Inhibition of the p38 MAPK pathway ameliorates renal fibrosis in an NPHP2 mouse model

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Abstract

Background. Nephronophthisis (NPHP), the most frequent genetic cause of end-stage kidney disease in children and young adults, is characterized by a variable number of renal cysts associated with cortical tubular atrophy and interstitial fibrosis. The p38 mitogen-activated protein kinase (MAPK) pathway is an important intracellular signaling pathway involved in the production of profibrotic mediators. The relationship between p38 MAPK and renal fibrosis in NPHP2 is unknown.

Methods. We administered a selective p38 MAPK inhibitor, FR167653, in a NPHP2 mouse model (inv/inv, invAC mice) from 3 to 6 weeks old, and the kidneys were examined at 6 weeks of age. Phosphorylation of p38 MAPK (p-p38 MAPK) protein levels, the degree of renal fibrosis, messenger RNA (mRNA) levels for extracellular matrix genes and mRNA levels for transforming growth factor in the kidneys were studied. Effect of an extracellular signal-regulated protein kinase (ERK) kinase (MEK) inhibitor on renal fibrosis was also evaluated.

Results. Expression of extracellular matrix genes and p-p38 MAPK were increased in the NPHP2 mouse model kidney. FR167653 successfully decreased p-p38 MAPK levels, the degree of fibrosis and extracellular matrix gene expressions. However, the FR167653 did not prevent cyst expansion, abnormal cell proliferation and acceleration of apoptosis and did not influence ERK activation. In contrast, MEK inhibition reduced both cyst expansion and fibrosis without affecting p38 MAPK activation.

Conclusions. These results suggest that inhibition of p38 MAPK reduced renal fibrosis but not cyst expansion, cell proliferation and apoptosis in NPHP2 model mice. Our results suggest that p38 MAPK and ERK signaling pathways independently affect renal fibrosis in inv mutant mice.

Keywords: cyst; fibrosis; inv; NPHP2; p38 MAPK

Introduction

Nephronophthisis (NPHP) is a genetic disorder of the kidney that affects children [1]. The disorder is inherited in an autosomal recessive fashion and is the most common genetic cause of childhood renal failure [2]. Eleven genes responsible for NPHP have been identified [3]. Characteristic features of NPHP are renal fibrosis and cyst formation [4]. Fibrosis is the deposition of collagen and other extracellular matrix (ECM) components in tissue. Pcy mutant mice, which carry a mutation in the nphp3 gene, is reported to show renal fibrosis and over-express ECM-related genes including collagen α1 (I), α1 (III) and α1 (IV) with cyst progression [5, 6]. Over-expression of many ECM-related genes has also been reported in advanced cyst stages in other animal renal cyst models [7–9]. The epithelial–mesenchymal transition (EMT) is known to play a role in renal fibrosis in ischemia-reperfusion injury. Previous reports showed that 36% of new fibroblasts originated from local EMT and that 15% were from bone marrow, the rest being local proliferation [10]. EMT was observed in human and mouse polycystic kidney disease [11–16].

The infantile form of NPHP (NPHP type 2) is caused by mutation in the human Inv/NPHP2 gene, which has been identified as the mouse inv gene homolog [17]. The inv gene was first discovered as the gene responsible for the mouse inv mutant [18]. The inv mouse shows a reversal of left–right asymmetry, jaundice and multiple renal cysts and mimics a phenotype in human infantile NPHP2 [19, 20]. Most inv mutant mice die before 7 days of age. The inv/inv, invAC: green fluorescent protein (GFP) (invAC) mouse was created by introduction of an inv gene that lacked a C-terminus fused with GFP into an inv mutant mouse [21]. The inv/inv, invAC mouse develops renal cysts, but it does not show any situs abnormalities or jaundice and can survive beyond 6 weeks of age [22]. Since renal cysts grow slowly in this mouse, inv/inv, invAC mice are suitable for investigating the mechanisms for development of cystic kidney disease.
The mitogen-activated protein kinase (MAPK) family of serine/threonine kinase includes extracellular signal-regulated protein kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK), which have been reported to be activated in human and mouse cystic kidneys [23–26]. In inv mutant mice, ERK and p38 MAPK are activated in an early phase of cyst development, and JNK is activated in a late phase of cyst development [27]. We previously reported that inhibition of the ERK signaling pathway resulted in inhibition of renal cyst enlargement and cell proliferation in inv mutant mice [27]. The p38 MAPK pathway and transforming growth factor (TGF)-β signaling are important intracellular signal transduction pathways for fibrosis, and previous reports have shown that pharmacological inhibition of p38 MAPK has beneficial effects in various animal models involving inflammatory disorders [28, 29] and obstructive nephropathy [30, 31].

