Exploring anti-fibrotic drugs
Luangmonkong, Theerut

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Precision-cut human kidney slices as a model to elucidate the process of renal fibrosis

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Precision-cut human kidney slices as a model to elucidate the process of renal fibrosis

Abstract

*Background and purpose*
Chronic kidney disease (CKD) is a major health concern and experimental models bridging the gap between animal studies and clinical research are currently lacking. Here, we evaluated precision-cut human kidney slices (PCKS) as a potential model for renal disease.

*Experimental approach*
PCKS were prepared from human cortical tissue obtained from tumor-nephrectomies and cultured up to 96h. Morphology, cell viability and metabolic functionality (i.e. UDP-glucuronosyltransferase and transporter activity) were determined to assess PCKS integrity. Furthermore, inflammatory- and fibrosis-related gene expression was characterized. Finally, to validate the model, renal fibrogenesis was induced using transforming growth factor beta 1 (TGF-β1).

*Key results*
Preparation of PCKS induced an inflammatory tissue response, while long-term incubation (96h) induced fibrogenesis as seen by an increased expression of collagen type 1A1 (COL1A1) and fibronectin (FN1). Importantly, PCKS remained functional for more than 48h as evidenced by active glucuronidation and phenolsulfonphthalein uptake. In addition, cellular diversity appeared to be maintained, yet we observed a clear loss of nephrin mRNA levels suggesting that our model might not be suitable to study the role of podocytes in renal pathology. Moreover, TGF-β1 exposure augmented fibrosis, as illustrated by an increased expression of multiple fibrosis markers including COL1A1, FN1 and alpha smooth muscle actin.

*Conclusion and implications*
PCKS maintain their renal phenotype during culture and appear to be a promising model to investigate renal diseases e.g. renal fibrosis. Moreover, the human origin of PCKS makes this model very suitable for translational research.

Key words: ex vivo; in vitro; human; renal fibrosis.

Abbreviations: 7-HCG, 7-hydroxycoumarin glucuronide; BCRP, breast cancer resistance protein; CKD, chronic kidney disease; COL1A1, collagen 1A1; ECM, extracellular matrix; ESRD, end-stage renal disease; FN1, fibronectin 1; HSP47, heat shock protein 47; OAT, organic anion transporter; OATP4C1, organic anion transporting polypeptide 4C1; PAI, plasminogen activator inhibitor; PCKS, precision-cut kidney slices; PDGFB, platelet-derived growth factor subunit B; TGF-β1, transforming growth factor beta 1; UGT, UDP-glucuronosyltransferase; αSMA, alpha smooth muscle actin.
Introduction

Chronic kidney disease (CKD) affects approximately 10% of the adult population in developed countries [1]. CKD is irreversible and can progress to end-stage renal disease (ESRD), demanding renal replacement therapy. Yet, at this time, no effective therapy exists to halt CKD progression. Loss of functional tissue due to glomerular and tubulointerstitial accumulation of extracellular matrix (ECM) proteins, i.e. fibrosis, is a key event in the development and perpetuation of CKD [2]. Currently, therapies for organ fibrosis in CKD patients solely focus on the origin of renal failure, e.g. diabetes or hypertension, and consequently have limited potential in halting fibrosis [3, 4]. Therefore, insight into renal fibrogenesis might aid in the development of therapeutic approaches to prevent loss of kidney function [5], or even reverse fibrosis.

Fibrosis is a complex pathophysiological process encompassing a myriad of cells and signaling pathways. A multitude of triggers can initiate the fibrotic response, including proteinuria and glomerular IgA deposition, yet irrespective of the initial insult, it will ultimately result in loss of organ architecture and function [4, 6]. Transforming growth factor beta 1 (TGF-β1) is one of the key factors involved in fibrogenesis, and activation of the TGF-β1 signaling pathway will increase the expression of, among others, plasminogen activator inhibitor (PAI)-1. This glycoprotein is a powerful promoter of renal fibrosis and is associated with many aggressive kidney diseases [7]. Even though there is great body of literature regarding fibrosis, the fibrotic process is still not understood completely. To elucidate the mechanisms of renal fibrosis, there is an urgent need for reliable models mimicking the human in vivo situation. Results obtained with existing animal models differ per strain [8], and in vitro models fail to replicate the multicellular nature of the fibrotic process. Recently, Poosti et al., reported the use of murine precision-cut kidney slices (PCKS) as a suitable model to investigate renal fibrosis [9]. PCKS are ideal to study multicellular (pathological) processes, e.g. fibrosis, since cellular diversity as well as organ architecture is maintained in the slices. In the current study, we aimed to improve the PCKS model by preparing slices from human tissue, and we established and characterized human PCKS as a unique ex vivo/in vitro model for renal disease.

