Cardiovascular, Pulmonary and Renal Pathology

Involvement of Hypoxia-Inducible Transcription Factors in Polycystic Kidney Disease

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In polycystic kidney disease (PKD), erythropoietin (EPO) production and interstitial vascularization are increased compared with other kidney diseases. EPO and several angiogenic factors are controlled by hypoxia-inducible transcription factors (HIFs), which are composed of a constitutive β -subunit and two alternative α -subunits (HIF-1 α , HIF-2 α). We hypothesized that cyst expansion may result in pericystic hypoxia and consecutive up-regulation of HIF and thus examined the expression of HIF- α and HIF target genes in human PKD and in a rodent PKD model. HIF-1 α and HIF-2 α were found to be up-regulated in cyst epithelium and cells of cyst walls, respectively. The distinct expression pattern of the HIF- α isoforms closely resembles the respective pattern in normal kidneys under systemic hypoxia. Pimonidazole staining, a marker for tissue hypoxia, confirmed the existence of regional hypoxia in polycystic kidneys. Immunohistochemistry for selected target genes implicated a role for HIF-1 α in vascular endothelial growth factor and Glut-1 activation and HIF-2 α in endoglin and EPO stimulation. Polycystin-deficient cells showed physiological, oxygen-dependent HIF- α modulation, excluding a direct influence of polycys-

tin deficiency on HIF- α regulation. In conclusion, HIF accumulation in human and rat PKD seems to be responsible for increased EPO production and pericystic hypervascularity and may have an impact on progression of PKD. (Am J Pathol 2007, 170:830–842; DOI: 10.2353/ajpath.2007.060455)

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease, with a prevalence of 1:1000. Approximately 5 to 10% of all patients on renal replacement therapy suffer from polycystic kidney disease (PKD).^{1,2} Mutations in the *PKD1* gene and defective in polycystin-1 protein are responsible for 85 to 90% of ADPKD, and mutations in the *PKD2* gene cause the disease in most of the remaining PKD patients.¹ Despite identification of the genetic cause, mechanisms of cyst development and disease progression remain incompletely understood.³ Interestingly, patients with ADPKD are frequently less anemic than patients with kidney disease of other etiology. Occasionally, ADPKD patients even develop polycythemia.^{4,5} In addition, serum erythropoietin (EPO) levels in ADPKD patients are higher compared with those in patients with other causes of kidney disease.⁶ In fact, significant differences in arteriovenous EPO concentrations have been demonstrated in cystic kidneys.⁷ Furthermore, cyst fluids can contain high concentrations of EPO and stromal cells in the cyst walls express EPO mRNA.⁷ An additional specific feature of polycystic kidneys is the pericystic hypervascularity. Increased new vessel formation in the cyst walls, which was found to be associated with enhanced expression of vascular endothelial growth factor (VEGF) and VEGF receptors, led to the hypothesis that angiogenesis is an important factor in the progression of cystic kidney disease, ensuring that the proliferation of epithelial cells is matched by an adequate supply of blood and nutrients.^{8,9} In con-

Supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 423: kidney injury: pathogenesis and regenerative mechanisms).

Accepted for publication December 5, 2006.

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Table 1. Characteristics of Han:SPRD Rats Used

Genetic status and grading for (cy/+)	Number of animals	Gender distribution (male:female)	Body weight (mean $\pm$ SD)	Total kidney weight (mean $\pm$ SD)	Two kidney-to-total body weight ratio (%; mean $\pm$ SD)
Wild type (+/+)	8	2:6	135.9 ($\pm$ 23.9)	1.34 ($\pm$ 0.35)	0.98 ($\pm$ 0.11)
(cy/+) low	13	3:10	141.0 ($\pm$ 20.0)	1.7 ($\pm$ 0.19)	1.22 ($\pm$ 0.09)
(cy/+) high	12	10:2	149.5 ($\pm$ 21.2)	2.7 ($\pm$ 0.76)	1.77 ($\pm$ 0.27)
(cy/cy)	5	3:2	41.4 ($\pm$ 11.5)	8.47 ($\pm$ 4.27)	18.5 ($\pm$ 9.2)

Median of two kidney-to-total body weight ratio of (cy/+): 1.36%. Animals with a two kidney-to-total body weight ratio less than or equal 1.36% were graded as low and more than 1.36% as high. SD, standard deviation.

trast, other types of kidney disease are usually characterized by rarefaction of peritubular capillaries.^{10–12}

EPO and several angiogenic factors are regulated by hypoxia-inducible transcription factors (HIFs).¹³ HIF is a heterodimer consisting of a constitutive β -subunit (HIF- β) and one of two alternative, oxygen-dependent α -subunits (HIF-1 α and -2 α). The production of HIF- α is primarily independent of oxygen, but HIF degradation is regulated by oxygen availability. In the presence of molecular oxygen, two proline residues of HIF- α are hydroxylated by a conserved family of prolyl hydroxylases. This is a prerequisite for subsequent binding of the von Hippel-Lindau protein, which targets the α -chains for rapid degradation via the ubiquitin-proteasome pathway.¹⁴ In hypoxia, HIF- α is not hydroxylated anymore, accumulates in the cell, and forms a heterodimer with HIF- β , which binds to a specific DNA sequence, the hypoxia response element. More than 100 HIF target genes have been identified to date, the vast majority of which improve oxygen delivery or hypoxia tolerance, including genes involved in angiogenesis [eg, VEGF, VEGF-receptor-1 (Flt-1), endoglin (CD105)], genes promoting anaerobic glucose utilization, eg, glucose-transporter-1 (Glut-1) or genes enhancing systemic oxygen transport (eg, EPO).¹⁵ We recently provided evidence that the HIF system is activated in the normal kidney under conditions of systemic and regional hypoxia, and that HIF expression is determined by the intrinsic capacity of different renal cell populations and local oxygen gradients.^{16–18}