In the present report, we determined if the p38 MAPK pathway affects renal cyst and fibrosis in inv mutant mice. We examined p38 MAPK activation in the inv/inv, inv/AC cystic kidney and then treated inv/inv, inv/AC mice with a selective p38 MAPK inhibitor, FR167653.

Materials and methods

Animals

The experimental protocols were performed according to the regulations of the Medicine Animal Care Committee of Kyoto Prefectural University. We used inv/inv, inv/AC mice in this study. The inv/inv, inv/AC mice used in this study were inv/inv mice carrying two alleles of inv/AC transgenes [22, 27].

FR167653 and PD184352 treatment protocol

A selective p38 MAPK inhibitor, FR167653 (Astellas Pharma Inc., Tokyo, Japan), was injected subcutaneously once a day at a dose of 33 mg/kg body weight diluted in 0.5% methylcellulose in phosphate-buffered saline (PBS) from 3 to 6 weeks of age [32, 33]. A control group was injected with vehicle (0.5% methylcellulose in PBS). Inv/inv, inv/AC mice treated with FR167653 (n = 23) and vehicle (n = 15) as well as control +/+ mice treated with FR167653 (n = 6) and vehicle (n = 6) were analyzed at 6 weeks of age. Administration of PD184352 as an MEK inhibitor was previously described [27, 34]. No difference was observed in survival rate between FR167653 and vehicle-treated groups as well as non-treated inv/inv, inv/AC mice group (supplementary table). Inv/inv, inv/AC mice treated with PD184352 (n = 3) and vehicle (n = 3) were subject for analysis.

Histological analysis

For morphological evaluation, kidneys were fixed in 4% paraformaldehyde in PBS for 8 h and embedded in paraffin wax. Sections (4 μm thick) were stained with hematoxylin and eosin stain and Heidenhain azan stain according to standard protocols.

Immunohistochemical staining for phospho-p38 (p-p38) MAPK was performed on frozen sections. Kidney samples for p-p38 MAPK staining were frozen in liquid nitrogen into Tissue Tek OCT compound (Sakura Finetechical Co. Ltd, Tokyo, Japan) and stored at −30°C until sectioned. Sections were cut at 10 μm and fixed in 4% paraformaldehyde in PBS at room temperature for 20 min monoclonal rabbit anti-p-p38 MAPK antibody (dilution 1:1,000, #9212; Cell Signaling Technology Inc.), polyclonal rabbit anti-p-p38 MAPK (Thr180/Tyr182) (dilution 1:1,000, #9211; Cell Signaling Technology Inc.), anti-actin (dilution 1:200, sc-1616; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-proliferating cell nuclear antigen ([PCNA]), dilution 1:500, VP-P980; Vector Laboratories, Inc., Burlingame, CA).

In p-p38 MAPK immunoblot analysis, we examined one every nine kidneys on time-course experiment of inv/inv, inv/AC mice, all kidneys on FR167653 administration experiment and one in every three kidneys on PD184352 treatment. In PCNA analysis, we examined one in every six kidneys of all groups. In ERK analysis, we examined three kidneys of all groups.

Biochemical analysis

Blood samples were obtained by cardiac puncture in all 6-week-old mice of administration experiment groups. Blood urea nitrogen (BUN) and serum creatinine (Cre) concentrations were measured by Mitsubishi Kagu Bio-Clinical Laboratories.

