Inhibition of Cdc25A Suppresses Hepato-renal Cystogenesis in Rodent Models of Polycystic Kidney and Liver Disease

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BACKGROUND & AIMS: In polycystic kidney disease and polycystic liver disease (PLD), the normally nonproliferative hepato-renal epithelia acquire a proliferative, cystic phenotype that is linked to overexpression of cell division cycle 25 (Cdc25)A phosphatase and cell-cycle deregulation. We investigated the effects of Cdc25A inhibition in mice and rats via genetic and pharmacologic approaches. **METHODS:** *Cdc25A*$^{+/-}$ mice (which have reduced levels of Cdc25A) were cross-bred with polycystic kidney and hepatic disease 1 (*Pkhd1*$^{del2/del2}$) mice (which have increased levels of Cdc25A and develop hepatic cysts). Cdc25A expression was analyzed in livers of control and polycystic kidney (PCK) rats, control and polycystic kidney 2 (*Pkd2*$^{ws25/-}$) mice, healthy individuals, and patients with PLD. We examined effects of pharmacologic inhibition of Cdc25A with vitamin K3 (VK3) on the cell cycle, proliferation, and cyst expansion in vitro; hepato-renal cystogenesis in PCK rats and *Pkd2*$^{ws25/-}$ *mice*; and expression of Cdc25A and the cell-cycle proteins regulated by Cdc25A. We also examined the effects of the Cdc25A inhibitor PM-20 on hepato-renal cystogenesis in *Pkd2*$^{ws25/-}$ mice. **RESULTS:** Liver weights and hepatic and fibrotic areas were decreased by 32%–52% in *Cdc25A*$^{+/-}$:*Pkhd1*$^{del2/del2}$ mice, compared with *Pkhd1*$^{del2/del2}$ mice. VK3 altered the cell cycle and reduced proliferation of cultured cholangiocytes by 32%–83% and decreased growth of cultured cysts by 23%–67%. In PCK rats and *Pkd2*$^{ws25/-}$ mice, VK3 reduced liver and kidney weights and hepato-renal cystic and fibrotic areas by 18%–34%. PM-20 decreased hepato-renal cystogenesis in *Pkd2*$^{ws25/-}$ mice by 15%. **CONCLUSIONS: Cdc25A inhibitors block cell-cycle progression and proliferation, reduce liver and kidney weights and cyst growth in animal models of polycystic kidney disease and PLD, and might be developed as therapeutics for these diseases.**

Keywords: Animal Model; Phosphatase; Therapeutic Strategy; Cell Division.

BASIC AND TRANSLATIONAL LIVER

Different genetic mutations in polycystic kidney disease (PKD) and polycystic liver disease (PLD) initiate the formation of hepato-renal cysts that continue to grow progressively.[1–3] Substantial evidence suggests that a switch from a nonproliferative normal epithelia to a proliferative cystic epithelia is associated, in particular, with accelerated cell proliferation (caused by increased cyclic adenosine monophosphate and decreased $[Ca^{2+}]_i$) and cell-cycle deregulation (caused by overexpression of the cell division cycle 25 [Cdc25]A phosphatase).[4–10] Indeed, suppression of cyclic adenosine monophosphate and restoration of $[Ca^{2+}]_i$ decreases abnormal cell proliferation and inhibits cyst growth.[4,5,8,9] Moreover, in PKD/PLD patients, treatment with somatostatin analogs halted progression of hepatic and renal cysts and improved quality of life.[11–14]

The cell-cycle regulator Cdc25A is of particular interest to us for the following reasons: (1) Cdc25A regulates all phases of the cell cycle,[15,16] (2) Cdc25A is overexpressed in cancers and its suppression reduces cancer cell growth,[17,18] and (3) elevated Cdc25A in polycystic kidney (PCK) rats, an animal model of PKD/PLD, is linked to decreased levels of its regulator, microRNA-15a; experimental increase of microRNA-15a in PCK cholangiocytes reduced Cdc25A expression, inhibiting cyst growth.[4] Taken together, these observations suggest that Cdc25A might represent a potential therapeutic target in the treatment of PKD/PLD.