It is intriguing to speculate that cyst expansion in the kidney results in regional hypoxia and consecutive up-regulation of HIF and that the activation of the HIF-pathway may increase pericycystic angiogenesis and EPO expression. To test this hypothesis, we investigated the expression of the oxygen-regulated α -subunits of the two HIF transcription factors HIF-1 and HIF-2 and of HIF target genes in kidneys of patients with PKD as well as in kidneys of wild-type, heterozygous, and homozygous Han:SPRD rats, a well-characterized model of hereditary PKD.^{19,20} To assess whether HIF regulation is directly influenced by polycystin, we also studied HIF- α regulation in polycystin-deficient epithelial cells *in vitro*.

Materials and Methods

Collection of Human Tissue and Patient Characteristics

Kidney specimens of seven patients [six males, one female; age, 55.6 ± 9.3 years (mean $\pm$ SD)] were obtained

immediately after nephrectomy and directly fixed in freshly prepared 3% paraformaldehyde (pH 7.4). Six patients were on hemodialysis at the time of nephrectomy with a mean hemoglobin of 10.3 g/dl ($\pm$ 0.45) 1 day before nephrectomy, whereas one patient was not yet on dialysis with a hemoglobin of 15.1 g/dl. Collection and analysis of tissue samples were approved by the local ethics committee.

Animals

Animal experiments were approved by the institutional review board for the care of animal subjects and performed in accordance with National Institutes of Health guidelines. Unaffected wild-type (+/+, $n = 8$), heterozygous (cy/+, $n = 25$), and homozygous (cy/cy, $n = 5$) Han:SPRD rats were used. All animals had free access to tap water and standard rat chow. The light cycle was 12 hours, humidity 55%, and room temperature 20°C. Heterozygous animals were sacrificed at days 36 to 41 and homozygous animals at day 23. On the day of sacrifice, body and total kidney weight (sum of left and right kidney weight) were determined, and the two kidney-to-total body weight ratio was calculated (Table 1). Normal Sprague-Dawley rats exposed to 0.1% carbon monoxide for 6 hours, as described previously,¹⁶ served as positive controls. Kidneys of Han:SPRD (cy/+) and (+/+) were either prepared for immunohistochemistry ($n = 22$) or snap-frozen for RNA analysis ($n = 11$). Kidneys of (cy/cy) animals were prepared for immunohistochemistry.

Induction of Anemia

Wild-type rats and heterozygous Han:SPRD rats ($n = 2$ each) were anesthetized with isoflurane. Repetitive phlebotomy was performed with substitution of blood by normal saline solution, and the hematocrit level was decreased to a value of $\sim$ 0.20. Animals were allowed to regain consciousness and were euthanized after 4 hours.

Tissue Preparation for Immunohistochemistry

Han:SPRD rats were anesthetized with pentobarbital, and kidneys were either perfusion-fixed *in situ* via cannulation of the abdominal aorta or fixed by immersion. Perfusion fixation was performed with freshly prepared 3% paraformaldehyde (pH 7.4) at 240 mm Hg for 1.5 minutes and at 100 mm Hg for 3.5 minutes and followed by perfusion with sucrose/phosphate-buffered saline to stop fixation.

Kidneys were then removed, cut into slices, and snap-frozen in liquid nitrogen-cooled isopentane or processed for paraffin embedding.

Detection of Tissue Hypoxia

Pimonidazole (Hypoxyprobe; Chemicon Europe, Hampshire, UK) was injected intravenously via a tail vein (60 mg/kg body weight) into heterozygously (cy/+) low-grade ($n = 2$) and high-grade affected ($n = 2$) or unaffected (+/+) animals ($n = 2$). Animals were sacrificed 45 minutes after the injection and tissue was processed for immunohistochemistry.