Real-time reverse transcription–polymerase chain reaction analysis

Real-time reverse transcription–polymerase chain reaction (PCR) was carried out on an Applied Biosystems 7300 real-time PCR system (Foster City, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the control gene for normalization. Each primer is shown in Table 1. Blood samples were obtained by cardiac puncture in all 6-week-old mice of administration experiment groups. Blood urea nitrogen (BUN) and serum creatinine (Cre) concentrations were measured by Mitsubishi Ka
gaku Bio-Clinical Laboratories.

Statistical analysis

Data are expressed as mean ± SE. Comparison of the data between the two groups was performed using an unpaired t-test as appropriate. Multiple group comparison of the data was performed using one-way analysis of variance with post-hoc Tukey–Kramer test. Survival rate was derived using the Kaplan–Meier method and was compared using log-rank test. Statistical analysis was performed using Excel (Microsoft, Redmond, WA) and Statcel 2 plug-in software (OMS Publishing, Saitama, Japan).
Table 1. Real-time reverse transcription–PCR primer list

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CAATGTGTCCGTCTGGATCT</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>Col alpha 1 (I)</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>ATCACCTGAAACAGCGTGC</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>Col alpha 1 (II)</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GTTGATGTGCACTGGCATT</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>Col alpha 1 (IV)</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CATTCACTGAGTGGCAGGG</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>Tgf beta 1</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CTCCGTTGGCTCTTAGTCG</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
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Results

Collagen gene expression and p38 MAPK activation in cystic kidneys of inv/inv mice

Expression levels of all collagen genes at 1 week of age were similar between control (+/+), inv/AC and inv/inv, inv/AC kidneys (Figure 1A). Expression levels of Col1a1 and Col3a1 in inv/inv, inv/AC cystic kidneys were significantly higher at 3 and 6 weeks of age compared with those in controls. Expression levels of Col4a1 in inv/inv, inv/AC cystic kidneys were similar to those in controls at 3 weeks of age, but they were significantly higher at 6 weeks of age compared with those in controls. Sircol collagen assay confirmed that the collagen content ratio in inv/inv, inv/AC cystic kidneys at 6 weeks of age (1 ± 0.07) was significantly higher than that of control (+/+, inv/AC) (6.4 ± 0.47), P < 0.01 (Figure 1C).

The total amount of p38 MAPK protein was not different between control (+/+), inv/AC and inv/inv, inv/AC kidneys at all examined ages (Figure 1B). Phosphorylated p38 MAPK of inv/inv, inv/AC kidneys was significantly higher in 3-week-old (P < 0.05) and 6-week-old (P < 0.01) mice compared with that in controls (Figure 1B). Intense p-p38 MAPK signals were detected by immunohistochemical staining of renal tissue in tubular epithelial cells of renal cysts in 6-week-old inv/inv, inv/AC kidneys (Figure 1D).

FR167653 decreases p38 MAPK phosphorylation but does not influence renal cyst progression, kidney function marker levels, cell proliferation and apoptosis

FR167653 and vehicle treatment did not affect p-p38 MAPK levels in +/+, inv/AC kidneys (Figure 2A and B). FR167653 treatment decreased p-p38 MAPK levels in inv/inv, inv/AC kidneys compared with those in vehicle-treated inv/inv, inv/AC kidneys (Figure 2A and B). The amount of p-p38 MAPK in FR167653 treated inv/inv, inv/AC kidneys was reduced to the almost identical level of that in controls (+/+, inv/AC kidney with or without FR167653). However, FR167653 administration failed to reduce the kidney size of inv/inv, inv/AC mice (Figure 2C). There was no difference in the kidney weight to body weight ratio (Figure 2D) and the distribution of tubular sizes (Figure 2E-G) between FR167653-treated (n = 23) and vehicle-treated (n = 15) inv/inv, inv/AC mice. Cre and BUN concentrations of FR167653-treated inv/inv, inv/AC mice were similar to those in the controls (Figure 2H).

