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ORIGINAL PAPER

Primary cultures of renal proximal tubule cells derived from individuals with primary hyperoxaluria

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Abstract The primary hyperoxalurias, PH1 and PH2, are inherited disorders caused by deficiencies of alanine:glyoxylate aminotransferase and glyoxylate reductase, respectively. Mutations in either of these enzymes leads to endogenous oxalate overproduction primarily in the liver, but most pathological effects are exhibited in the kidney ultimately leading to end-stage renal failure and systemic oxalosis. To provide a non-invasive means of accessing kidney cells from individuals with primary hyperoxaluria, we have derived primary cultures of renal proximal tubule cells from the urine of these patients. The cells stain positively for the epithelial markers pan-cytokeratin and zonula occludens 1 and the proximal tubule marker γ-glutamyl transpeptidase. Mutation analysis confirmed that the cultured cells had the same genotype as the leucocytes of the patients and also expressed glyoxylate reductase at the mRNA level, illustrating their potential value as a source of renal material from these individuals.

Keywords Primary hyperoxaluria · Kidney · Human proximal tubular cell · Cell culture

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Introduction

Primary hyperoxaluria type 1 (PH1; OMIM 259900) and type 2 (PH2; OMIM 260000) arise from inherited deficiencies of two enzymes; alanine:glyoxylate aminotransferase (AGT; E.C 2.6.1.44) [1] and glyoxylate reductase/hydroxypyruvate reductase (GR/HPR; E.C 1.1.1.79) [2] respectively, resulting in a failure to metabolise glyoxylate. Although AGT is liver-specific [1], GR/HPR has a widespread tissue distribution although the bulk of the enzyme is in the liver [3, 4]. Thus, the two disorders can be considered as failures of hepatic glyoxylate detoxification. The diseases typically present in childhood and cause severe morbidity and poor life expectancy in affected patients due to the overproduction of endogenous oxalate from glyoxylate and with pathological effects in the kidney ultimately leading to the end-stage renal failure and systemic oxalosis.

Although the kidney is the organ mainly affected by PH, renal biopsies are not usually performed. Therefore, as an alternative strategy, we aimed to isolate renal cells from PH patients which could be studied in vitro. Our data demonstrates that primary cultures of human proximal tubule cells can be obtained from urine of patients with PH, providing the means with which to study the impact of these diseases on the kidney.

Materials and methods

Patients

Fresh urine samples were collected without preservative in 30 ml universal tubes from PH1 patients ($n = 7$) and PH2 patients ($n = 10$), during routine visits to the clinic. All the PH1 patients were male with a mean age of 9.3 years (range

1–21 years). In the group of PH2 patients, three were male and seven female with a mean age of 11.4 years (range 7–21 years). Ethical permission for this study was obtained from South Birmingham Ethics committee (reference 2002/050).

Cell culture

A 25 ml aliquot of urine was centrifuged (450 *g* for 5 min) within 2 h of collection. The cell pellet was re-suspended in Renal Epithelial Basal Medium supplemented with 0.5% foetal calf serum (FCS), growth factors, and antibiotics (Cambrex Bio Science, Wokingham, UK). All cells were cultured on tissue culture flasks coated with type I collagen from calf skin (Sigma, Poole, UK) at 37°C in a 5% CO_2 incubator. The growth medium was changed twice weekly and cells were passaged 1:3 or 1:5 on confluence. The cells were characterised in monolayer culture at passages 4–6.