Methods

Ethics statement

This study was approved by the Medical Ethical Committee of the University Medical Centre Groningen (UMCG), according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research (www.federa.org), refraining the need of written consent for ‘further use’ of coded-anonymous human tissue. The procedures were carried out in accordance with the experimental protocols approved by the Medical Ethical Committee of the UMCG.
**Renal tissue**

Macroscopically healthy renal cortical tissue from tumor-nephrectomies was obtained and kept in ice-cold University of Wisconsin solution. Cold ischemia time between nephrectomy and culturing was limited to 2h. Patient demographics are presented in Table 1.

**Table 1: Patient demographics.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (% female)</td>
<td>50.0</td>
</tr>
<tr>
<td>Age (mean, years)</td>
<td>55.1 ± 21.3</td>
</tr>
<tr>
<td>Nephrectomy side (% right)</td>
<td>100.0</td>
</tr>
<tr>
<td>Serum creatinine before nephrectomy (mean, μmol/L)</td>
<td>80.2 ± 11.3</td>
</tr>
<tr>
<td>eGFR before nephrectomy (mean, mL/min/1.73 m²)*</td>
<td>77.8 ± 9.4</td>
</tr>
</tbody>
</table>

*Calculated using the Modification of Diet in Renal Disease (MDRD) formula. Values are presented as means ± standard deviation or otherwise if indicated (n=6).

**Preparation/characterization of PCKS**

Workflow of PCKS preparation is shown in Figure 1. In short, cylindrical cores, 6 mm in diameter, were obtained from human renal tissue using a biopsy punch. Kidney slices were subsequently prepared in ice-cold Krebs-Henseleit buffer, supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO₃ (Merck), 10 mM HEPES (MP Biomedicals, Aurora, US) and saturated with carbogen (95% O₂/5% CO₂), using a Krumdieck tissue slicer as previously described [10]. PCKS, with a wet weight of 4-6 mg and an estimated thickness of 250-300 μm, were incubated individually in 1.3 mL of Williams medium E with Glutamax (Life Technologies, Carlsbad, US) containing 10 μg/mL ciprofloxacin and 2.7 g/L D-(+)-Glucose solution (Sigma, Saint Louis, US) at 37 °C in a 80% O₂/5% CO₂ atmosphere, while gently shaken [10]. Medium was refreshed every 24h.

**Figure 1:** Workflow preparation of precision-cut kidney slices. Cylindrical cores, 6 mm in diameter, were obtained from human renal cortical tissue using a biopsy punch (Step 1, 2). PCKS were prepared using the Krumdieck tissue slicer, slices with a wet weight of 4-6 mg had an estimated thickness of 250-300 μm (Step 3, 4). Slices were subsequently incubated in optimized medium at 37 °C and 80% O₂/5% CO₂ in an incubator shaking at 90 rpm (amplitude 2 cm).
For immunohistochemistry, PCKS were fixed in 10% formalin, embedded in paraffin and sectioned (2 μm) followed by PAS staining to assess morphology or Sirius red staining to visualize collagen protein expression. PCKS diameter was measured using ImageJ (US National Institutes of Health).

Viability was determined by both ATP content as previously described [11] and LDH release using the CytoTox-ONE Homogenous Membrane Integrity assay (Promega, Madison, US).

**PCKS functionality**

UGT activity was measured by HPLC. Following incubation, PCKS were incubated with 0.5 mM 7-hydroxycoumarin (7-HC) for 3h at 37°C. Afterwards, aliquots of culture medium were centrifuged (12,470 g, 5 minutes) and injected into the HPLC system (PE-Sciex API 3000, Concord, Canada) equipped with a C18 column (150 x 2.1 mm, 5 μm, Alltech Associates, Deerfield, US). 7-HC metabolites were measured as previously described [12].

To determine phenolsulfonphthalein uptake, slices were cultured as described above and subsequently homogenized in sonication solution, containing 70% ethanol and 2 mM EDTA, using a Mini-Beadbeater, and extinction of the solution was measured at 558 nm using a spectrophotometer (Synergy HT BioTek, Winooski, US).

