Impaired ERK signaling in renal cystic cells
Chapter 5

Down-regulation of *PKD1* and *PKD2* after DNA Damage

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Abstract

PKD1 and PKD2, the genes mutated in Autosomal Dominant Polycystic Kidney Disease, have been previously reported to be involved in cell cycle regulation. RNA subtractive hybridization performed by us to identify differentially regulated genes in normal fibroblasts (VH10 and VH25), revealed that PKD1 and PKD2 themselves were down-regulated after UV-C irradiation. Northern blotting and RT-PCR showed that PKD1 and PKD2 were down-regulated after cellular stresses induced by UV-C, X-ray, and the alkylating agent methyl methanesulfonate (MMS) in diploid fibroblasts and renal epithelial cells (RCTEC, PKD9-7WT, MDCK, and mIMCD3). The induced damage caused apoptosis in approximately 20% of cells, as measured by FACS analysis of FITC-annexin-V labeled cells which coincided with activation of caspase-3. The remainder of cells is capable of recovering from this damage. In ADPKD one of the PKD1 or PKD2 alleles is already inactivated by a germ-line mutation. Therefore, further lowering of gene expression by exposure of kidneys to normally accumulated sub-lethal DNA damage present in the kidney may just be sufficient to cause cyst formation and progression.

Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a common inherited disorder that predominantly manifests with progressive development of fluid-filled cysts in both kidneys. Cyst formation ultimately results in chronic renal failure. ADPKD is one of the major causes of chronic renal failure. Of all patients requiring renal dialysis and transplantation, 8-10% is represented by patients suffering from chronic renal failure due to Autosomal Dominant Polycystic Kidney Disease (ADPKD; 1). In the majority of patients, the disease can be accounted for by a mutation in the PKD1 gene (2;3). Approximately 15% of patients suffer from a mutation in the PKD2 gene (4-6). The precise function of polycystin-1 and polycystin-2, the proteins encoded by the PKD1 and PKD2 gene respectively, remains to be elucidated. Therefore, it is still unclear how a mutation in polycystin-1 or polycystin-2 results in cyst formation. Due to an as yet unknown mechanism, a subset of renal epithelial cells undergoes a significant change that enables them to escape the regulatory mechanisms that normally tightly regulate their function (Figure 4, Chapter 1, page 13). These cells expand and grow out to form an isolated cyst. Cyst enlargement then occurs via fluid accumulation in the cyst lumen due to mislocalization of ion channels. Progressive development of cysts and accompanying fibrosis of the surrounding tissue disrupts renal function and ultimately results in chronic renal failure.

Two major theories have been postulated to describe the mechanism of cyst formation. The ‘two-hit’ model predicts that both alleles of PKD1 or PKD2 need to be affected to result in ADPKD. Since all cells have inherited one affected allele (germ-line mutation), a somatic mutation in the other normal allele is required for cyst formation. Somatic mutations in kidney epithelial cells are relatively frequent and increase exponentially in life (7). The ‘two-hit’ model predicts that cysts will
develop only from cells that have acquired a somatic mutation in the second allele. This hypothesis adequately explains the phenotypic variation observed within families, since individuals can acquire different second hit mutations during life (8-10). It also fits well in line with the observation that cysts originate from less than 1% of total nephrons. Furthermore, Qian et al. have demonstrated that cyst-lining cells derived from a single cyst are of clonal origin (8). However, where the two-hit model predicts that cysts are devoid of any functional polycystin-1 or polycystin-2, expression of both polycystin-1 and polycystin-2 have been reported in cysts. Whether these proteins are functional or abnormally expressed mutant products remains to be determined. The second model, the ‘haplo-insufficiency’ model, postulates that levels of polycystin-1 and polycystin-2 need to be tightly regulated for proper function. Inactivation of one allele due to an inherited mutation may result in increased stochastic variations in expression levels (11). These variations in expression level may ultimately lead to late-onset disease manifestations, as is the case in ADPKD. The haplo-insufficiency model adequately reconciles with the observation that polycystin-1 and polycystin-2 can be detected in cysts. Moreover, mouse models have shown that both too low (knock-out) and too high expression (transgenic) of polycystin-1 results in ADPKD (12;13).

Several lines of investigation suggest that PKD1 and PKD2 are involved in cell cycle regulation (14-19). Interestingly, RNA subtractive hybridization performed by us to identify differentially regulated genes in normal human diploid fibroblasts (VH10 and VH25), revealed that PKD1 and PKD2 themselves were down-regulated after UV-C irradiation. Based on this observation, we set out to study the expression of PKD1 and PKD2 after DNA damage in more detail. Northern blotting and RT-PCR showed that PKD1 and PKD2 were down-regulated after UV-C induced DNA damage in human diploid fibroblasts and renal epithelial cells. The same effect was achieved with X-ray and the alkylating agent MMS, indicating that this down-regulation is not damage specific. Down-regulation of PKD1 and PKD2 after DNA damage may account for the variations in expression level described in the ‘haplo-insufficiency’ model for cyst formation. Sub-lethal DNA damage is normally accumulated during life. In the case of ADPKD, germ-line inactivation of one allele in combination with down-regulation of PKD1 and PKD2, may just be sufficient to enhance cyst formation and progression.