Multiple agents including analogs of natural vitamin K (VK) have been developed as pharmacologic inhibitors of Cdc25 phosphatases. Vitamin K3 (VK3) (2-methyl-1, 4-naphthoquinone, or menadione), in particular, has antiproliferative capacity irreversibly inhibiting Cdc25A through covalent modification of its active site.[19–21] Another Cdc25A inhibitor, N-[4-biphenyl]-3, 4-bis-[2-hydroxy-ethylsulphanyl]-maleimide (PM-20), acts as a phosphatase antagonist binding to the Cdc25A active site and subsequently inhibiting cancer cell growth in vitro and in vivo.[20,22] Although VK3 and PM-20 as anticancer agents had been tested in vitro and in vivo and recently in clinical trials,[19,23,24] their role in benign hyperproliferative condi-

Abbreviations used in this paper: ADPKD, autosomal-dominant polycystic kidney disease; ARPKD, autosomal-recessive polycystic kidney disease; Cdc25, cell division cycle 25; Cdk, cyclin-dependent kinase; PCK, polycystic kidney; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PKD, polycystic kidney disease; *Pkd2*, polycystic kidney 2; *Pkhd1*, polycystic kidney and hepatic disease 1; PLD, polycystic liver disease; Rb, retinoblastoma; 3-D, 3-dimensional; VK3, vitamin K3.

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0016-5085/$36.00
doi:10.1053/j.gastro.2011.11.036

tions in general and in PKD/PLD in particular has not been explored.

In the present study, we used genetic and pharmacologic approaches to assess the pathogenic role of Cdc25A in hepatocystogenesis further and to provide evidence supporting its therapeutic potential in PKD/PLD. First, we cross-bred *Cdc25A*$^{+/-}$mice (characterized by reduced levels of Cdc25A)[25] with our own model of PKD/PLD, polycystic kidney and hepatic disease 1 (*Pkhd1*$^{del2/del2}$) mice (characterized by progressive hepatic but not renal cyst growth and increased Cdc25A levels).[26] We hypothesized that if Cdc25A is involved in hepatic cystogenesis, the cross-bred mice would have less severe cystic disease owing to decreased Cdc25A levels. Second, we examined the effects of pharmacologic targeting of Cdc25A by VK3 on: (1) cell cycle and proliferation in vitro; (2) cyst expansion in 3-dimensional (3-D) cultures; (3) hepato-renal cystic areas and fibrosis in vivo in the PCK rat and polycystic kidney 2 (*Pkd2*$^{ws25/-}$) mice; and (4) the expression of Cdc25A and the cell-cycle proteins known to be regulated by Cdc25A (ie, cyclin-dependent kinase [Cdk]1, 2, 4/6; cyclin A, B, D, and E; retinoblastoma [Rb]; and proliferating cell nuclear antigen [PCNA]). We also tested the effect of PM-20 on progression of hepato-renal cystic disease in vivo in *Pkd2*$^{ws25/-}$ mice. Both the PCK rat and *Pkd2*$^{ws25/-}$ mice resemble human pathology in PKD/PLD and have been used in preclinical trials.[2,6] The PCK rat (a model of autosomal-recessive PKD/PLD) develops liver and kidney cysts at birth that gradually expand over time. *Pkd2*$^{ws25/-}$ mice (a model of autosomal-dominant PKD/PLD) have progressive hepato-renal cystic disease with onset at 5–6 months of age. We recently described the phenotype of PCK rats and *Pkd2*$^{ws25/-}$ mice in detail.[27,28]

We showed that hepatic cystogenesis is inhibited in *Cdc25A*$^{+/-}$:*Pkhd1*$^{del2/del2}$ cross-bred mice compared with their *Pkhd1*$^{del2/del2}$ counterparts, providing support for an essential role of Cdc25A in cyst growth. VK3 reduced the proliferation of cystic cholangiocytes, caused cell-cycle arrest, suppressed cyst growth in 3-D cultures, inhibited progression of hepato-renal cystic disease in vivo in animal models of PKD/PLD, and affected the expression of cell-cycle proteins. Targeting of Cdc25A with PM-20 also attenuated hepato-renal cystogenesis in *Pkd2*$^{ws25/-}$ mice. These data suggest that Cdc25A represents a potential molecular target in PKD/PLD and its inhibitors might be beneficial in the treatment of these pathologic conditions.

