kidneys. *Kidney Int* 17: 372–381, 1980
13. Schafer K, Bader M, Gretz N, Oberbaumer I, Bachmann S: Focal overexpression of collagen IV characterizes the initiation of epithelial changes in polycystic kidney disease. *Exp Nephrol* 2: 190–195, 1994
14. Schafer K, Gretz N, Bader M, Oberbaumer I, Eckardt KU, Kriz W, Bachmann S: Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. *Kidney Int* 46: 134–152, 1994
15. Nagao S, Yamaguchi T, Kusaka M, Maser RL, Takahashi H, Cowley J BD, Grantham JJ: Renal activation of extracellular signal-regulated kinase in rats with autosomal-dominant polycystic kidney disease. *Kidney Int* 63: 427–437, 2003
16. Sullivan LP, Wallace DP, Grantham JJ: Chloride and fluid secretion in polycystic kidney disease. *J Am Soc Nephrol* 9: 903–916, 1998
17. Sullivan LP, Wallace DP, Grantham JJ: Epithelial transport in polycystic kidney disease. *Physiol Rev* 78: 1165–1191, 1998
18. Yamaguchi T, Nagao S, Wallace DP, Belibi FA, Cowley BD, Pelling JC, Grantham JJ: Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys. *Kidney Int* 63: 1983–1994, 2003
19. Burton CJ, Combe C, Walls J, Harris KP: Secretion of chemokines and cytokines by human tubular epithelial cells in response to proteins. *Nephrol Dial Transplant* 14: 2628–2633, 1999
20. Grandaliano G, Gesualdo L, Bartoli F, Ranieri E, Monno R, Leggio A, Paradies G, Caldarulo E, Infante B, Schena FP: MCP-1 and EGF renal expression and urine excretion in human congenital obstructive nephropathy. *Kidney Int* 58: 182–192, 2000
21. Noris M, Bernasconi S, Casiraghi F, Sozzani S, Gotti E, Remuzzi G, Mantovani A: Monocyte chemoattractant protein-1 is excreted in excessive amounts in the urine of patients with lupus nephritis. *Lab Invest* 73: 804–809, 1995
22. Rovin BH, Doe N, Tan LC: Monocyte chemoattractant protein-1 levels in patients with glomerular disease. *Am J Kidney Dis* 27: 640–646, 1996
23. Ota T, Tamura M, Osajima A, Doi Y, Kudo H, Anai H, Miyazaki M, Nishino T, Nakashima Y: Expression of monocyte chemoattractant protein-1 in proximal tubular epithelial cells in a rat model of progressive kidney failure. *J Lab Clin Med* 140: 43–51, 2002