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## Increased renal expression of monocyte chemoattractant protein-1 and osteopontin in ADPKD in rats

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### **Increased renal expression of monocyte chemoattractant protein-1 and osteopontin in ADPKD in rats.**

**Background.** Human autosomal-dominant polycystic kidney disease (ADPKD) is variable in the rate of deterioration of renal function, with end-stage renal disease (ESRD) occurring in only approximately 50% of affected individuals. Evidence suggests that interstitial inflammation may be important in the development of ESRD in ADPKD. Han:SPRD rats manifest ADPKD that resembles the human disease. Homozygous cystic (Cy/Cy) rats develop rapidly progressive PKD and die near age 3 weeks. Heterozygous (Cy/+) females develop slowly progressive PKD without evidence of renal dysfunction until the second year of life, whereas heterozygous (Cy/+) males develop more aggressive PKD with renal failure beginning by 8 to 12 weeks of age.

**Methods.** To examine the relationship between proinflammatory chemoattractants and the development of interstitial inflammation and ultimately renal failure in ADPKD, we evaluated monocyte chemoattractant protein-1 (MCP-1) and osteopontin mRNAs and proteins in kidneys from Han:SPRD rats.

**Results.** MCP-1 and osteopontin mRNAs, expressed at low levels in kidneys from normal (+/+) animals at all ages, were markedly elevated in kidneys from 3-week-old Cy/Cy animals. In kidneys from heterozygous (Cy/+) adults of either gender, MCP-1 and osteopontin mRNAs were more abundant than normal; MCP-1 mRNA was more abundant in Cy/+ males than in females. Thus, chemoattractant mRNA expression correlated with the development of renal failure in Cy/Cy and Cy/+ rats. Osteopontin mRNA, localized by *in situ* hybridization, was moderately expressed in the renal medulla of normal animals; however, this mRNA was expressed at very high levels in the cystic epithelia of Cy/+ and Cy/Cy animals. MCP-1 and osteopontin proteins, localized by immunohistochemistry, were weakly detected in +/+ kidneys but were densely expressed in Cy/Cy and in adult Cy/+ kidneys, primarily over cystic epithelium. Increased expression of chemoattractants was associated with the accumulation of ED-1 positive cells (macrophages) in the interstitium of cystic kidneys.

**Key words:** autosomal-dominant PKD, interstitial inflammation, renal failure, inherited disease, cysts, fibrosis.

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**Conclusions.** We suggest that proinflammatory chemoattractants have a role in the development of interstitial inflammation and renal failure in ADPKD.

The polycystic kidney diseases (PKDs) are characterized by the development of fluid-filled, epithelial-lined cysts derived from renal tubules. PKD can be inherited or acquired. Autosomal-dominant (AD) PKD, the most common form seen in humans [1, 2], has a highly variable onset and renal failure occurs in approximately 50% of affected individuals [1]. There is growing awareness that the rate of disease progression is dependent to a significant extent upon epigenetic factors that contribute to cyst growth, interstitial inflammation and progressive fibrosis.

In 1989, Kaspareit-Rittinghausen et al described a rodent model of PKD, the Han:SPRD, which arose spontaneously in Sprague-Dawley rats [3]. PKD in the Han:SPRD rat is inherited as an autosomal dominant trait, but there is a gene dose effect. Rats of either gender that inherit two abnormal genes develop rapidly progressive PKD and die with renal failure at approximately three weeks of age [3, 4]. Heterozygous animals develop slowly progressive PKD that resembles in many respects human ADPKD [4, 5]. In heterozygous Han:SPRD rats, the gender effect is exaggerated in comparison to the human counterpart. Male heterozygotes develop severe renal cystic changes and renal insufficiency soon after sexual maturity, and die with renal failure by 12 to 14 months of age [4, 5]. Extensive interstitial inflammation and fibrosis develops in conjunction with the onset of renal insufficiency in male heterozygotes [4, 5]. In contrast, the renal cysts and interstitial changes progress more slowly in female heterozygotes [4] and death from renal insufficiency does not occur until late in the second year of life [6]. Interstitial inflammation and fibrosis are thought to have important roles in the development and/or progression of renal dysfunction in a variety of diseases, including those in which glomeruli are the primary targets [7–9].

Monocyte chemoattractant protein-1 (MCP-1) and osteopontin are chemoattractant mediators with specificity for macrophages. These proteins are expressed at high levels by tubular epithelial cells in several models of renal disease associated with pronounced interstitial abnormalities [7, 8, 10–17]. To examine the potential relationship of these proinflammatory factors to the development of interstitial inflammation and renal failure in hereditary PKD, we determined the abundance and localization of MCP-1 and osteopontin mRNAs and proteins and the distribution of macrophages in the kidneys of Han:SPRD rats.

## METHODS

### Animals

Han:SPRD rats were maintained as an inbred colony in the University of Kansas Medical Center Animal Care Facility as previously described [4]. At selected ages, animals of each genotype were anesthetized and weighed. The abdomen was opened, and right kidneys were dissected free of other tissues, removed, weighed, frozen in dry ice, and stored at  $-80^{\circ}\text{C}$  (right kidneys were processed subsequently for nucleic acid analysis as described below). Left kidneys were dissected free of other tissues, removed, weighed, serially sectioned, and fixed by immersion in 10% buffered formalin. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

### RNA isolation

RNA was isolated from right kidneys using a modification of the technique of Chomczynski and Sacchi [4, 18]. Briefly, right kidneys were homogenized in GTC solution (GTC solution is 4 mol/L guanidine thiocyanate, 25 mmol/L trisodium citrate, 0.1 mol/L  $\beta$ -mercaptoethanol, 0.1% Antifoam A, pH 7.0) using a polytron tissue homogenizer. GTC homogenates were treated by sequential addition of 2 N sodium acetate (pH 4), phenol, and chloroform with vortexing after each addition. After centrifugation, the aqueous layer was transferred to a fresh tube, and RNA was precipitated by isopropanol precipitation. RNA was pelleted by centrifugation, redissolved, chloroform extracted, and ethanol precipitated. RNA was again pelleted by centrifugation, redissolved, and quantitated by spectrophotometry. After a final ethanol precipitation, RNA was stored at  $-20^{\circ}\text{C}$  until use.