Immunohistochemistry

Paraffin sections (2 to 4 μm) were dewaxed in xylene and rehydrated in a series of ethanol washes. Slides were coated with 3-aminopropyl-tri-ethoxysilane. For detection of HIF- α -isoforms, a monoclonal mouse anti-human HIF-1 α antibody ($\alpha 67$; Novus Biologicals, Littleton, CO) and polyclonal rabbit anti-mouse HIF-2 α antibodies (PM8 for human and PM9 for rat tissue, obtained from two different rabbits immunized with a peptide containing amino acids 337 to 439 of mouse HIF-2 α) were used at a dilution of 1:10,000 as described.^{16,21} Additional primary antibodies were monoclonal mouse anti-rat and anti-human VEGF (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-rat and human HO-1 (1:60,000; Stressgen, Victoria, BC, Canada), polyclonal rabbit anti-human glucose-transporter-1 (Glut-1, 1:10,000; Biotrend, Golden, CO), monoclonal mouse anti-human endoglin (CD105, 1:5000; DAKO, Hamburg, Germany) and a monoclonal mouse anti-pimonidazole antibody (Hypoxyprobe mAB1). Biotinylated secondary anti-mouse or anti-rabbit antibodies were used. For signal amplification and visualization, a signal amplification system (DAKO) was used according to the manufacturer's instructions. Antigen retrieval was performed for 6 minutes for paraffin-embedded tissue and 3 minutes for frozen tissue in preheated DAKO target retrieval solution, using a pressure cooker. For pimonidazole stainings, sections were cooked in citrate buffer. All incubations were performed in a humidified chamber. Between incubations, specimens were washed two to four times in buffer (50 mmol/L Tris-HCl, 300 mmol/L NaCl, and 0.1% Tween 20, pH 7.6). For peroxidase reaction, peroxidase-linked streptavidin was used with diaminobenzidine (DAKO) as chromogen. For alkaline phosphatase-linked streptavidin fast red or fast blue solution (Sigma-Aldrich, Taufkirchen, Germany) was used as chromogen. Control samples included samples from normoxic wild-type rats, which are known not to express HIF at a detectable level,¹⁶ and human kidneys or samples prepared with the use of preimmune serum from animals immunized against HIF-2 α . Slides were partially counterstained with hematoxylin or Richardson staining solution. For double-staining procedures, HIF-2 α was stained as described above, followed by immunohistochemistry using a CD68 antibody [1:200, mouse anti-human CD68 antibody

(clone PG-M1); DAKO] and an alkaline-phosphatase antibody using fast blue as chromogen. Signals were analyzed with a Leica DMRB microscope (Leica, Bensheim, Germany) using differential interference contrast. Photographs were digitally recorded by means of a Visitron system (Visitron, Puchheim, Germany).

Scoring of Immunohistochemically Detected Renal HIF- α Protein

The number of HIF-1 α - and HIF-2 α -positive cells per visual field was counted in 20 randomly selected visual fields per renal cross-section for $n = 4$ (+/+), $n = 10$ (cy/+) low, $n = 8$ (cy/+) high, and $n = 5$ (cy/cy) animals, the phlebotomized animals, and for the human ADPKD samples.

RNase Protection Assays

Total RNA was extracted from frozen tissue samples with RNazol B (Biogenesis, Poole, UK), and RNase protection assays were performed essentially as described.²² ³²P-labeled human riboprobes were generated using SP6 or T7 RNA polymerase (Roche Diagnostics, Mannheim, Germany), with the following protected fragments: rat HO-1, protected fragment 490 to 676 bp (accession no. NM_012580.2); mouse Glut-1, protected fragment 1073 to 1208 (accession no. M23384); mouse VEGF, rat EPO, protected fragment 386 to 578 (accession no. NM017001), and U6 small nuclear RNA, protected fragment 1 to 107 (human U6sn; accession no. X01366). Radiolabeled riboprobes were protected from RNase digestion by hybridizing to the following amounts of total RNA: HO-1 and EPO, 50 μg ; Glut-1, 30 μg ; and U6sn, 1 μg . The latter was used as internal control for each sample, adding 1/10 (ie, 100 ng) of each reaction to its appropriate sample after RNase digestion. After resolution on polyacrylamide gels, signals were quantified by phosphorimaging (BAS 2000; Fujix, Fuji, Japan). Values were normalized to the control (U6sn).

Cell Culture

HeLa cells and three types of renal tubular cells were used: Ox161, human renal tubular cells deficient for PKD-123; HKC-8 cells, an SV40-transformed human proximal tubular cell line (kindly provided by Prof. L. Racusen, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD) and primary human proximal tubular epithelial cells (hPTs) isolated after informed consent of patients undergoing tumor nephrectomy. The cystic epithelial cells (Ox161) were isolated from superficial cysts of a polycystic kidney removed because of persistent infection. Primary cultured cells were transduced sequentially at early passages with retroviral vectors containing a temperature-sensitive T antigen and human telomerase, selected for antibiotic resistance (G418 and hygromycin). Ox161 cells were cultured at 33°C in Dulbecco's modified Eagle's medium (DMEM)/

Figure 1. Evidence for hypoxia in kidneys of (cy/+) rats. Pimonidazole staining (black) of kidneys of heterozygously affected Han:SPRD rats (cy/+) revealed hypoxia in cortical cyst epithelia (**arrows**) in animals with low-grade [(cy/+) low] as well as high-grade PKD [(cy/+) high], whereas wild-type animals (+/+) stained negative for pimonidazole in the renal cortex. In the renal medulla, the extent of hypoxia as indicated by pimonidazole staining seems to be more severe in (cy/+) animals in comparison to wt animals. Adjacent to vascular bundles pimonidazole was not detectable (VB, vascular bundles). Original magnifications: $\times 200$ (top, middle); $\times 400$ (top, right).