Characterisation of the proximal tubular cell phenotype

Immunohistochemistry

5×10^3 cells/well were plated out in 200 µl of growth medium onto eight-well Lab-Tek chamber slides (Nunc, Hereford, UK) coated with type I collagen from calf skin. After 48 h, the cells were fixed with 4% paraformaldehyde and non-specific binding sites were blocked by incubation with 10% FCS, 0.2% bovine serum albumin (BSA; Sigma) and 0.1% Tween-20 in phosphate-buffered saline (PBS; Invitrogen, Paisley, UK) for 30 min. Cells were then incubated overnight at 4°C with antibodies against the epithelial markers pan-cytokeratin (1:50, Abcam, Cambridge, UK)[5], zonula occludens 1 (ZO-1) (1:20; Invitrogen) [6] and the proximal tubule marker γ-glutamyl transpeptidase (1:50; Santa Cruz Biotechnology, Heidelberg, Germany) [5]. Where required, slides were washed and then exposed to their corresponding FITC-conjugated secondary antibodies (1:100, Dako UK Ltd, Ely, UK) for 1 h at room temperature, counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Peterborough, UK) and visualised under a fluorescence microscope. The immortalised human proximal tubule cell line, HK-2 (American Type Culture Collection, Manassas, VA, USA [7] was used as a positive control and cells incubated without primary antibody as a negative control.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was isolated from PH and control patient cells with Tri-Reagent, according to providers' instructions (Sigma). cDNA was prepared from 1 µg total RNA and a 25 µl final volume PCR mixture was set up as previously described [8], for aminopeptidase A, uromodulin, and aquaporin-3. The house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as a loading control. The sequence of the primers used and expected amplicon sizes were as follows: aminopeptidase A (length 164 bp) sense: 5′-TGG AGA ACT GGG GAC TCA TC-3′ antisense: 5′-CCA CAA TGC TTC CCA CCA GT-3′; uromodulin (length 200 bp) 5′-CCA ATG ACA TGA AGG TGT CG-3′; antisense: 5′-GCT GTA AGT GGC ATG GGT TT-3′; aquaporin 3 (length 781 bp) sense: 5′-ACC CTC ATC CTG GTG ATG TTT G-3′ antisense 5′-TCT GCT CCT TGT GCT TCA CAT-3′; GAPDH (length 173 bp) sense: 5′-CTG ACT TCA ACA GCG ACA CC-3′ antisense: 5′-TTA CTC CTT GGA GGC CAT GT-3′. PCR amplifications were performed on a DNA Engine Dyad (MJ Research, Waltham, MA). Negative controls of reactions without cDNA template were included. The results shown are representative agarose gels of at least three independent experiments.

Confirmation of patient genotyping

Genomic DNA was isolated from cell pellets using Qiagen DNA blood mini kit (Qiagen, Crawley, UK) and amplified using primers as previously described [4, 9]. In addition, cDNA was used to amplify the GRHPR gene using primers HPRA and Ex9R3 as previously described [4]. PCR products were purified using the QIA PCR purification system (Qiagen) prior to sequencing on an ABI 3100 genetic analyser (Applied Biosystems, Warrington, UK).

Results

Cell culture was attempted from 26 fresh urine collections of 17 primary hyperoxaluric patients. Nine of the samples (35%) became contaminated with bacterial infection within 7 days of culture, despite showing an initial negative result on urinalysis strips. Of the remaining 17, small colonies were observed in 11 of the samples (64.7%) after 7–10 days in culture, while 6 (35.3%) had no growth even after 3 weeks in culture. Of the 11 samples that demonstrated colonies, 4 (36%) samples developed focal monolayers only, while the remaining 7 (64%) grew to confluent monolayers within 2–4 weeks. Subculture of these cultures was successful in five of the samples (71%), and all five were maintained in culture for a minimum of six passages. The cells displayed an epithelial morphology, with a characteristic "cobblestone" appearance, which did not alter on subculture. The growth pattern and morphology of the cell lines derived from the two PH types did not differ to that of the unaffected controls or the HK-2 cell line, as assessed by light microscopy (Fig. 1).