### Gel electrophoresis and Northern blots

Denaturing agarose gel electrophoresis was performed as previously described [4] to assess the integrity of RNA samples and prior to Northern blotting. Briefly, RNA samples were denatured in 2.2 mol/L formaldehyde, 50% formamide and electrophoresed in formaldehyde, agarose gels. Gels were stained with acridine orange, destained,

and photographed to assess the relative intensities of the 28S and 18S ribosomal RNA bands and to assure that equal amounts of RNA were loaded in each lane. After electrophoresis, destaining, and photography, RNA in gels was transferred to Nytran (Schleicher & Schuell) filters by capillary transfer and fixed to the filters by baking at  $80^{\circ}\text{C}$  *in vacuo* for two hours.

### Northern hybridizations and autoradiography

For hybridization with osteopontin or MCP-1 DNA probes, filters were prehybridized at  $55^{\circ}\text{C}$  in  $3 \times \text{SET}$  [ $20 \times \text{SET}$  is 3 mol/L NaCl, 0.04 mol/L ethylenediaminetetraacetic acid (EDTA), 0.6 mol/L Tris-HCl, pH 8), 5% sodium dodecyl sulfate (SDS),  $10 \times \text{Denhardt's}$  solution [ $10 \times \text{Denhardt's}$  solution is 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin (BSA)], 40 mmol/L sodium phosphate buffer, pH 7.8, 100  $\mu\text{g}/\text{mL}$  sonicated salmon sperm DNA for one hour or longer. Hybridizations were performed overnight at  $55^{\circ}\text{C}$  in  $3 \times \text{SET}$ , 5% SDS, 40 mmol/L sodium phosphate buffer, pH 7.8, 100  $\mu\text{g}/\text{mL}$  sonicated salmon sperm DNA, 10% dextran sulfate, with 1 to  $3 \times 10^6$  cpm of [ $^{32}\text{P}$ ]-radio-labeled DNA probe (see below) per mL of hybridization solution. Filters were washed at  $55^{\circ}\text{C}$  in  $1 \times \text{SSC}$  ( $20 \times \text{SSC}$  is 3 mol/L sodium chloride, 0.3 mol/L trisodium citrate, pH 7.0), 0.1% SDS for one hour,  $1 \times \text{SSC}$ , 0.1% SDS for one hour, and  $1 \times \text{SSC}$ , 0.1% SDS (for MCP-1) or  $0.3 \times \text{SSC}$ , 0.1% SDS (for osteopontin) for one hour. Filters were then exposed to Fuji RX film (Fuji, Tokyo, Japan) at  $-80^{\circ}\text{C}$ .

Renal MCP-1 RNA abundance was assessed by Northern analysis from one litter (1 +/+, 1 Cy/+, 1 Cy/Cy, both male and female) at two weeks of age, one litter at three weeks of age (1 +/+, 1 Cy/+, 1 Cy/Cy, both male and female), two litters (2 +/+, 2 Cy/+, both male and female) at eight weeks of age, and two litters (2 +/+, 5 Cy/+, both male and female) at 24 weeks of age. Renal osteopontin RNA abundance was assessed by Northern analysis from two litters (2 +/+, 2 Cy/+, 2 Cy/Cy, both male and female) at two weeks of age, two litters at three weeks of age (2 +/+, 2 Cy/+, 2 Cy/Cy, both male and female), two litters (2 +/+, 2 Cy/+, both male and female) at eight weeks of age, and three litters (3 +/+, 6 Cy/+, both male and female) at 24 weeks of age. Northern transfers and hybridizations were performed at least twice with all RNA samples; all results were qualitatively similar. For more accurate comparisons, the most recent blots were rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

After radiolabeled probes for osteopontin or MCP-1 had decayed, filters were rehybridized with a  $^{32}\text{P}$ -labeled antisense RNA probe for rat GAPDH. Filters were prehybridized at  $66^{\circ}\text{C}$  in  $3 \times \text{SET}$ , 0.1% SDS,  $10 \times \text{Denhardt's}$  solution, 250  $\mu\text{g}/\text{mL}$  for one hour or longer. Hybridizations were performed overnight at  $66^{\circ}\text{C}$  in  $3 \times$

SET, 0.1% SDS, 20 mmol/L sodium phosphate buffer, pH 7.8,  $10 \times$  Denhardt's, 250  $\mu\text{g}/\text{mL}$  tRNA, 10% dextran sulfate, with  $10^6$  cpm of [ $^{32}\text{P}$ ]-radiolabeled antisense RNA probe (see below) per mL of hybridization solution. Filters were washed at  $66^\circ\text{C}$  in  $1 \times$  SSC, 0.1% SDS for one hour,  $0.3 \times$  SSC, 0.1% SDS for one hour, and  $0.1 \times$  SSC, 0.1% SDS for one hour. Filters were then exposed to Fuji RX film at  $-80^\circ\text{C}$ .

The intensity of autoradiographic signals was quantitated using a Macintosh computer, a flat-bed scanner, and the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Autoradiographic signals for osteopontin and MCP-1 were expressed relative to the GapDH signal. For each filter, values were normalized to the 24-week-old male normal (+/+) that was arbitrarily assigned a value of 1.