F12 supplemented with 5% Nuserum. HeLa cells were maintained in DMEM (Seromed, Biochrom, Berlin, Germany), supplemented with 10% fetal calf serum, glutamine (2 mmol/L), penicillin (50 IU/ml), and streptomycin sulfate (50 $\mu\text{g/ml}$). HKC-8 cells were cultured in DMEM/Ham's F-12 (Seromed, Biochrom) in a 1:1 ratio containing 10% fetal calf serum (PAA), 2 mmol/L L-glutamine, 5 $\mu\text{g/ml}$ insulin, 5 $\mu\text{g/ml}$ transferrin, 5 ng/ml selenium, and 50 $\mu\text{g/ml}$ gentamicin. Monolayer cell cultures of hPTs were generated by outgrowth from 1-mm³ pieces of the normal cortex on a matrix of fetal calf serum. For maintenance and experiments, cells were cultured in serum-free DMEM/F12 supplemented with 2 mmol/L L-glutamine, 5 $\mu\text{g/ml}$ insulin, 5 $\mu\text{g/ml}$ transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 4 pg/ml triiodo-L-thyronine, 10 ng/ml epidermal growth factor, and 50 $\mu\text{g/ml}$ gentamicin. Passages 3 to 4 were used for the study, ~ 5 to 6 weeks after tissue preparation.

Cells were grown at 37°C at 21% O₂ and 5% CO₂ (normoxic conditions) or exposed to 6 hours of hypoxia (1% O₂, 5% CO₂, and 94% N₂). Hypoxic experiments were performed in a NuAire incubator (Zapf, Sarstedt, Germany).

Immunoblotting

Protein extraction and blotting were performed as described previously.²⁴ Protein content of extracts was quantified with the Bio-Rad DC protein assay (Bio-Rad, Munich, Germany), and 60 μg of each extract were resolved on polyacrylamide gels. Proteins were transferred onto an Immobilon P membrane (Millipore, Bedford, MA)

overnight and probed with anti-HIF-1 α monoclonal antibody (Transduction Laboratories, Lexington, KY) at a concentration of 1 $\mu\text{g/ml}$ each with NB 100-132 (1:2000; 190b; Novus Biologicals) or HIF-2 α . Signals were detected with horseradish peroxidase-conjugated antibodies (DAKO) and enhanced chemiluminescence (Super-Signal West Dura Extended; Pierce, Rockford, IL). After analysis, membranes were stained with Coomassie Blue (Sigma) to verify equal protein loading and transfer.

Statistics

All results are expressed as mean $\pm$ SD. A *P* value < 0.05 was considered significant. Statistical analysis was performed using a covariate analysis and nonparametric analysis for independent samples (Mann-Whitney *U*-test) using SPSS Software for Windows (version 14.0).

Results

Animal Characteristics

From the 38 Han:SPRD rats examined, eight were wild-type (+/+) and five were homozygous (cy/cy) animals (Table 1). The remaining 25 heterozygous (cy/+) animals were graded according to their two kidney-to-total body weight ratio. Animals with a ratio less than or equal to 1.36% (median ratio) were graded as low and animals more than this as high (Table 1). Whereas wild-type animals have a normal life span, heterozygous animals die at 12 to 21 months and homozygous animals die at 3 to 4

Figure 2. HIF- α accumulates in the renal cortex of (cy/+) and (cy/cy) rats. Top: Representative H&E-stained kidney sections of unaffected (+/+) and heterozygously (cy/+) and homozygously affected (cy/cy) Han:SPRD rats. In (+/+) animals, HIF- α was not detectable (left). Comparable focal staining of both HIF- α -isoforms occurred in (cy/+) and (cy/cy) rat-kidneys (middle and right columns). Cell populations expressing HIF-1 α and HIF-2 α protein were consistently different: HIF-1 α was expressed in epithelial cells of the cysts, whereas HIF-2 α occurred in pericystic stromal and endothelial cells. Original magnifications: $\times 12.5$ (top); $\times 400$ (left, middle and bottom rows); $\times 1000$ (middle and right).

weeks because of uremia.¹⁹ The top of Figure 2 shows representative hematoxylin and eosin (H&E) stains of (+/+), (cy/+), and (cy/cy) kidneys. Wild-type animals have normal kidney morphology. In (cy/+) animals, the number of cysts per section increases with a higher grading but normal renal tissue is still existent at the time of sacrifice (days 36 to 41). The kidneys of the very severely affected (cy/cy) animals are completely interspersed with cysts without any remaining normal renal tissue.

Evidence for Pericystic and Medullary Hypoxia in Kidneys of Han:SPRD (cy/+) Rats

To identify regions of severe hypoxia in cystic kidneys, pimonidazole was injected intravenously into wild-type (+/+) and heterozygous (cy/+) Han:SPRD rats. The grading of the injected animals revealed that two of the (cy/+) animals suffered from low-grade PKD and two from high-grade PKD. Pimonidazole staining was negative in the cortex of wild-type animals (Figure 1, top),

whereas in kidneys of low- and high-grade (cy/+) animals pimonidazole was strongly detectable within the epithelium of cortical cysts. In addition, focal staining of the papilla was evident, with a staining intensity that increased with increasing distance from vascular bundles (VBs) (Figure 1, bottom).