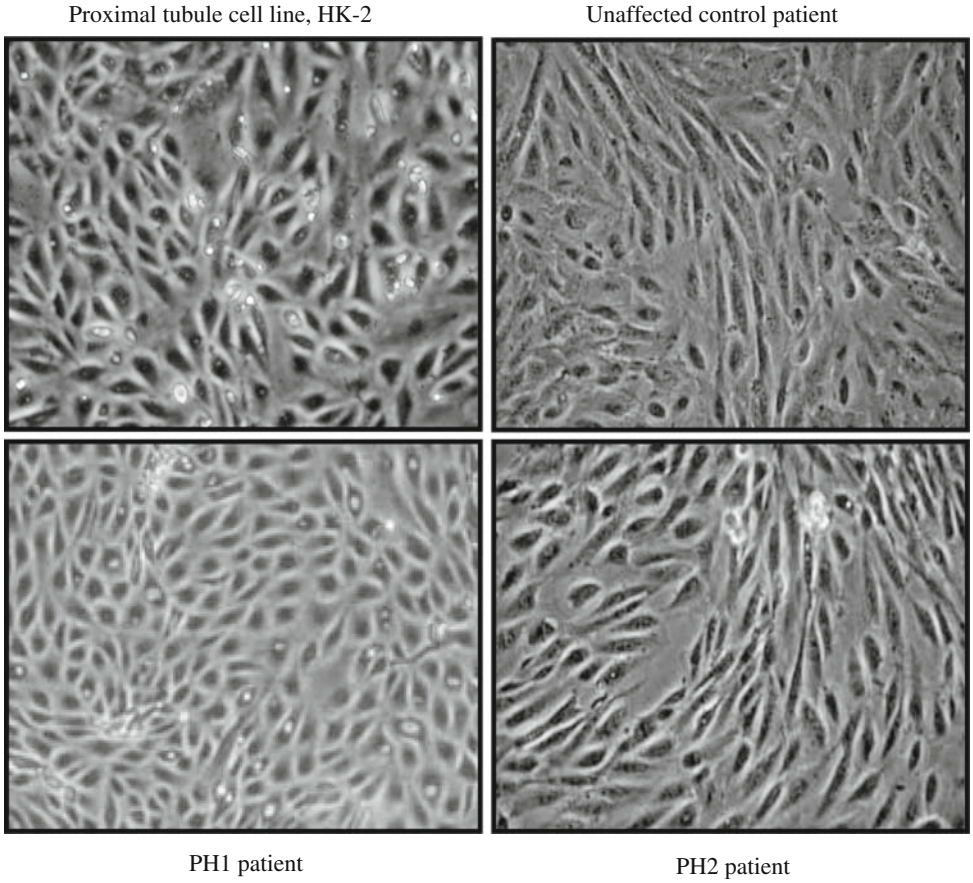

Fig. 1 Cultured epithelial cell monolayers from the proximal tubule cell line, HK-2 (*top left*); an unaffected control sample (*top right*); a PH1 patient urine sample (case #4) (*bottom left*); and a PH2 patient urine sample (case #1) (*bottom right*). Cells demonstrated a classical epithelial morphology with all cells having the characteristic "cobblestone" appearance, which remained unchanged on subculture (magnification ×100)

To characterise the cells, immunocytochemistry for the epithelial cell markers pan-cytokeratin [5] and the tight junction marker, ZO-1 [6] was performed. For each of the samples examined, positive immunofluorescence was observed in all of the cells for pan-cytokeratin (Fig. 2, left-hand panel). In addition, positive staining for ZO-1 localised to the regions of intercellular contact was observed for all cells, consistent with the known site of expression [6] (Fig. 2, centre panel). However, these markers do not distinguish between the origin of different epithelial cells. We, therefore, performed staining against the brush border enzyme γ-glutamyl transpeptidase [5]. Confocal microscopy demonstrated that over 90% of the cells showed strong positive staining distributed throughout the plasma membrane and cytoplasm (Fig. 2, right-hand panel), as previously reported [10]. In addition, PCR analysis demonstrated positive expression of the proximal tubule marker aminopeptidase A but no detectable expression was observed for uromodulin or aquaporin 3 (Fig. 3), markers of the distal convoluted tubule and the principal cells of the collecting duct, respectively [11]. Nevertheless, the expression of uromodulin and aquaporin 3 was present in human kidney mRNA used as a positive control.

Mutation analysis of DNA prepared from the five samples maintained in long-term culture demonstrated the same genotype as the leucocytes of the patients (Table 1) with one exception (case 5, Table 1). This patient was in receipt of a combined liver/kidney transplant and the exfoliated cells were therefore presumably those of the donor.

Unlike the liver-specific AGT, GRHPR expression has been shown in the kidney [3, 4]. We therefore analysed mRNA expression of GRHPR in our proximal tubule cells using RT-PCR (Fig. 4). As shown, the samples obtained from a PH1 patient (case 4, Table 1) and a representative control patient demonstrated positive expression of GRHPR at the expected band size. Interestingly, a PH2 patient (case 1, Table 1) showed an alternative sized message, which on sequencing was shown to be heterozygous for 540delT mutation and encode an aberrantly spliced transcript in which exon 1 was fused to exon 5.

Discussion

Although PH is not primarily a renal disease, the kidney is the main target of chronic oxalate exposure. A source of renal tissue is therefore invaluable for analysis of kidney-derived gene expression in these patients. Renal biopsies are invasive and rarely performed in this disorder and the isolation of renal tubular cells from urine seems to be a