### In situ hybridizations

In situ hybridizations were performed on 10% neutral buffered formalin-fixed paraffin sections using established protocols [19]. Midcoronal sections were alcohol dehydrated and incubated with prehybridization solution containing 1.2 mol/L NaCl, 0.02 mol/L Tris, 0.04% Ficoll, 0.04% BSA, 0.04% polyvinylpyrrolidone, 0.002 mol/L EDTA, 0.1% salmon sperm DNA, and 0.1 mg/mL yeast tRNA. Tissue sections were hybridized at  $50^\circ\text{C}$  overnight in an identical solution containing 25% formamide, 10 mmol/L dithiothreitol (DTT), 0.1% SDS, and  $4 \times 10^4$  cpm/ $\mu\text{L}$   $^{35}\text{S}$ -labeled RNA probe (see below). Slides were washed in  $2 \times$  SSC for two hours at  $60^\circ\text{C}$ , and autoradiograms were generated by exposure to Kodak Biomax film (Eastman Kodak, Rochester, NY, USA) for five days.

After exposure to the BioMax film, slides were dipped in nuclear track silver emulsion (Kodak NTB2) diluted 1:1 with distilled water warmed to  $45^\circ\text{C}$  in a water bath. When the emulsion was dry, slides were placed in light-proof containers and stored at  $4^\circ\text{C}$  for five days. Slides were developed, fixed and counterstained with hematoxylin. Slides were then viewed on a Nikon microscope equipped with epifluorescence.

As a negative control, prior to incubation in prehybridization solution sections were incubated in 0.1 mg/mL RNase in RNase buffer for 1.5 hours at room temperature. Slides were then rinsed in RNase buffer followed by  $2 \times$  SSC for two hours. Sections were dehydrated in ethanol before the addition of the prehybridization solution and subsequent hybridized as above. As a second negative control, slides were hybridized with  $^{35}\text{S}$ -labeled sense probe as above.

### Nucleic acid probe generation

Plasmid containing rat osteopontin cDNA (insert size  $\sim 1.1$  kb) in pBluescript SK/– was obtained from C. Gia-

chelli [20]. For generation of radiolabeled nucleic acid probes for Northern hybridizations, the insert ("2B7") from this plasmid was isolated and radiolabeled with  $\alpha$ -[ $^{32}\text{P}$ ]-cytidine triphosphate (DuPont/NEN, Wilmington, DE, USA) using a random primed cDNA labeling kit (Promega, Madison, WI, USA). For generation of radiolabeled RNA probes for in situ hybridizations, the osteopontin plasmid was linearized with Bgl I (for antisense probes) or Mam I (for sense probes) and in vitro transcriptions were performed in the presence of  $\alpha$ -[ $^{35}\text{S}$ ]-uridine triphosphate (DuPont/NEN) using T7 (antisense) or T3 (sense) RNA polymerase (Promega); RNA probes were separated from unincorporated precursors using G-50 Quick Spin columns (Boehringer-Mannheim, Mannheim, Germany).

Plasmid DNA containing MCP-1 cDNA (insert size  $\sim 0.66$  kb) in pBluescript SK/– was obtained from T. Yoshimura [21]. The MCP-1 specific portion of this plasmid was isolated and radiolabeled with  $\alpha$ -[ $^{32}\text{P}$ ]-cytidine triphosphate (DuPont/NEN) using a random primed cDNA labeling kit (Promega).

Plasmid pRcGAP123 containing rat GAPDH cDNA was obtained from R. Wu [22]. An EcoR I/Xho II fragment of this cDNA encompassing approximately 758 bases of 5' coding sequence was subcloned into pGEM-3Z (Promega). This plasmid was linearized with EcoR I prior to transcription with SP6 RNA polymerase. Radiolabeled antisense RNA probes were prepared by in vitro transcription of linearized plasmid in the presence of  $\alpha$ -[ $^{32}\text{P}$ ]-guanosine triphosphate (DuPont/NEN).

### Immunohistochemistry

Immunohistochemistry was performed as previously described [12, 13, 23]. Briefly, midcoronal sections were obtained from left kidneys, embedded in paraffin, and sectioned at  $\sim 4$   $\mu\text{m}$  thickness. Sections were then serially deparaffinized with xylene. Endogenous peroxidase activity was quenched with 4:1 methanol: $\text{H}_2\text{O}_2$ , and endogenous biotin was blocked with avidin D and biotin blocking solutions (Vector, Burlingame, CA, USA). Subsequently, sections were incubated in either normal horse or goat serum (1:20, Vector) for 50 minutes at  $25^\circ\text{C}$ , followed by a five-minute rinse in phosphate buffered saline (PBS). Sections were then incubated for 60 minutes at  $25^\circ\text{C}$  with one of the following primary antibodies: (1) MPIIB10, a mouse monoclonal IgG antibody to rat osteopontin (dilution 1:8000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA); (2) polyclonal rabbit anti-murine MCP-1 (dilution 1:4800; kindly provided by B. Rollins, Dana-Farber Cancer Institute, Boston, MA, USA); and (3) ED-1, a mouse monoclonal immunoglobulin G (IgG) antibody that recognizes cytoplasmic antigens in monocytes/macrophages (dilution 1:2000; Serotec, Oxford, UK). Next, sections were rinsed for five minutes in PBS and were then incubated with secondary