Expression of HIF- α in Kidneys of Han:SPRD (+/+, cy/+, cy/cy) Rats

Staining for HIF-1 α and HIF-2 α in the cortex and medulla of unaffected (+/+), heterozygous (cy/+), and homozygous (cy/cy) Han:SPRD rats are shown in the middle and bottom panels of Figures 2 and 3. Neither HIF-1 α nor HIF-2 α was detectable in kidneys of (+/+) animals. In contrast, in cystic kidneys of (cy/+) and (cy/cy) rats, HIF-1 α and HIF-2 α were focally and strongly expressed within the renal cortex (Figure 2) and the renal medulla (Figure 3). The cellular distribution of the two HIF- α isoforms was markedly consistent between (cy/+) and (cy/cy) rats and well comparable with normal rat kidneys

Figure 3. HIF- α accumulates in the renal medulla of (cy/+) and (cy/cy) rats. As in the renal cortex, HIF- α clearly accumulates in the renal papilla of (cy/+) and (cy/cy) rats with the same cellular distribution pattern of HIF-1 α and -2 α , whereas no HIF- α was detectable in the renal papilla of wild-type (+/+) rats. Original magnifications, $\times 400$.

studied under systemic or regional hypoxia: HIF-1 α was found in tubular epithelial cells and HIF-2 α in peritubular interstitial cells, including endothelial cells.^{16–18} There was some variability between kidneys, which seemed to be related to disease severity: low-grade (cy/+) showed less HIF- α accumulation than high-grade (cy/+) or (cy/cy) kidneys (Figure 6). In heterozygous rats with low-grade PKD, HIF- α was predominantly detectable in the inner medulla and only sporadically in the renal cortex (data not shown), whereas in more severe cases, HIF- α was also detect-

able in the renal cortex. In kidneys of these more severely affected heterozygous as well as in (cy/cy) animals, HIF-1 α accumulated predominantly in epithelial cells of the cysts (Figure 2) and in medullary collecting ducts (Figure 3). In contrast, strong staining of HIF-2 α was found in interstitial cells of the renal medulla (Figure 3) and in pericystic stromal cells (Figure 2) of the renal cortex. The expression of HIF- α in kidneys of normal rats exposed to hypoxia (carbon monoxide 0.1% for 6 hours) revealed the same distribution pattern as described previously (data not shown).¹⁶

Table 2. Summary of HIF- α Distribution in PKD

	HIF-1 α				HIF-2 α			
	(+/+)	(cy/+)	(cy/cy)	ADPKD*	(+/+)	(cy/+)	(cy/cy)	ADPKD*
Cortex								
Glomeruli	–	–	–	–	–	–	–	++
Non-CE	–	–	n.a. [†]	+ [‡]	–	–	–	–
CE	n.a.	+	+++	+++	n.a.	–	–	–
EC	–	–	–	–	–	+	++	++
IC	–	–	–	–	–	+	++	+++
Outer medulla								
Non-CE	–	+ [‡]	n.a. [†]	n.a.	–	–	–	–
CE	n.a.	+	++	++	n.a.	–	–	–
EC	–	–	–	–	–	+	+	++
IC	–	–	–	–	–	+	++	+++
Papilla								
Non-CE	–	++	+	n.a.	–	–	–	n.a.
CE	n.a.	n.a.	++	n.a.	n.a.	–	–	n.a.
EC	–	–	–	n.a.	–	+	+	n.a.
IC	–	–	–	n.a.	–	++	+++	n.a.

CE, cystic epithelium; EC, endothelial cells; IC, interstitial cells; n.a., not applicable.

*Not all sections contained medulla/glomeruli, no section contained papilla.

[†]In kidneys of homozygous animals there is almost no noncystic epithelium left.

[‡]Only sporadically (<5%).

ADPKD

HIF-1 α

HIF-2 α

Figure 4. HIF- α accumulates in ADPKD. Both HIF- α isoforms are expressed in human ADPKD. The cellular distribution of HIF-1 α (epithelial cells, left) and HIF-2 α [pericycystic stromal cells and endothelial cells (**arrowhead**), right] resembles the distributional pattern in PKD-rats. Original magnifications: $\times 100$ (top); $\times 1000$ (bottom).

The distribution of the two HIF- α isoforms in the different renal cell compartments is summarized in Table 2.

Expression of HIF- α in Human ADPKD

The human kidneys that were analyzed represent an advanced stage of ADPKD with a high degree of fibrosis and a broad inflammatory infiltrate compared with the kidneys of Han:SPRD rats. HIF- α accumulation in these kidneys was more prominent and more widely distributed than in PKD rats (Table 2, Figure 6). Consistent with the findings in the rat model, a clear staining for HIF-1 α in epithelial cells of the cysts (Figure 4, top), within the renal medulla (Figure 4, bottom) and sporadically in infiltrating leukocytes was also seen in human PKD. Marked staining for HIF-2 α was found in interstitial cells (Figure 4) and in endothelial cells of human kidneys (Figure 4, arrowhead). To determine the origin of the interstitial cells that were

positive for HIF-2 α , we performed double staining for HIF-2 α and CD68, a marker of human neutrophils, and found a relevant part of interstitial cells staining positive for both HIF-2 α and CD68 (Figure 5).

Relationship between the Number of HIF- α -Positive Cells and the Severity of PKD

Scoring for HIF- α revealed for both isoforms an increase in the mean number of positive cells with increasing severity of the disease (Figure 6), with no detectable HIF- α in wt (+/+) and the highest number of HIF- α -positive cells in ADPKD kidneys. Differences between the groups were significant, with the only exception that the mean number of HIF-2 α -positive cells of (cy/+) high rats was not significantly different from that of (cy/cy) rats (Figure 6).