Materials and Methods

Cell culture

Human diploid fibroblasts, VH10 and VH25, were cultured in D-MEM with 4500 mg/l glucose, 100 U/ml penicillin and streptomycin, 1 mM sodium-pyruvate, 0.1 mM HEPES, 2 mM glutaMAX™-I , and 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cell culture reagents were purchased from Invitrogen Ltd. (Paisley, Scotland) and disposables from Greiner Bio-One GmbH. (Frickenhausen, Germany). Madin-Darby Canine Kidney (MDCK) and mouse inner medullary collecting duct 3 (mIMCD3) renal
epithelial cells, obtained from the American Type Culture Collection (ATCC), and the human control and ADPKD renal cystic epithelial cell lines, RCTEC and PKD9-7 respectively (20), were maintained in D-MEM/F12 and supplements as stated above. Cells were cultured on 15 cm² petri dishes until confluence and serum-starved by replacing culture medium with medium containing 0.5% heat-inactivated fetal bovine serum. Three days after serum-starvation, cells were irradiated with UV-C (15-30 J/m²), X-ray (2 Gy), or the alkylation agent MMS (1mM), as described (21).

**RNA isolation and Northern blotting**

Total RNA was isolated 1 h or 20 h after treatment as described by Van Laar et al. (21). RNA subtractive hybridization was performed using the PCR-Select™ cDNA subtraction and differential screening kits (BD Biosciences, San Jose, CA, USA). Northern blotting was performed using 20 µg total RNA per lane as described. Briefly, samples were transferred to Hybond N filters (Uppsala, Sweden). Filters were then incubated with specific ³²P-probes over night at 42°C. PKD1 was detected using a full-length polycystin-1 cDNA probe and PKD2 was detected using a C-terminal probe (EcoR V fragment from cTM4-FL). Filters were then washed in 2xSSC/0.1%SDS at 55°C for 60 minutes followed by 2x 30 minutes washing with 1xSSC/0.1%SDS. Expression was detected on Kodak Biomax XAR film (Kodak, Chalon sur Saone, France).

**Real-Time Polymerase Chain Reaction**

Real-Time PCR (RT-PCR) was performed using SYBR® Green PCR master mix and the ABI PRISM 7700 SDS according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). PKD1 was detected with 0.2 µM hPKD1 exon34F (5’-CAAGACACCCCACATGAAACG-3’) and hPKD1 exon35R (5’-CGCAGCGTCTCTGTGTTT-3’) primers. PKD2 was detected with hPKD2-F (5’-CCGTGGATGACATTTCAGAGAGT-3’) and hPKD2-R (5’-CATCAGTATGGGCCCTTCC-3’) primers. Denaturation was performed at 94°C for 5 minutes, followed by 38 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 40 seconds, with a final elongation at 72°C for 7 min. Each sample was analyzed in triplicate. Expression was normalized to 18S ribosomal RNA using 5’-GCCGCTAGAGGTAAATT-3’ and 5’-CATTCTGGCAAATGCTTT-3’ primers.

**Fluorescence Activated Cell Sorter (FACS) and caspase-3 activity assays**

Fluorescence Activated Cell Sorter (FACS) analysis of FITC-annexin-V labeled cells, and caspase-3 activity assays were performed as described (22). Briefly, cells were trypsinized, washed twice with ice-cold PBS. Cells were labeled with 25 µg/ml FITC-annexin-V and 25 µg/ml propidium iodide (PI) in 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2, pH 7.4 containing annexin V-FITC and PI (25 µg/ml) for 30 minutes and subsequently analyzed.
using a FACSCalibur flow cytometer (BD Biosciences). For each measurement 10,000 events were counted. Caspase-3 activity assays were performed in parallel to FACS annexin-V analysis using the Caspase Activity Assay kit according to the manufacturer’s instructions (BioVision Inc., New Minas, Canada).