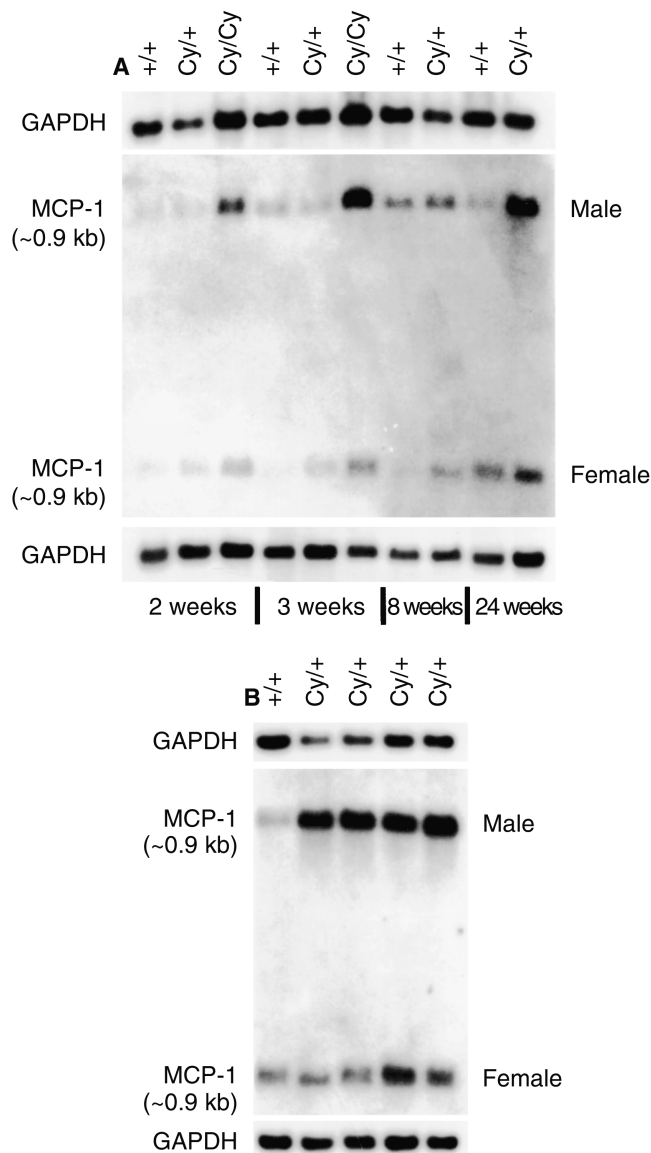
antibody [either a biotinylated horse anti-mouse (rat adsorbed) IgG or a biotinylated goat anti-rabbit IgG antibody (dilution 1:150; Vector)] for 60 minutes at 25°C. Sections were then incubated in an avidin biotinylated-horseradish peroxidase complex for 60 minutes at 25°C followed by 0.1 mol/L Tris, pH 7.5, 0.0225% H<sub>2</sub>O<sub>2</sub> for five minutes. Sections were counterstained with 1% methyl green solution for 20 minutes. As negative controls, separate sections were treated as above with either no primary antibody or with an irrelevant primary antibody. Sections of kidneys from rats treated to cause urinary tract obstruction were used as positive controls for MCP-1, osteopontin, and ED-1 immunostaining.

## RESULTS

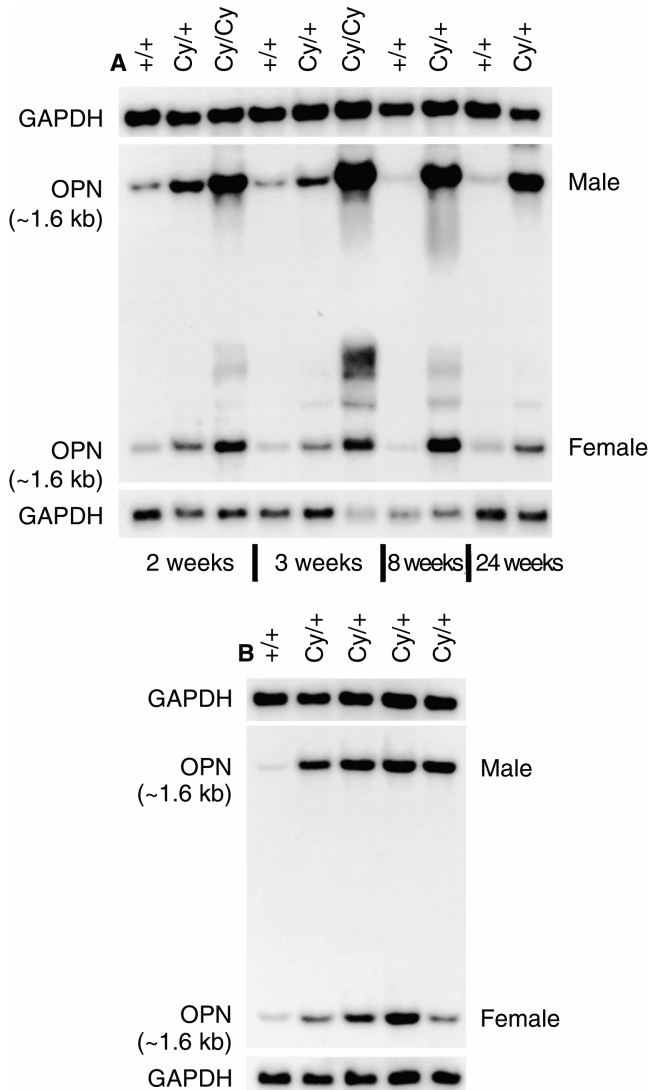
Whole kidney MCP-1 mRNA levels from homozygous (Cy/Cy) animals of either gender were elevated at two and three weeks of age, but were only faintly detected in normal (+/+) rats (Fig. 1A). Compared to the expression of GAPDH, a "housekeeping" RNA, the increases in MCP-1 were highly selective. Cy/Cy animals died soon after three weeks of age from renal failure. Renal MCP-1 mRNA levels were also increased in 8- and 24-week-old heterozygous (Cy/+) rats in comparison to +/+ animals. MCP-1 mRNA levels, determined in several litters, were increased in Cy/Cy animals and in Cy/+ animals older than three weeks. Moreover, in adult animals MCP-1 mRNA levels were increased to a greater extent above +/+ in male Cy/+ than in female Cy/+ animals (Fig. 1B). The mean  $\pm$  SE increase (Fig. 1) in renal MCP-1:GAPDH ratio for 24-week-old Cy/+ rats relative to 24-week-old +/+ males (used for normalization within the same blot) was  $5.6 \pm 0.5$  for Cy/+ males and  $2.8 \pm 0.3$  for Cy/+ females ( $P = 0.016$ , Mann-Whitney U test).