HIF-2 α /CD68

Figure 5. HIF-2 α is expressed in neutrophils in ADPKD. Double labeling was performed for HIF-2 α (brown) and the leukocyte marker CD68 (blue), revealing that HIF is stabilized in human neutrophils in the interstitium of ADPKD kidneys. Original magnifications, $\times 630$.

Increase in Renal HIF- α Stabilization in Response to Phlebotomy in Han:SPRD (cy/+) Rats

To assess whether HIF- α stabilization in PKD rats is still influenced by its physiological stimulus, hypoxia, we used repetitive phlebotomy in male (cy/+) and normal Sprague-Dawley rats to decrease the hematocrit to 20%. Anemia led to a further increase in HIF- α accumulation, with HIF-1 α becoming detectable in noncystic tubular epithelial cells (Figure 7, left column) and HIF-2 α staining in cortical and medullary interstitial cells of the (cy/+) rats (Figure 7, right column) compared with kidneys of non-phlebotomized (cy/+) rats (Figures 2 and 3). Scoring of the number of HIF- α -positive cells revealed an increase in both normal and (cy/+) rats [HIF-1 α from 18.8 ± 4.8 positive cells per visual field in (cy/+) high kidneys (Figure 6) to 32.3 ± 3.7 in anemic (cy/+) kidneys; HIF-2 α from 10.1 ± 4.7 to 38.6 ± 1.7 in anemic (cy/+) kidneys]. Phlebotomized normal rats were strongly HIF- α -positive, but the mean number of HIF- α -positive cells was lower than in the anemic (cy/+) rats (HIF-1 α : 26.1 ± 8.5 ; HIF-2 α : 17.2 ± 1.9).

Figure 6. Scores for HIF- α in rat and human PKD. The mean number of HIF-1 α - and HIF-2 α -positive cells increases with increasing severity of the disease in rats. In kidneys of wt animals, HIF- α is absent. In human ADPKD kidneys, the highest numbers for HIF- α -positive cells were found, with higher scores for HIF-2 α than for HIF-1 α .

HIF Target Gene Expression in Kidneys of Han:SPRD (cy/+) and (cy/cy) Rats

RNA expression of HO-1, Glut-1, VEGF, and EPO was quantified by RNase protection assays of whole kidney extracts of (cy/+) and (+/+) rats and normoxic and hypoxic normal Sprague-Dawley rats (Figure 8, A and B). In addition, immunostaining for VEGF, Glut-1, and HO-1 was performed (Figure 8C). Compared with kidney extracts from normoxic and hypoxic Sprague-Dawley rats, HO-1, Glut-1, and EPO mRNA were elevated in cystic kidneys, albeit to a variable extent. Although EPO expression in kidneys of normal hypoxic Sprague-Dawley rats was stronger than in cystic kidneys, the expression of Glut-1 and HO-1 in cystic kidneys was comparable with that observed under systemic hypoxia (Figure 8B). HO-1 expression levels corresponded well with the severity of cystic disease, with the lowest expression in wild-type Han:SPRD rats (+/+), intermediate expression in kidneys of (cy/+ low), and the highest expression levels in (cy/+ high) animals. RNA levels of Glut-1 did not vary significantly between animals (Figure 8, A and B), and EPO expression was relatively low in cystic kidneys and did not correlate with the grading of (cy/+) animals (Figure 8, A and B). VEGF mRNA was lower in PKD kidneys than in hypoxic and control kidneys (Figure 8, A and B), and there was no significant difference in VEGF expression between wild-type and the different grades of cystic kidney of the Han:SPRD rats.

At the protein level, HO-1 was detectable in the cytoplasm of epithelial cells [Figure 8C, (cy/+)]. With increasing severity of the disease, the number of infiltrating leukocytes increased, partially staining positive for HO-1 as well [Figure 8C, (cy/cy)], and possibly contributing to the higher HO-1 mRNA levels in kidneys of more severely affected animals. Glut-1 was detectable by immunohistochemistry in epithelial cells of the cyst wall of human PKD and in (cy/+) and (cy/cy) rat kidneys (Figure 8C, top), which corresponds to HIF-1 α localization. Notably, this localization of Glut-1 differed from that in normal rat kidneys, where Glut-1 was confined to medullary thick ascending limbs and collecting ducts, as observed previously (data not shown).²⁵

In control kidneys, VEGF was detectable in medullary and cortical thick ascending limbs as well as in some glomerular cells, as found previously (data not shown).²⁶ In cystic kidneys of (cy/+) and (cy/cy) rats, VEGF was also detectable in epithelial cells of the cysts and in collecting ducts (Figure 8C). The localization of VEGF signals thus partly corresponds to HIF-1 α expression.