**Quantitative PCR**

Total RNA from untreated or exposed (5 ng/mL human TGF-β1; Roche, Basel, Switzerland) PCKS was isolated with the RNeasy mini kit (Qiagen, Venlo, the Netherlands), using a Mini-Beadbeater for homogenization. RNA (1 μg) was reverse transcribed using the Reverse Transcription System (Promega). Subsequently, cDNA was used for RT-qPCR performed with a 7900HT RT-qPCR system (Applied Biosystems). Relative expression values were expressed as percentage compared to GAPDH (100%) or as fold induction using the 2-ΔΔCt method. Used primers are listed in Table 2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
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<tbody>
<tr>
<td>αSMA</td>
<td>AGGGGGTGATGGTGAGGA</td>
<td>ATGATGCATGTCTCTAGG</td>
<td>GGTGTGCCAGAGCCAGACAGCA</td>
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<tr>
<td>FN1</td>
<td>AGGCTTGAACCAACTACGGATGA</td>
<td>GCCTAAGCAGCTTGCAACAGTTT</td>
<td>ATGCCCTTGGAGATGAGTGAGGA</td>
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<tr>
<td>GAPDH</td>
<td>ACCAGGGCTGCTTTAATCTTCT</td>
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<tr>
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<td>GCCACACGGTGTCGCCGCA</td>
<td>GCCAGGGCAGGGTCCTCCAGGAG</td>
<td>TTCCTGCCCTCTCTTACAGCG</td>
</tr>
<tr>
<td>COL1A1</td>
<td>CAATCACCTGAGGAGAGCC</td>
<td>CGCCGGGCTGGTTTTC</td>
<td>CAGGTACCATGAGGCAGAGTG</td>
</tr>
<tr>
<td>COL1A1</td>
<td>CACGAGTTCTGGACAGCCA</td>
<td>GAGCACTAGCTCTTTTC</td>
<td>_</td>
</tr>
</tbody>
</table>

**Table 2:** Primers used in quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Gene</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCRP</td>
<td>Hs01053790_m1</td>
<td>OAT1</td>
<td>Hs00537914_m1</td>
</tr>
<tr>
<td>CD31</td>
<td>Hs00169777_m1</td>
<td>OATP4C1</td>
<td>Hs00698884_m1</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Hs01023894_m1</td>
<td>PDGFB</td>
<td>Hs00966522_m1</td>
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<tr>
<td>IL-1β</td>
<td>Hs015505410_m1</td>
<td>TGF-β1</td>
<td>Hs00998133_m1</td>
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<td>IL-6</td>
<td>Hs00985639_m1</td>
<td>UGT1A1</td>
<td>Hs02511055_s1</td>
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<tr>
<td>IL-8</td>
<td>Hs0174103_m1</td>
<td>UGT1A9</td>
<td>Hs02516855_sH</td>
</tr>
<tr>
<td>Nephrin</td>
<td>Hs0190446_m1</td>
<td>Vimentin</td>
<td>Hs0185584_m1</td>
</tr>
</tbody>
</table>
Western blot

Total protein (100 μg) was separated via SDS/PAGE using 10% gels and blotted onto PVDF membranes. Antibodies used are listed in Table 3. Proteins bands were visualized using the VisiGlo Prime HRP Chemiluminescent Substrate Kit (Amresco, Solon, US).

Table 3: Antibodies used in Western blotting.

<table>
<thead>
<tr>
<th>Antibody and dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-collagen type I (COL1), 1:1000</td>
<td>Rockland, Pennsylvania, US</td>
</tr>
<tr>
<td>Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1:5000</td>
<td>Sigma, Saint Louis, US</td>
</tr>
<tr>
<td>Polyclonal goat anti-rabbit immunoglobulins/HRP, 1:2000</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-mouse immunoglobulins/HRP, 1:5000</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

Statistics

Statistics were performed using Graphpad Prism 6.0 by either a Kruskal-Wallis test followed by Dunn’s multiple comparisons test or the Mann-Whitney test as appropriate. Differences between groups were considered to be statistically significant when \( p<0.05 \).

Results and discussion

Characterization of PCKS

Figure 2A shows the morphology of PCKS during incubation. As demonstrated, general morphology remains good between 3-72h with only minor signs of cellular damage (e.g. pyknosis and anucleosis). Closing of tubuli was also observed probably due to a lack of pre-urine flow. Furthermore, PCKS became smaller during culture (Figure 2B and 2C).