Osteopontin mRNA levels was detected in kidneys from all +/+ animals (Fig. 2A), but were markedly elevated in Cy/Cy kidneys and in 8- and 24-week-old Cy/+ animals of either gender. Osteopontin mRNA levels tended to be increased to a greater extent in male than in female Cy/+ animals. Additional animals studied at 24 weeks of age (Fig. 2B) confirmed higher levels of osteopontin mRNA in male than in female Cy/+ animals. The mean  $\pm$  SE increase (Fig. 2) in the renal osteopontin:GAPDH ratio for 24-week-old Cy/+ rats relative to 24-week-old +/+ males (used for normalization within the same blot) was  $10.6 \pm 2.3$  for Cy/+ males and  $6.5 \pm 0.8$  for Cy/+ females ( $P = 0.076$ , Mann-Whitney U test).

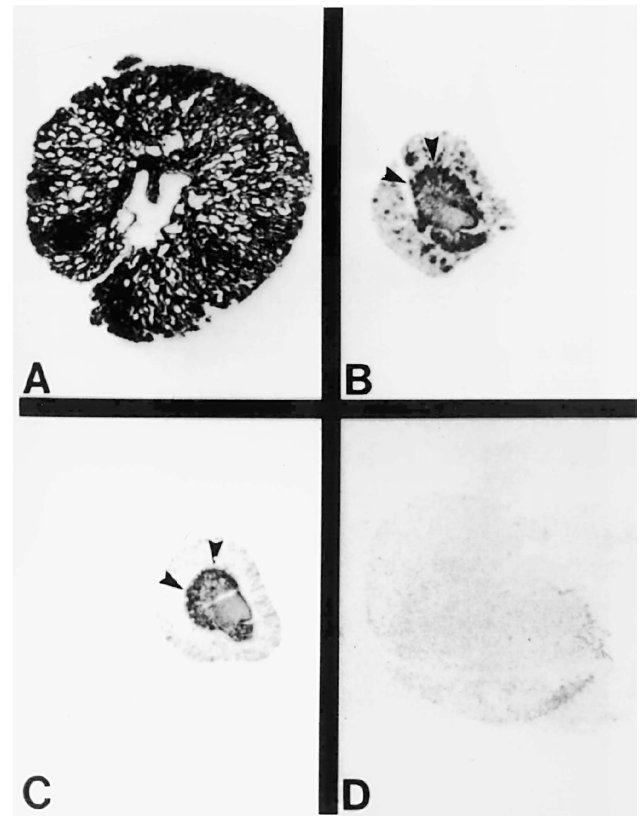
In situ hybridization was used to localize the major sites of osteopontin mRNA expression in cystic kidneys. At three weeks of age, osteopontin mRNA could not be detected in renal cortex of normal animals (Figs. 3C), but was strongly expressed in renal medulla (Fig. 3C), as previously reported [24]. In contrast, osteopontin mRNA



**Fig. 1. (A) Autoradiogram of a Northern blot showing monocyte chemoattractant protein-1 (MCP)-1 mRNA levels in kidneys from Han:SPRD rats of the indicated genotypes, genders, and ages.** Also shown are corresponding autoradiograms obtained after blots were rehybridized with a GAPDH probe. Each lane contained 10  $\mu$ g total RNA. Densitometric ratios of MCP-1 autoradiographic signals to GAPDH autoradiographic signals were determined as described in the **Methods** section. Values were normalized to the 24-week-old male normal (+/+) rat, which was arbitrarily assigned a value of 1. Densitometric ratios for male lanes 1 to 10 were: 0.8, 0.9, 3.7, 1.9, 2.0, 4.3, 2.0, 2.8, 1.0 and 3.6. Densitometric values for female lanes 1 to 10 were: 1.1, 1.2, 2.3, 0.4, 1.5, 4.2, 0.7, 1.9, 2.0, and 2.5. **(B)** Autoradiogram of a Northern blot showing MCP-1 mRNA levels in kidneys from 24-week-old male and female Han:SPRD rats of the indicated genotypes. Also shown are corresponding autoradiograms obtained after blots were rehybridized with a radiolabeled GAPDH probe. Each lane contained 10  $\mu$ g total RNA. Densitometric ratios of MCP-1 autoradiographic signals to GAPDH autoradiographic signals were determined as described in **Methods**; values were normalized to the male normal (+/+) rat, which was arbitrarily assigned a value of 1. Densitometric ratios for male lanes 1 to 5 were: 1.0, 6.6, 6.4, 5.5, and 6.1. Densitometric values for female lanes 1 to 5 were: 2.2, 2.4, 2.4, 3.8, and 3.1.



**Fig. 2. (A)** Autoradiogram of a Northern blot showing osteopontin mRNA levels in kidneys from Han:SPRD rats of the indicated genotypes, genders, and ages. Also shown are corresponding autoradiograms obtained after blots were rehybridized with a GAPDH probe. Each lane contained 10  $\mu$ g total RNA. Densitometric ratios of osteopontin autoradiographic signals to GAPDH autoradiographic signals were determined as described in the **Methods** section. Values were normalized to the 24-week-old male normal (+/+) rat, which was arbitrarily assigned a value of 1. Densitometric ratios for male lanes 1 to 10 were: 2.2, 8.1, 18.6, 1.8, 4.8, 26.0, 1.1, 24.3, 1.0, and 19.9. Densitometric values for female lanes 1 to 10 were: 1.2, 4.1, 7.5, 0.8, 2.4, 12.4, 1.0, 17.0, 1.0, and 4.3. **(B)** Autoradiogram of a Northern blot showing osteopontin mRNA levels in kidneys from 24-week-old male and female Han:SPRD rats of the indicated genotypes. Also shown are corresponding autoradiograms obtained after blots were rehybridized with a radiolabeled GAPDH probe. Each lane contained 10  $\mu$ g total RNA. Densitometric ratios of osteopontin autoradiographic signals to GAPDH autoradiographic signals were determined as described in the **Methods** section. Values were normalized to the male normal (+/+) rat, which was arbitrarily assigned a value of 1. Densitometric ratios for male lanes 1 to 5 were: 1.0, 8.2, 8.8, 7.9, 8.3. Densitometric values for female lanes 1 to 5 were: 2.1, 5.9, 7.8, 9.0, 5.5.