HIF Target Gene Expression in Human ADPKD

Comparable with polycystic rat kidneys, Glut-1 and VEGF were detectable in epithelial cells of human polycystic kidneys, which also corresponds to HIF-1 α accumulation (Figure 9). In addition, the angiogenic factor endoglin, which is known to be expressed in newly formed vessels,²⁷ was strongly expressed in pericystic vessels (Figure 9), which corresponds to HIF-2 α localization. HO-1

Figure 7. Increased HIF- α accumulation in phlebotomized (cy/+) rats. Cellular specificity of HIF- α is conserved under anemic conditions, but the number of cells staining positive for HIF- α increases in anemic PKD rats (bottom). In addition to the cystic epithelium (#), HIF-1 α strongly stained positive in unaffected tubular epithelial cells (*). The number of interstitial cells reacting positive for HIF-2 α also increases under anemic conditions in kidneys of (cy/+) rats. Phlebotomized healthy Sprague-Dawley rats were used as controls (top). Original magnifications, $\times 400$.

was strongly expressed in infiltrating macrophages (Figure 9), whereas epithelial cells were negative (not shown).

HIF- α Regulation in Polycystin-Deficient Cells

In the majority of patients with ADPKD, an inactivating mutation of the PKD-1 gene results in defective polycystin-1, the gene product of PKD-1. To evaluate whether HIF- α stabilization or regulation is influenced by polycystin-1 deficiency, we investigated if HIF- α in polycystin-1-deficient Ox161-cells displays a physiological oxygen-dependent regulation. In comparison to HeLa cells (known to have a normal regulation of both HIF- α isoforms) and the proximal tubular cell line HKC-8 and hPT, there was no difference in the pattern of regulation of HIF- α in polycystin-deficient cells (Ox161); HIF-1 α and HIF-2 α were both barely detectable under normoxic conditions but accumulated strongly under hypoxic conditions (Figure 10).

Discussion

This study demonstrates significant areas of regional hypoxia in cystic kidneys, identifies the accumulation of HIF transcription factors in this disease entity, demonstrates parallel up-regulation of HIF target genes and, thereby overall, provides evidence for regional hypoxia as a novel and potentially important pathomechanism in the progression of PKD. Several lines of evidence suggest that the accumulation of HIF results from regional hypoxia. First, in Han:SPRD rats, an established model of PKD, we found staining for the bioreductive dye pimonidazole, which labels tissues with pO_2 values of less than 10 mm Hg.²⁸ Pimonidazole staining in the medulla was consistent with known oxygen gradients in normal kidneys, but pimonidazole staining was also found in the epithelium lining many cysts. Second, the cellular distribution of the two HIF isoforms, with HIF-1 α expression in epithelial cells and HIF-2 α expression in interstitial and peritubular endothelial cells, is consistent with the pattern

Figure 8. Expression of HIF target genes in (+/+), (cy/+), and (cy/cy) rats. The mRNA levels of the HIF target genes HO-1, Glut-1, EPO, and VEGF were determined by RNase protection assay (**A**) and quantified by densitometry (**B**). RNase protection assay revealed different levels of EPO mRNA in kidneys of Han:SPRD rats independent of the genetic status (**A**, **B**), whereas HO-1 mRNA levels correlated with the grade of disease (**A**, **B**), with a significant increase in mRNA in (cy/+) and (cy/cy) kidneys compared with wild-type (+/+) animals (**B**). Glut-1 levels were comparable with hypoxia but were not significantly different between (+/+), (cy/+), and (cy/cy) animals (**A**, **B**). Renal RNA extracts from normoxic (control) and hypoxic normal Sprague-Dawley rats served as controls. **C**: Glut-1 and VEGF protein were detectable in the cytoplasm of epithelial cells of the renal cysts of heterozygous (cy/+) and homozygous (cy/cy) rats. HO-1 stained positive in cyst epithelium and in (cy/cy) kidneys in addition to interstitial leukocytes. * $P < 0.05$.

of HIF induction previously observed in normal kidneys under conditions of systemic or regional hypoxia.^{16–18} Third, additional systemic hypoxia, resulting from phlebotomy of Han:SPRD rats led to a further up-regulation of HIF in both cystic and morphologically normal regions of the kidney, which indicates sustained physiological regulation of HIF- α . Conversely, anemia cannot solely account for HIF induction in cystic kidneys under baseline conditions. Heterozygous Han:SPRD rats are only mildly anemic,¹⁹ and although the majority of patients whose kidneys were analyzed had different degrees of moderate anemia, one patient had a normal hemoglobin level and nevertheless showed HIF expression in his kidney. Thus, a disruption of tissue architecture including the fibrosis of pericystic tissue and a possible compression of peritubular capillaries probably impairs oxygen delivery in cystic kidneys, an effect that would be enhanced by a reduction in blood oxygen-carrying capacity. Fourth, a very similar pattern of HIF induction was observed across species and thus independent of the genetic defect. At the same time, findings in rats and humans were also consistent in that HIF induction appeared to increase with disease progression. Fifth, the mean number of HIF-

α -positive cells increases with increasing cyst formation. Finally, we were able to exclude that a polycystin gene defect induces HIF in an oxygen-independent manner because hypoxic HIF stabilization was normal in polycystin-deficient cells and no obvious deviation in HIF- α regulation, such as in VHL-deficient cell lines, was found. Nevertheless, we cannot exclude that additional nonoxygen-dependent mechanisms of HIF induction may also play some role in HIF induction and regulation of its activity. Particularly in human kidneys, we observed a marked inflammatory infiltrate. Cytokines released by infiltrating leukocytes have been shown to affect HIF stability and/or activity. For example, interleukin-1 and tumor necrosis factor- α were shown to facilitate stabilization and DNA binding of HIF.²⁹