Next, viability of PCKS was assessed by ATP content and LDH leakage. ATP levels greatly increased at the start of incubation, from 4.1 (0h) to 16.6 pmol/μg (3h; Figure 2D), in line with previous observations in liver slices [13]. Furthermore, ATP levels remained fairly stable during culture with a content of 11.2 pmol/μg at 96h. LDH levels in the culture medium were also constant (Figure 2E) indicating that PCKS remained viable. These finding are in agreement with the study from Poosti et al. showing that murine PCKS are viable up to 72h [9].

Metabolic activity and cell marker expression during incubation

The kidney contributes greatly to metabolism and (transporter-mediated) solute clearance [14]. Therefore, we studied transporter activity and UDP-glucuronosyltransferase (UGT) functionality. Uptake of phenolsulfonphthalein, an organic anion transporter (OAT) substrate [15], was observed at 3-48h and OAT activity time-dependently decreased with 94% between 3-96h (Figure 3A), in line with the observed OAT1 gene expression \((r=0.68, \ p<0.001)\). Furthermore, we observed mitigated expression of organic anion transporting polypeptide 4C1 (OATP4C1) and breast cancer resistance protein (BCRP) at the start of culture (0-24h), after which levels remained stable. These findings are in agreement with in vitro studies performed with primary human proximal tubular epithelial cells (PTECs), in which functional expression of OATs can be sustained for a limited time [16].
Figure 2: General morphology and viability of PCKS. (A) PAS staining of PCKS during culture, magnification 10X; Insets, magnification 20X. (B, C) Representative figures and quantification of PCKS size during incubation, quantified using ImageJ (n=3). (D, E) Viability of PCKS was assessed by ATP content and LDH leakage. Data are presented as means ± SEM of minimally five independent experiments performed in triplicate. Statistical analysis was performed via a Kruskal-Wallis test followed by Dunn’s multiple comparisons, compared to first column. *p<0.05.
Figure 3: Functionality and cellular changes during culture of PCKS. (A) Representative figures and quantification of phenolsulfonphthalein uptake in PCKS determined by a spectrophotometer at 558 nm. (B) Enzyme activity was determined by HPLC. Slices were incubated with 7-HC (0.5 mM) for 3h at 37 °C. (A, B, C) Gene expression was studied by RT-qPCR. Relative expression was calculated using the housekeeping gene GAPDH (100%). Data are presented as means ± SEM of six independent experiments performed in triplicate. Statistical analysis was performed via a Kruskal-Wallis test followed by Dunn’s multiple comparisons test, compared to first column. *p<0.05.
Furthermore, 7-hydroxycoumarin glucuronide (7-HCG) formation was observed in PCKS for more than 48h. Glucuronidation time-dependently decreased from 40.1 (24h) to 13.2 pmol/3h/μg at 96h (Figure 3B). Moreover, UGT activity clearly correlated with UGT1A9 gene expression ($r=0.61$, $p=0.004$), a main contributor to renal glucuronidation [17]. In addition, similar to OATP4C1 and BCRP, UGT1A1 expression diminished from 0-24h, after which it stayed constant. Previously, de Kanter et al. demonstrated UGT activity in human PCKS during short-term (3h) incubation [12]. Here, we show long-term functionality of UGTs in PCKS, suggesting that the model is suitable for metabolism studies.
Moreover, no clear differences were observed in gene expression of vimentin, e-cadherin and CD31 during culturing (Figure 3C), suggesting that (myo)fibroblasts, epithelial cells and endothelial cells, respectively, were not lost during incubation. Of note, even though CD31 is a well-established marker for endothelial cells [18], under pathological conditions pro-fibrotic renal fibroblasts can also express CD31 [19]. Furthermore, we observed a marked decrease in the expression of nephrin, a podocyte marker and key component of the slit diaphragm (Figure 3C) [20]. Lower nephrin levels indicate dedifferentiation or damage of podocytes, possibly induced by platelet-derived growth factor [21], yet it does not necessarily correlate with podocyte apoptosis [22]. Thus, the fate of podocytes in our model remains to be fully characterized.

Taken together, PCKS behave similar to PTECs during culture, yet the major advantage of our model is that it appears to mimic and retain the cellular and architectural complexity of the kidney.