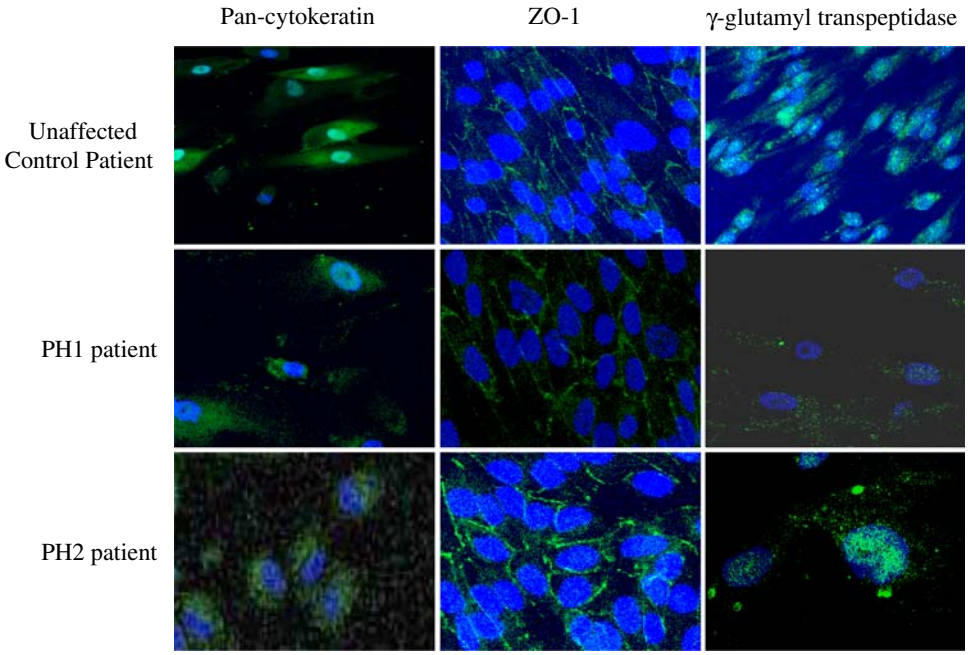

Fig. 2 Immunocytochemistry for a panel of epithelial and proximal tubule markers of cultures grown from urine samples of unaffected control samples (*top row*), a PH1 patient (*middle row*), and a PH2 patient (*bottom row*). Immunofluorescence was observed in all of the cells for the epithelial markers pan-cytokeratin (*left-hand panel*) and ZO-1 (*centre panel*), which was localised to the regions of intercellular contact. Over 90% of the cells showed strong positive staining against the brush border enzyme γ-glutamyl transpeptidase distributed throughout the plasma membrane and cytoplasm (*right-hand panel*) (magnification ×60)

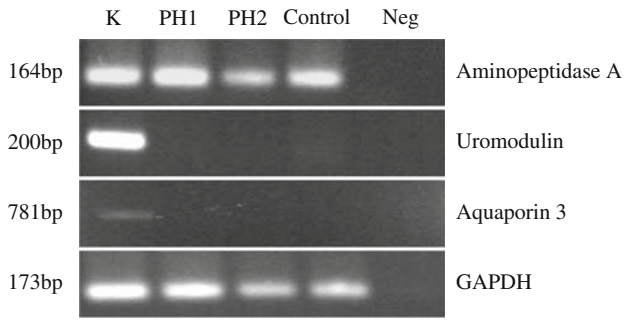

Fig. 3 RT-PCR characterisation of the exfoliated proximal tubule cells. RNA isolated from the cultured cells of a PH1 patient urine sample (*case #4*); a PH2 patient urine sample (*case #1*) and an unaffected control sample expressed the proximal tubule marker, aminopeptidase A, but not uromodulin (distal tubule marker) or aquaporin 3 (collecting duct marker). Expression of GAPDH was used as a house-keeping gene. Kidney RNA was used as a *positive control*. *Negative controls* which contained no cDNA are also shown. Results shown are representative agarose gels of at least three independent experiments

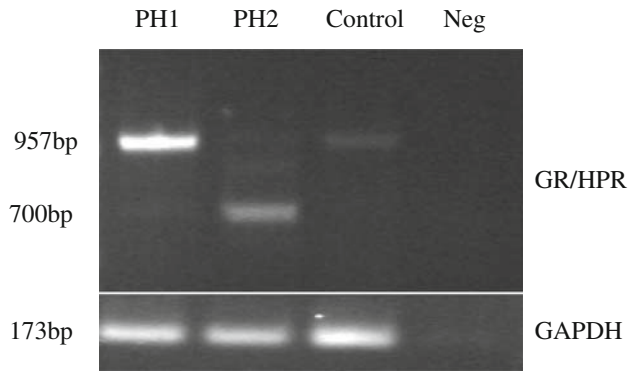

Fig. 4 mRNA expression of the GRHPR gene in the exfoliated proximal tubule cells. RT-PCR demonstrated GRHPR gene expression in the cultured cells of a PH1 patient urine sample (*case #4*); a PH2 patient urine sample (*case #1*) and an unaffected control sample. Expression of GAPDH was used as a house-keeping gene. Negative controls which contained no cDNA are also shown. Results shown are representative agarose gels of at least three independent experiments

reasonable alternative strategy. The technique also has the potential to provide patient-specific cell lines which may be useful for physiological studies. For example, the identification of the proximal tubular SLC26A6 oxalate transporter [12] and its role in renal oxalate transport [13] is one such candidate for analysis in patients with PH.