The rather marked and widespread accumulation of HIF in both rat and human cystic kidneys is remarkable because in other models of renal hypoxia there seems to be a down-regulation of HIF with time, despite persistent compromise of tissue oxygenation.^{17,18} The mechanisms for this down-regulation are yet poorly understood but may involve the induction of HIF prolyl-hydroxylases,³⁰ which promote the destabilization of HIF. How such

Figure 10. Normal hypoxic up-regulation of HIF- α in polycystin-1-deficient cells. Immunoblots showing no differences in HIF- α regulation between HeLa cells, HKC-8 cells and primary proximal tubular cells (hPT) and polycystin-1-deficient Ox161-cells: strong increase in HIF-1 α (top row) and HIF-2 α (bottom row) under hypoxic conditions.

mechanisms are apparently overcome in cystic kidneys, remains unclear. On the other hand, partial and temporary down-regulation of the HIF response could explain why at a given time point only a subset of cells and cysts were found to accumulate HIF.

A fundamental principle of tumor biology is that diffusion limitation for oxygen and nutrients occurs when tumor cell clusters begin to form and that further progression depends critically on metabolic adaptation of the tumor and the formation of new vessels. These adaptive responses are believed to be primarily mediated by HIF.³¹ HIF up-regulation has been demonstrated in a large spectrum of different tumors, and the experimental blockade of HIF induction in tumor xenografts, at least in most cases, is associated with reduced tumor growth.^{32,33} On the basis of our observations, we propose that similar mechanisms may play an important role in the growth of renal cysts. Five different target genes investigated, involved in different aspects of cellular or systemic adaptation to hypoxia, were found to be expressed in PKD. Localization of these target gene products reveals a partial overlap with either one of the two HIF- α isoforms. Thus, the expression of HO-1, Glut-1, and VEGF in the cyst epithelium corresponds to HIF-1 α expression. In the case of Glut-1 and VEGF, a difference from the normal expression pattern was evident, in which staining is restricted to specific tubular segments.^{25,26} Endoglin, a marker of neovascularization, stained positive in endothelial cells of pericycystic blood vessels. In addition, pericycystic stromal cells were previously shown to express EPO.⁷ Thus, endoglin and EPO localization correspond well to HIF-2 α protein localization. Such target gene specificity is in line with other recent findings demonstrating that HIF-2 α is the HIF isoform responsible for EPO regulation.²² Pericycystic HIF-2 α induction may thus provide an explanation for the well-known phenomenon of comparatively higher hemoglobin concentrations in PKD patients. Additional evidence also suggests that EPO has proangiogenic as well as anti-apoptotic effects on nonhematopoietic cells.^{34,35} It is possible therefore that pericycystic neovascularization results from a concerted action of both HIF- α -isoforms with angiogenic factors, including VEGF released from the cyst epithelium,

Figure 9. Expression of HIF target genes in ADPKD. Glut-1 and VEGF are strongly positive in the cytoplasm of epithelial cells of the renal cysts. Endoglin, involved in neovascularization, is expressed in pericycystic and interstitial vessels. HO-1 stained positive in macrophages. Original magnifications: $\times 400$ (Glut-1, endoglin); $\times 630$ (VEGF, HO-1).

EPO secretion from interstitial cells in the cyst walls, and direct up-regulation of angiogenic factors such as endoglin in endothelial cells.

Apart from facilitating cyst growth by adaptation to a hypoxic microenvironment, HIF might also play a more direct role in the uncoordinated proliferation of tubular epithelial cells. Patients affected by the von-Hippel Lindau (VHL) disease are at increased risk to develop renal cancer, and their kidneys typically show a spectrum of epithelial disorders, including dysplastic foci, cystic lesions, and multilocular clear cell carcinoma.³⁶ The VHL protein plays a critical role in the oxygen-dependent degradation of HIF- α and nonfunctional VHL leads to oxygen-independent HIF activation.³⁷ A systematic analysis of kidneys from patients with the VHL syndrome has shown that HIF activation is an early event in the development of renal lesions and co-localizes with epithelial dysplasia and cystic malformation of renal tubules.³⁸ Mice with conditional VHL inactivation in renal proximal tubular cells develop renal cysts, which could be prevented by additional inactivation of HIF- β .³⁹ In addition, it has very recently been shown that VHL inactivation is associated with abrogation of the primary cilium,⁴⁰ an extracellular appendage that transmits calcium signals after mechanical bending and the function of which is altered in different types of PKD.⁴¹ However, the precise mechanisms by which HIF and its target genes are involved in dysregulation of tubular epithelial cell proliferation remain to be elucidated.

Because of inheritance, patients with ADPKD can frequently be identified at an early stage. However, therapeutic approaches to retard cyst formation have so far not been established. Experimental approaches, which await testing in clinical trials, include the use of anti-proliferative and immunosuppressive mTOR inhibitors.^{42–44} Interestingly, mTOR has recently been demonstrated to be upstream of HIF, and HIF responses are reduced under mTOR inhibition.^{45,46} Based on our findings, suggesting that HIF may play an important role in determining the phenotype and progression of PKD, it is possible that disruption of this pathway by mTOR or HIF inhibitors retards disease progression.⁴⁷

Acknowledgments

We thank Simone Baumann and Gertrud Gruber for their excellent technical assistance.

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