Effect of FR167653 treatment on renal fibrosis and TGF-ß1 expression in inv/inv, inv/AC cystic kidneys

We then examined FR167653 treatment in inhibiting renal fibrosis. Treatment with FR167653 resulted in a significant decrease in the fibrosis index in inv/inv, inv/AC kidneys (n = 12) at 6 weeks of age compared with that of the vehicle-treated group (n = 16) (43 ± 4.47 versus...
Fig. 2. Effect of the p38 MAPK inhibitor, FR167653, on renal cysts in invAC mice. (A) Immunoblot analysis of p-p38 MAPK and total p38 MAPK proteins of vehicle-treated and FR167653-treated +/+ , invAC and inv/inv, invAC mice. (B) Densitometric quantitation of three independent immuno- blots. Results are expressed as a ratio of the density of p-p38 MAPK to total p38 MAPK. The obtained values were further compared with the mean value from vehicle-treated +/+ , invAC mice (*P < 0.01). (C) Gross appearance of representative kidneys from vehicle-treated inv/inv, invAC (left) and FR167653-treated (right) inv/inv, invAC mice. (D) Percentage of kidney weight to body weight (n = 24 in vehicle-treated inv/inv, invAC and n = 38 in FR167653-treated inv/inv, invAC). (E) Hematoxylin-and eosin-stained kidney in vehicle-treated (E) and FR167653-treated inv/inv, invAC (F) mice. (G) Tubule diameter in vehicle-treated and FR167653-treated inv/inv, invAC mice. At least 200 tubules in eight kidneys from each group were examined. (H) Cre: serum creatinine (upper) and BUN concentrations (lower). (I) Immunoblot analysis of PCNA and actin proteins. (J) Percentage of apoptotic cells in total renal tubular epithelial cells.
p38 induces fibrosis in NPHP2 kidneys

FR167653 reduced the high p-p38 MAPK/p38 MAPK ratio and renal fibrosis in inv/inv, inv/AC kidneys to control levels. The p38 MAPK-activated cells were tubular epithelial cells. It has been reported that activation of p38 MAPK makes renal epithelial cells express smooth muscle actin (SMA) [36, 37]. In the present study, SMA-positive cells were observed not only in the fibrotic area but also in cyst lining cells (Supplementary figure 2), although the number of positive cells is low. Studies have shown that TGF-β1 increases activation of p38 MAPK, which induces EMT in renal epithelial cells [36, 37]. Our results showed that TGF-β1 mRNA expression levels were increased in inv/inv, inv/AC kidneys. However, TGF-β1 expression levels were not affected by administration of FR167653. Thus, p38 MAPK is likely to act on downstream signaling of TGF-β1 in renal fibrosis in the inv/inv cystic kidney. Our studies suggested that EMT of tubular cells contributed the fibrosis at least in this particular NPHP mouse model. However, recent reports showed that renal epithelial cells did not become myofibroblasts in vivo [38] and that pericytes and perivascular fibroblasts were the primary source of collagen-producing cells [39]. We do not deny a possibility that p38 MAPK may induce secretion of some fibrogenic cytokines from renal tubular cells and induce activation of collagen-producing cells in inv/inv cystic kidney.