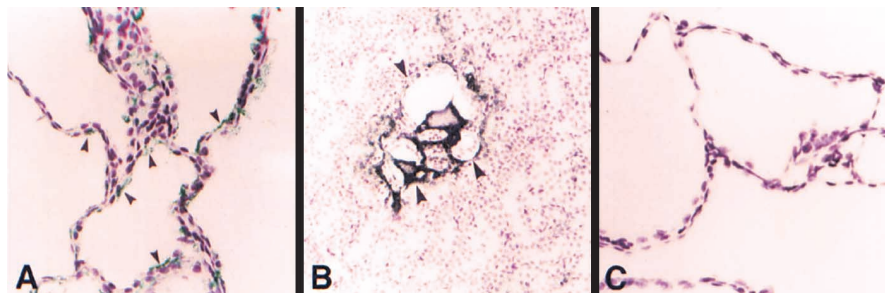


**Fig. 3. Whole kidney autoradiograms of in situ hybridizations for osteopontin mRNA in representative sections from Han:SPRD rats.** Magnification for all panels is 4 $\times$ . **(A)** Three-week-old homozygous cystic (Cy/Cy) rat, antisense riboprobe. **(B)** Three-week-old male heterozygous (Cy/+) rat, antisense riboprobe. Osteopontin was expressed diffusely in the medulla (arrowheads) and focally in the cortex. **(C)** Three-week-old male normal (+/+) rat, antisense riboprobe. Osteopontin mRNA was diffusely expressed in renal medulla (arrowheads), as previously reported [24], but was not detected in the cortex. **(D)** Twenty-four-week-old male heterozygous (Cy/+) rat, sense riboprobe.

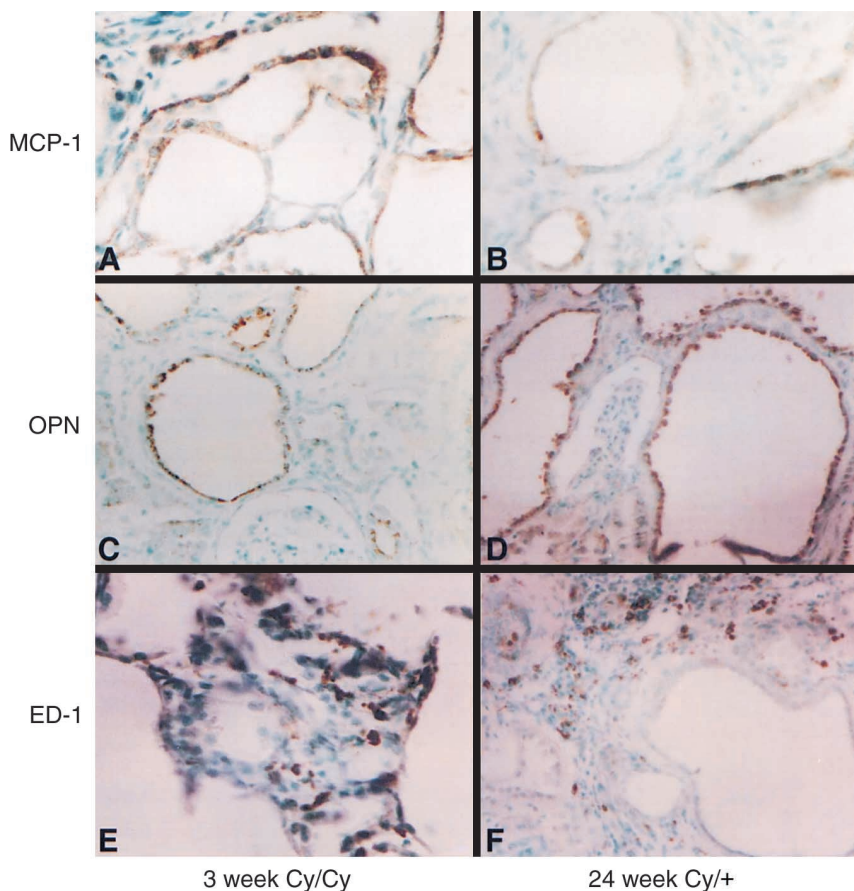
was intensely expressed in cortex and medulla of Cy/Cy (Figs. 3A and 4A). Osteopontin mRNA was also focally expressed in cystic epithelium from Cy/+ animals (Figs. 3B and 4B), but was not detected to an appreciable extent in non-cystic parenchyma (Fig. 3B and 4B). Hybridization with a sense riboprobe was not associated with detectable expression in either normal or cystic kidneys (Figs. 3D and 4C). An examination of MCP-1 by in situ hybridization was not done.

Renal MCP-1 was abundant in 3-week-old homozygous (Cy/Cy) kidneys, primarily in cystic epithelial cells (Fig. 5A), although it was only weakly detected in cystic epithelia from 3-week-old Cy/+ animals (data not shown). Renal MCP-1 was easily detected in the cystic epithelia of 24-week-old Cy/+ rats (Fig. 5B). MCP-1 could not be detected in +/+ kidneys by immunohistochemistry (data not shown).