Materials and Methods

Animals and Cell Culture

Animals (number of rats and mice used is indicated for each experimental condition) were maintained on a standard diet after Mayo Institutional Animal Care and Use Committee approval. They were anesthetized with pentobarbital (50 mg/kg). Blood was collected from PCK rats by cardiac puncture. Liver and kidneys were fixed and embedded in paraffin for histology. For in vitro study, cholangiocytes were isolated from normal and PCK rats and cultured as described.[7] Normal and diseased human liver tissue were obtained from the Mayo Clinical Core and National Disease Research Interchange.

Development of Cdc25A$^{+/-}$:Pkhd1$^{del2/del2}$ Mice

The *Cdc25A*$^{+/-}$ mice were bred to *Pkhd1*$^{del2/del2}$ mice (both of c57 black 6 background) and offspring (ie, *Cdc25A*$^{+/-}$:*Pkhd1*$^{+/del2}$) were back-crossed with *Pkhd1*$^{del2/del2}$ mice to produce *Cdc25A*$^{+/-}$:*Pkhd1*$^{del2/del2}$ animals. Mice were genotyped by polymerase chain reaction (PCR). The primers and PCR conditions are described in the Supplementary Materials and Methods section. We analyzed body, liver, and kidney weights, and cystic and fibrotic areas, in age-matched 7- to 9-month-old littermates of *Cdc25A*$^{+/-}$ (n = 3), *Pkhd1*$^{del2/del2}$ (n = 5), and *Cdc25A*$^{+/-}$:*Pkhd1*$^{del2/del2}$ (n = 3) mice.

Flow Cytometry

Normal (n = 5) and PCK cholangiocytes (n = 6) were treated with 50, 100, and 200 μmol/L of VK3 for 24 hours, fixed in ethanol, and suspended in 50 μg/mL propidium iodide containing 0.1 mg/mL RNase. Cell-cycle analysis was performed at the Mayo Advanced Genomics Technology Center.

Immunofluorescence Confocal Microscopy

Microscopy was performed with a Zeiss LSM-510 microscope (Carl Zeiss, Thornwood, NY) using tissue of normal human beings (n = 5) and patients with autosomal-dominant PKD (ADPKD), autosomal-recessive PKD (ARPKD), and congenital hepatic fibrosis (n = 5 for each condition); normal (n = 5) and PCK (n = 6) rats; and normal (n = 4) and *Pkd2*$^{ws25/-}$ (n = 5) mice. Liver sections were incubated with antibodies against Cdc25A, Cdk4 and Cdk6, cyclin D1 and D3, Rb protein (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50); Cdk1 and cyclin B (Cell Signaling, Danvers, MA; 1:50); cyclin A and D2 (Abcam, Cambridge, MA; 1:50); Cdk2 (BD Transduction Laboratories, Lexington, KY; 1:50); cyclin E (Upstate; Lake Placid, NY; 1:50); and PCNA (Santa Cruz; 1:100). Respective secondary antibodies (Invitrogen, Carlsbad, CA; 1:200) were applied. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Billerica, MA) was used to detect apoptosis. For mitotic and apoptotic indices (calculated as a percentage of PCNA- or terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling–positive cells, respectively), 1000 nuclei in randomly selected fields of liver and kidney sections were counted.

Western Blot

Cholangiocytes isolated from livers of normal human beings (n = 3) and ADPKD patients (n = 3); normal (n = 5) and PCK rats (nontreated [n = 5] or VK3-treated [n = 4]); normal (n = 6), *Pkd2*$^{ws25/-}$ (n = 6), Cdc25A$^{+/-}$ (n = 3), *Pkhd1*$^{del2/del2}$ (n = 6) and Cdc25A$^{+/-}$:*Pkhd1*$^{del2/del2}$ cross-breed mice (n = 3); and cultured normal (n = 3) and PCK (n = 3) cholangiocytes were used. The details are described in the Supplementary Materials and Methods section.

Cell Proliferation

Normal (n = 5) and PCK (n = 5) cholangiocytes (5000 cells/well) were grown in regular Dulbecco's modified Eagle medium: nutrient mixture F-12 medium at 37°C (5% CO_2 and 100% humidity) for 48 hours before treatment. VK3 (50, 100, and 200 μmol/L) was added daily for a total of 5 days. Cell proliferation was determined every 24 hours by CellTiter 96

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