Results

**Down-regulation of PKD1 and PKD2 after DNA damage**

RNA subtractive hybridization performed by us to identify differentially regulated genes in normal human diploid fibroblasts (VH10 and VH25), revealed that PKD1 and PKD2 were down-regulated after UV-C irradiation. Based on this observation, we set out to study the expression of PKD1 and PKD2 after DNA damage in more detail. Northern blotting was used to confirm the down-regulation of PKD1 and PKD2 after UV-C irradiation. Human diploid fibroblasts, VH10 and VH25, and renal epithelial cells, RCTEC (Figure 1A-B), MDCK, and mIMCD3 (not depicted) all showed consistent down-regulation of PKD1 and PKD2. p21Waf1, a positive control for UV-C treatment, was up-regulated whereas the loading control GAPDH was unaffected, indicating that differentially expressed genes can be identified using these methods. In addition, PKD2 was also found to be down-regulated in the human ADPKD renal cystic epithelial cell line, PKD9-7 (not depicted). Quantitative Real-Time PCR analysis confirmed that both PKD1 and PKD2 were down-regulated after UV-C treatment in RCTEC and in the ADPKD renal cystic epithelial cell line, PKD9-7 (Figure 1C, left and middle panel). Moreover, down-regulation was detected as early as 1 hour post UV-C irradiation and was detected up to 20 hours after UV-C irradiation (Figure 1C, right panel). FACS analysis using annexin-V labeled cells, an early marker for apoptosis, showed that irradiation with UV-C (15 J/m²) is a sub-lethal dose inducing apoptosis in approximately 20% of cells at 20 hours, which further increased to 27% at 40 hours (Figure 2, upper panel). This early onset of apoptosis at 20 hours after exposure was associated with an increased activity of caspase-3, an essential factor in execution of apoptosis, (Figure 2, lower panel). At higher dosage (30 J/m² UV-C) approximately 70% of cells were apoptotic at 20 hours after exposure, which correlated with a higher caspase-3 activity compared to the lower UV dosage.

To further investigate expression of PKD1 and PKD2 after stress induction, we exposed human renal epithelial RCTEC cells to UV-C, X-ray irradiation and the alkylating agent, MMS. Expression of PKD1 and PKD2 was tested by Quantitative Real-Time PCR. Results showed that in addition to UV-C, both X-ray and MMS treatment resulted in down-regulation of PKD1 and PKD2 suggesting that this could be a general response to certain types of cellular stress induced by genotoxic agents (Figure 3).
Discussion

Here we report the downregulation of both PKD1 and PKD2 after cellular damage. This response seems to be a general phenomenon and occurred after exposure to different types of genotoxic agents. Down-regulation of PKD1 and PKD2 after UV-C is an early event and persists up to 20 hours after induction. Down-regulation may thus be part of a general response to cellular stress. Our data are seemingly contradictory with two previous reports on PKD2 expression after renal ischemic injury. Obermüller et al. used mouse and rat models for polycystic kidney disease to induce acute and chronic renal ischemic injury (23). Expression of PKD2 was increased in some sections and decreased in other sections, depending on type and severity of damage. Using the same renal ischemic injury model in normal Sprague-Dawley rats, Zhao et al. reported up-regulation of PKD2 in all nephron segments (24). However, they may have induced such severe damage that all nephron segments showed up-regulation of PKD2. Overall, these data suggest that after extensive high dosage of damage PKD2 is up-regulated, whereas at lower dosages of cellular damage PKD2 is decreased. In this respect, our data are in line with Obermüller et al., since we show that the cellular damage induced in our system does not lead to massive apoptosis (Figure 2).

We hypothesize that down-regulation of both PKD1 and PKD2 is a common response for a variety of cell types after sub-lethal cellular damage normally encountered during life, since this effect is not only detected after UV-C but also after a variety of other cellular stresses (Figure 3). The cell is equipped with a broad range of mechanisms to repair the damage during a temporarily cell cycle arrest or, when the damage is irreversible, senescence or programmed cell death. Our data indicate that down-regulation of PKD1 and PKD2 is an integral part of the response after cellular stress. In support of this, Maser et al. previously reported that mouse and rat polycystic kidneys showed decreased levels of renal anti-
oxidant enzymes that normally protect against oxidative stress (25). In the case of ADPKD where one allele of the \textit{PKD1} or \textit{PKD2} gene has already been affected by a germ-line mutation, down-regulation of the second allele after sub-lethal damage may have undesirable/negative effects on renal cell function. Eventually, this may lead to renal cyst formation. These data fit well in line with the `haplo-insufficiency' model for cyst formation. We propose that sub-lethal cellular damage may be a non-genetic environmental factor in ADPKD cyst formation. It would be interesting to assess the effect of cellular damage on mouse models for \textit{Pkd1} and \textit{Pkd2}. Heterozygous mice only develop a few small cysts at an old age, presenting with a fundamental difference from human ADPKD patients. Since these mice are kept in a well controlled environment, these animals may accumulate only low cellular damage. To test this hypothesis, we propose to induce cellular damage to heterozygous knock-out mice or to cross heterozygous mice with DNA-repair deficient mice. Based on our data, we expect more severe or progressive polycystic kidney disease.

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References