Osteopontin was abundant in 3-week-old homozygous (Cy/Cy) kidneys (Fig. 5C), primarily in the cystic epithe-



**Fig. 4.** In situ hybridizations for osteopontin mRNA in representative sections from 3-week-old male Han:SPRD rats. Bright-field photomicrographs of paraffin sections are shown. All sections were counterstained with Mayer's hematoxylin. (A) Section from a homozygous cystic (Cy/Cy) rat kidney reacted with an  $^{35}\text{S}$ -labeled antisense osteopontin riboprobe ( $\times 400$ ). Arrowheads denote the intense localization of osteopontin mRNA (green silver granules) within cystic tubular epithelium. (B) Heterozygous (Cy/+) rat kidney hybridized with an  $^{35}\text{S}$ -labeled antisense osteopontin riboprobe ( $\times 200$ ). Arrowheads denote localization of osteopontin mRNA nearly exclusively within cystic epithelium. Normal renal cortical parenchyma surrounds cysts. (C) A cortical section from a homozygous cystic (Cy/Cy) rat kidney reacted with the  $^{35}\text{S}$ -labeled sense osteopontin riboprobe ( $\times 400$ ) showing only a faint background staining.



**Fig. 5.** Immunoperoxidase labeling for MCP-1 (A and B), osteopontin (C and D), and ED-1 (E and F). All magnifications are  $\times 800$ . (A) Kidney section from a 3-week-old homozygous cystic (Cy/Cy) rat showing marked immunolabeling for MCP-1 within cystic epithelium. (B) Kidney section from a 24-week-old heterozygous cystic (Cy/+) rat showing MCP-1 immunolabeling within cystic epithelium. (C) Kidney section from a 3-week-old homozygous cystic (Cy/Cy) rat showing diffuse immunolabeling for osteopontin within cystic epithelium. (D) Kidney section from a 24-week-old heterozygous cystic (Cy/+) rat showing osteopontin immunolabeling within cystic epithelium. (E) Kidney section from a 3-week-old homozygous cystic (Cy/Cy) rat showing diffuse interstitial infiltration with ED-1 positive cells. (F) Kidney section from a 24-week-old heterozygous cystic (Cy/+) rat showing diffuse interstitial infiltration with ED-1 positive cells.

lial cells, although it was only weakly detected in cystic epithelia from 3-week-old Cy/+ animals (data not shown). Renal osteopontin was abundantly expressed in the cystic epithelia of 24-week-old male Cy/+ rats (Fig. 5D) but not in females (data not shown). In both homozygous and heterozygous cystic rats, immunodetectable renal

osteopontin was localized most intensely within markedly cystic epithelium, while osteopontin expression by non-dilated, non-cystic tubules was relatively limited. Osteopontin was not detected in the renal cortex of 3- and 24-week-old +/+ rats (data not shown).

Previous studies have described interstitial infiltrates

in male *Cy/+* rats [4, 5]. To determine if the cellular infiltrates in Han:SPRD rats included macrophages, we labeled renal sections with antibodies to ED-1, a macrophage cell surface marker. ED-1 positive cells were readily detected in the renal interstitium of *Cy/+* rats 24 weeks of age (Fig. 5F). ED-1 positive cells could be detected as early as 3 weeks of age in male *Cy/+* animals. Kidneys from homozygous cystic (*Cy/Cy*) animals also contained significant numbers of ED-1 positive cells (Fig. 5E). Normal kidneys had no detectable ED-1 positive cells (data not shown).

## DISCUSSION

Autosomal-dominant polycystic kidney disease is a slowly progressive disorder in humans characterized by the development of progressively enlarging cysts from tubules and the development of renal failure in approximately 50% of individuals. This disease is associated with the appearance in the renal interstitium of inflammatory cells that may contribute to progressive deterioration of renal function. The Han:SPRD rat is similar to human ADPKD, even though defects in different genes cause these conditions. The Han:SPRD rat has proven useful for studies of cyst progression, gender dimorphism, and renal functional deterioration. The primary goal of the current studies was to examine the expression of MCP-1 and osteopontin in relation to the development and progressive enlargement of renal cysts and the intrusion of renal interstitial inflammation. In heterozygous (*Cy/+*) animals, the progressive development of cysts was associated with expression of MCP-1 and osteopontin mRNAs and proteins that was clearly greater in both male and female *Cy/+* than in normal littermates. Furthermore, the increase in these mRNAs in *Cy/+* kidneys was greater in males than females. *In situ* hybridization showed that osteopontin mRNA was expressed in the medullae of normal animals, but in *Cy/+* rats it was also prominently displayed in focal patches within the cortex, which at high magnification proved to be cysts lined with epithelial cells containing abundant osteopontin mRNA. Immunohistochemistry indicated that the increased levels of MCP-1 and osteopontin mRNAs were accompanied by increased abundance of the corresponding proteins. Antibodies to MCP-1 and osteopontin were localized to the epithelial cells lining cysts. The current studies are consistent with a previous report of increased MCP-1 mRNA in Han:SPRD rats [25]; in this study, amelioration of PKD by altered dietary protein was associated with less severe interstitial abnormalities and a reduction in renal MCP-1 mRNA.

Monocyte chemoattractant protein-1 is induced by PDGF in cultured fibroblasts [26] and is also a potent chemotactic factor for monocytes/macrophages. It is expressed by a variety of cell types, is induced by numerous

growth factors and cytokines [26], and is inhibited by glucocorticoids [27]. MCP-1 expression is increased acute renal failure, including that caused by obstruction [12] or ischemia [10], and in chronic renal failure associated with tubulointerstitial nephritis [15, 28].

Osteopontin was originally described as having a role in bone mineralization [29], however it is also expressed in multiple other tissues, especially those with epithelial components. Similar to MCP-1, osteopontin is a potent monocyte/macrophage chemoattractant [29]. Osteopontin is induced by several different growth factors and cytokines [29, 30], as well as other mediators [29–34]. Osteopontin, also called uropontin, inhibits calcium oxalate precipitation and has been proposed as an important inhibitor of renal stone formation [35]. Similar to MCP-1, osteopontin expression is increased in acute renal failure caused by obstruction [13] or ischemia [14] and in chronic renal failure associated with interstitial nephritis [7, 15].