Children with PH have great variation in the onset of renal disease, even within individuals in the same family and with the same mutation. Clearly as yet unidentified genes influence susceptibility to stone development, and our goal is to identify such genes. Our hypothesis is that genes expressed by proximal tubule cells control susceptibility to the development of oxalate stones. The implication is that the response to oxalate differs between individuals as a result of differences in the expression of these gene(s) and is responsible for the differences in susceptibility to the development of calcium oxalate stones. Therefore, isolating cells from patients with PH provide an opportunity to study alterations in gene expression which would not be possible using proximal tubule cells cultured from urine of control subjects or obtained from existing cell lines.

A number of protocols are in existence for the culture of proximal tubular cells from freshly collected urine samples and primary cultures have been used to investigate cystinosis and Fanconi's syndrome [14, 15] and renal tubular transport [16]. We encountered difficulties which appeared

Table 1 Genotype of leucocyte and exfoliated proximal tubule cells

Case number	Leucocyte		Proximal tubule cells		Type	Gender	Age (years)	Clinical presentation
	Allele 1	Allele 2	Allele 1	Allele 2				
1	403_405 + 2delAAGT	540delT	403_405 + 2delAAGT	540delT	PH2	F	10	Bilateral stones
2	403_405 + 2delAAGT	540delT	403_405 + 2delAAGT	540delT	PH2	M	7	Bilateral stones
3	403_405 + 2delAAGT	403_405 + 2delAAGT	403_405 + 2delAAGT	403_405 + 2delAAGT	PH2	M	12	Stones
4	481T	614T	481T	614T	PH1	M	14	Family studies (sibling of patient 5)
5	481T	614T	481**G**	614**C**	PH1	M	21	Renal failure, subsequently transplanted

The cells demonstrated the same genotype as that of the leucocytes of the patients, with the exception of case #5 who had received a kidney transplant and the genotype is presumably that of the donor kidney

Numbering based on cDNA sequences NM_000030 (AGXT) and NM_012203 (GRHPR) with nucleotide #1 denoting the first coding base

Nomenclature based on HUGO/HGVS recommendations (http://www.genomic.unimelb.edu.au/mdi/mutnomen/)

to be related to PH patients in particular. First, bacterial infection was a major problem with 35% of the samples contaminated within 7 days of culture, despite showing an initial negative result on urinalysis strips. Whether this reflects a greater tendency to urinary tract infections in PH patients is not known, although these are documented to make a significant contribution (10%) to presentation of these diseases [17]. In addition, it should be noted that a potential caveat in our study could be that the cells isolated from urine samples may not represent healthy, normal cells.

However, owing to the non-invasive method of harvesting the cells it is possible to obtain repeat samples and this was done in nine cases with subsequent success. Exfoliation of viable cells has been noted to be increased in patients with clinical renal dysfunction [14, 18] and it may be that a lesser degree of exfoliation occurs in PH patients compared with cystinosis and other proximal tubule disorders. Oxalate crystals were noted in some of our cultures although this did not appear to have any impact on whether or not the cells were viable.

The isolated cells displayed an epithelial morphology, which did not alter on subculture, and expressed the epithelial markers pan-cytokeratin and ZO-1. The proximal tubular nature of the isolated cells was confirmed by immunocytochemistry with positive staining for γ-glutamyl transpeptidase and RT-PCR for the expression of aminopeptidase A. Furthermore, the cells were negative for the expression of the distal tubular and collecting duct markers, uromodulin and aquaporin 3, respectively, as assessed by RT-PCR.

Genomic DNA from the cells demonstrated the same genotype as leucocytes from the patients. Of particular interest to us was the finding of normal and mutant transcripts of GRHPR mRNA which confirmed our previous observations of tissue differences in expression of mutant and normal transcripts from this gene in patients with PH2 [19].

In conclusion, proximal tubule cells have been successfully cultured from patients with PH and have enabled mRNA expression studies in renal tissue. We anticipate that such cell lines will enable studies of potential oxalate-influenced genes identified in the mouse model of PH [20] to be tested in human disease.

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