**Expression of inflammatory and fibrosis markers during incubation**

To assess the effect of slicing on cell stress, IL-6, IL-8 and IL-1β gene expression was determined. Expression of all three interleukins markedly increased from 4.5% to more than 130% in the beginning of culturing (3-24h), whereafter gene expression decreased (Figure 4A). These findings suggest that an early inflammatory response is evoked in PCKS, still the trigger for the observed increase remains unclear.

With regards to fibrogenesis, we observed a marked increase in the gene expression of collagen 1A1 (COL1A1) and fibronectin 1 (FN1) as well as elevated type I collagen protein levels during culture (Figure 4B and 4C). The observed effect was concurrent with an increased gene expression of TGF-β1. Furthermore, we detected elevated gene levels of platelet-derived growth factor subunit B (PDGFB) at 3h. Thus, the observed fibrotic response in PCKS is likely caused by a concerted action of the TGF-β1 and PDGF pathways possibly activated by interleukins. This notion is in line with previous studies showing that mechanical stress and cytokines, among other factors, drive a fibrogenic response [23]. In contrast, gene expression of alpha smooth muscle actin (αSMA), a marker for matrix producing myofibroblasts [24], first diminished from 38.4% (0h) to 5.0% (24h) and subsequently increased (12.4%; 96h), while no changes were observed in the expression of heat shock protein 47 (HSP47). A similar expression profile is shown in human liver slices [13]. Thus, the collagen-producing cells in PCKS remain to be identified.

**Figure 4: Continued on next page.**
Precision-cut human kidney slices as a model to elucidate the process of renal fibrosis

Figure 4: Expression of inflammatory and fibrosis markers during incubation of PCKS. (A, B) Gene expression was studied by RT-qPCR. Relative expression was calculated using the household gene GAPDH (100%). Data are presented as means ± SEM of six independent experiments performed in triplicate. Collagen type 1 protein expression was studied using Western blot (n=3). Statistical analysis was performed via a Kruskal-Wallis test followed by Dunn’s multiple comparisons test, compared to 0h. *p<0.05. (C) Sirius red staining of PCKS during culture, magnification 20X.
**Figure 5:** Induction of renal fibrosis with TGF-β1. PCKS were treated with 5 ng/mL human TGF-β1 for either 48 or 72h. Subsequently, gene expression was studied by RT-qPCR, expressed as fold induction using the $2^{-\Delta\Delta CT}$ method, and viability was assessed by ATP content of the slices. Data are presented as means ± SEM of four independent experiments performed in triplicate. Groups were compared to control using the Mann-Whitney test. *p<0.05.
PCKS as a model for renal fibrosis

Lastly, we investigated whether the fibrotic response could be augmented in PCKS. Therefore, we exposed PCKS to TGF-β1, a key mediator of fibrosis [25, 26]. As shown in Figure 5, treatment with TGF-β1 for 48h significantly increased the fibrogenic response, resulting in a more than 1.8-fold increase in gene expression of COL1A1 and FN1, similar to the observations in murine PCKS [9]. Moreover, exposure to TGF-β1 for 72h significantly augmented the gene levels of all the tested fibrosis markers namely COL1A1, FN1, HSP47 and αSMA, without affecting PCKS viability (Figure 5). Moreover, we observed an increased expression of PAI-1, a downstream signaling molecule of the TGF-β1 pathway, indicating successful activation of the TGF-β1 signaling cascade in human PCKS. Therefore, our model can be used to investigate renal fibrogenesis and the underlying molecular pathways.

Conclusion

In conclusion, we have characterized a unique ex vivo/in vitro model to investigate human renal disease, viz. precision-cut kidney slices. Merits of this model are the human origin of the tissue and the fact that original organ architecture is maintained. A limitation of the model is that circulating bone marrow-derived cells that contribute to the pathogenesis of fibrosis, e.g. macrophages [27], are absent. In addition, the observed loss of differentiated podocytes indicates that our model is not suitable to study podocyte injury and loss as factors in renal pathology. Still, the current study with human PCKS paves the way for a myriad of novel avenues of research including unraveling the molecular mechanism of human renal fibrosis and CKD progression as well as identifying potential anti-fibrotics. The latter might even be tested in PCKS prepared from diseased (i.e. fibrotic) instead of healthy renal tissue.

Acknowledgements

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References