The abnormal expression of these proteins in PKD may be linked to increased tubular epithelial cell proliferation or abnormal epithelial cell development that characterizes renal cyst formation and growth. PKD and acute renal failure are both characterized by increased epithelial cell proliferation and less than fully differentiated tubular epithelial cells as manifest by immature histomorphology and fetal patterns of proto-oncogenes and other developmentally regulated genes [4, 36–39]. In acute renal failure, both epithelial cell dedifferentiation and increased MCP-1 and osteopontin expression are transient; however, as shown in the current study, in PKD they persist. It has even been suggested that PKD results in part from a defect in normal cellular differentiation [40]. If so, then abnormal expression of MCP-1 and osteopontin in PKD could be a reflection of abnormal or incomplete epithelial cell differentiation. In preliminary studies there was no elevated expression of MCP-1 or osteopontin mRNA in fetal and neonatal kidneys (data not shown); thus, the expression of these chemoattractants in PKD is probably not simply a manifestation of a developmental arrest but more a reflection of the abnormal cystic phenotype.

Renal histopathologic and functional abnormalities seen in ureteral obstruction are ameliorated by angiotensin-converting enzyme (ACE) inhibition, concomitant with reductions in chemoattractant production [41]. Analogous improvements in renal histopathology and function are seen in Han:SPRD rats with ACE inhibition [42–44], but changes in chemoattractants have not been reported. A recent study showed increased MCP-1 and decreased EGF expression in human obstructive uropathy [45]. These findings are analogous to the findings of the current study and our previous study showing decreased epidermal growth factor (EGF) expression in Han:SPRD rats [46]. In the Han:SPRD rat, focal tubular obstruction has been observed, however most cysts do not show evi-

dence of obstruction [47]. Although obstruction of some nephrons could possibly lead to increased production of osteopontin and MCP-1, as seen in ureteral obstruction [12], we found MCP-1 and osteopontin expressed in the vast majority of cysts examined, most of which would not be obstructed. Thus obstruction does not appear an adequate explanation for the expression of these chemoattractants in this type of PKD.

One clue to the cause of increased production of MCP-1 and osteopontin may lie in their extremely high levels in Han:SPRD rats with homozygous cystic disease (Cy/Cy). These animals have inherited abnormal genes from both parents leading to dramatic enlargement of the kidneys due to cysts. It is reasonable to assume that the homozygous condition impairs epithelial cell function to such an extent that compensatory processes that seem to hold the Cy/+ disease within certain bounds are no longer adequate. Thus, it is possible that robust cellular proliferation may be an inciting factor in the expression of MCP-1 and osteopontin at such extreme levels. This suggestion is in keeping with observations that the renal expression of MCP-1 and osteopontin is increased in association with the burst of proliferative repair following acute renal injury [10, 14].

The gender difference in Han:SPRD heterozygotes may provide insight into mechanisms responsible for development of renal insufficiency in PKD. Males, which develop renal insufficiency in early adulthood, have prominent interstitial inflammatory infiltrates and interstitial fibrosis, whereas females, which maintain normal renal function until late in life, have minimal interstitial changes [4, 5]. Interstitial abnormalities similar to those seen in male Han:SPRD heterozygotes also have been described in human ADPKD [48]. Studies have implicated an important role for tubulointerstitial changes in the development of renal insufficiency in human ADPKD [48, 49], in drug-induced animal models of cystic disease [50], and in hereditary animal models of cystic disease [4, 51]. In each of these instances, renal failure develops in conjunction with the accumulation of interstitial inflammatory cells and fibrosis. A role for interstitial inflammation and fibrosis in the pathogenesis of renal cystic disease and consequent renal dysfunction is supported by the ameliorating effect of methylprednisolone in the Han:SPRD rat and *pcy* mouse models of slowly progressive renal cystic disease [52]. However, it should be noted that attempts to alter the progression of cystic disease or renal dysfunction in Han:SPRD rats with other anti-inflammatory agents including azathioprine or cyclosporine have been unsuccessful (unpublished observations). While interstitial changes appear to progress in parallel with the development of renal failure in human and animal models of PKD, the potential linkage between renal insufficiency and the development of interstitial changes remains to be verified.

Whereas interstitial changes may be closely correlated with the development of renal failure in human and animal models of PKD, the pathophysiologic pathways leading to the development of interstitial changes remain to be defined. Gardner et al demonstrated the presence of several cytokines in the fluid of patients with ADPKD [53] and also demonstrated a potential role for cytokines in drug induced PKD [54]. In the current study, elevated expression of chemoattractant mRNA was seen in adult Cy/+ rats compared to +/+ rats. Additionally, chemoattractant mRNA levels were greater in Cy/+ males than Cy/+ females and thus correlated with the extent of interstitial changes and the severity of renal insufficiency in Cy/+ rats. The presence of ED-1 positive cells in the interstitium of kidneys in association with increased abundance of chemoattractants is consistent with the concept that these proteins may have a role in the development and/or perpetuation of interstitial inflammation in progressive PKD. However, it is also possible that increased production of these proteins is a response to cyst formation, to some other stimulus, or to cellular stress, and thus these proteins may serve protective roles. A potentially analogous protective role for osteopontin has been suggested by studies in osteopontin knockout mice, in which acute renal failure is more severe than in genetically normal mice [55–57].

This study provides evidence to indicate that the increased expression of MCP-1 and osteopontin in Cy/+ rats correlates generally with the progression of renal dysfunction. It is not clear, however, if MCP-1 and osteopontin are uniquely responsible for the interstitial changes seen in this animal model of hereditary PKD. Osteopontin was present in the renal medullae of normal animals where inflammation does not appear to be a significant factor. In addition, both MCP-1 and osteopontin mRNAs and protein were increased in the kidneys of female heterozygous (Cy/+) animals that would not be destined for renal failure until much later than in males, although the increases were greater in male than female heterozygotes. Thus, MCP-1 and osteopontin may be only links in a larger chain of events that results in renal interstitial inflammation, interstitial fibrosis, and ultimately functional decline in selected individuals with slowly progressive PKD.

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