**Discussion**

FR167653 reduced the high p-p38 MAPK/p38 MAPK ratio and renal fibrosis in inv/inv, inv/AC kidneys to control levels. The p38 MAPK-activated cells were tubular epithelial cells. It has been reported that activation of p38 MAPK makes renal epithelial cells express smooth muscle actin (SMA) [36, 37]. In the present study, SMA-positive cells were observed not only in the fibrotic area but also in cyst lining cells (Supplementary figure 2), although the number of positive cells is low. Studies have shown that TGF-β1 increases activation of p38 MAPK, which induces EMT in renal epithelial cells [36, 37]. Our results showed that TGF-β1 mRNA expression levels were increased in inv/inv, inv/AC kidneys. However, TGF-β1 expression levels were not affected by administration of FR167653. Thus, p38 MAPK is likely to act on downstream signaling of TGF-β1 in renal fibrosis in the inv/inv cystic kidney. Our studies suggested that EMT of tubular cells contributed the fibrosis at least in this particular NPHP mouse model. However, recent reports showed that renal epithelial cells did not become myofibroblasts in vivo [38] and that pericytes and perivascular fibroblasts were the primary source of collagen-producing cells [39]. We do not deny a possibility that p38 MAPK may induce secretion of some fibrogenic cytokines from renal tubular cells and induce activation of collagen-producing cells in inv/inv cystic kidney.
Although FR167653 treatment effectively inhibited p38 MAPK activation and renal fibrosis, it did not inhibit the increase in kidney weight, expansion in renal tubules, cell proliferation and apoptosis in renal epithelial cells. Thus, intracellular signaling pathways that induce cell proliferation, apoptosis and cyst formation were p38 MAPK independent. We previously reported that small cysts were already found in some inv/inv, invDC mice at 1 week of age, and that all the inv/inv, invDC mice at 3 weeks of age had many enlarged renal cysts [27]. The present results showed that collagen α1 (I), α1 (III) and α1 (IV) gene expression was increased after cyst development in inv/inv, invDC kidneys, suggesting that renal fibrosis develops after cyst formation.

Inhibition of fibrosis by FR167653 treatment reduced fibrosis ~60% of vehicle control but not complete. The results suggest that there are other pathways to induce renal fibrosis in inv/inv, invDC kidneys. PD184352, a MEK inhibitor, suppressed both renal cyst and fibrosis and ameliorated renal function [27]. FR167653 treatment did not alter ERK activation. Conversely, PD184352 did not affect p38 MAPK activation. These results suggest that both p38 MAPK and ERK pathways are likely to be independently involved in renal fibrosis of inv/inv, invDC mice.

Reduction of fibrosis with FR167653 had no effect on renal function and survival rate in our study. Although reduction of fibrotic index with PD184352 treatment (12.2%) was lower than that with FR167653 treatment (19.2%), we

Fig. 4. Effect of PD184352 on renal fibrosis in inv/inv, invDC mice. (A) Fibrosis index assayed by the point-counting method on Azan-stained sections. (B) Quantitative analysis of three collagen mRNA expression levels. P < 0.01, Error bar = SE (C) Immunoblot analysis of p-p38 MAPK and total p38 MAPK proteins of vehicle-treated and PD184352-treated +/-, invDC mice and inv/inv, invDC mice. Right graph is densitometric quantitation of three independent immunoblots. Results are expressed as a ratio of the density of p-p38 MAPK to total p38 MAPK. The obtained values were further compared with the mean value from vehicle-treated +/-, invDC mice (*P < 0.01). (D) Immunoblot analysis of phospho-ERK and total ERK proteins of vehicle-treated and FR167653-treated +/-, invDC mice and inv/inv, invDC mice. Right graph is densitometric quantitation of three independent immunoblots. Results are expressed as a ratio of the density of phospho-ERK to total ERK. The obtained values were further compared with the mean value from vehicle-treated +/-, invDC mice (*P < 0.01).
had observed improvement in renal functions of PD184352-treated group [27]. These results suggest that renal fibrosis in \textit{inv/inv, invAC} mice is not an important factor for renal function. Renal volume was reported to be inversely correlated to renal function in autosomal polycystic kidney disease [40]. Therefore, suppression of the cyst expansion but not fibrosis may be important for improvement of renal function in \textit{inv/inv, invAC} mice.

In summary, the present results support that the primary lesion of the \textit{inv/inv, invAC} mutant kidney is located in renal epithelial cells and that interstitial fibrosis is a secondary manifestation. Although both ERK and p38 MAPK signaling pathways are activated in an early phase of cyst formation, the two pathways have different roles in the \textit{inv} kidney. Activation of ERK contributes to cyst expansion as well as to fibrosis, cell proliferation and apoptosis, whereas activation of p38 MAPK signaling promotes renal fibrosis only.

**Supplementary data**

Supplementary data are available online at http://ndt.oxfordjournals.org.

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**Conflict of interest statement.** None declared.

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