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Constitutive activation of nf- κ b causes tubule enlargement and cyst formation in cultured mammalian collecting duct cells

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ABSTRACT

Polycystic kidney disease (PKD) is characterized by progressive enlargement of renal cysts. It is widely accepted that cystogenesis is mostly due to the disturbance in the balance between the proliferation and apoptosis of the tubular epithelial cells. We have previously demonstrated an increase in nuclear factor κB (NF-κB) in fibrocystin-decreased kidney cells. The aim of the present study was to determine the effect of NF-κB signaling on cyst formation, tubular cell apoptosis and proliferation in the mCCD cells, an in vitro model of tubulogenesis that would be suitable for experimental induction of cyst formation. Selective activation of NF-κB pathway using TNF-α resulted in progressive dilatation of existing tubules, leading to the formation of cyst-like structures. The caspase-3 inhibitor reduced tubular apoptosis and proliferation and prevented tubule enlargement and cyst formation. We conclude that NF-κB signaling plays a key role in renal cyst formation, at least in part by inhibiting caspase-3 activity. These observations *provide a potential platform for the future treatment of the renal manifestations of PKD.*

KEYWORDS

Polycystic kidney; Cystogenesis; NF-κB pathway; Caspase-3.

INTRODUCTION

Polycystic kidney disease (PKD) is a systemic hereditary human disorder characterized by progressive enlargement of cysts in the kidneys, often leading to end-stage renal disease. Moreover, multiple organs other than the kidney are usually affected by the disease. Cystogenesis in PKD is a multi-step process associated with aberrant levels of cellular proliferation, apoptosis, increased fluid secretion, dedifferentiation, matrix remodeling, and alteration in cellular polarity [1, 2]. Growing evidence suggests that PKD is a developmental disorder [3, 4].

Inherited PKD includes both autosomal dominant and autosomal recessive forms. Autosomal dominant polycystic kidney disease (ADPKD), the most common polycystic kidney disease, is caused by mutations in either PKD1 or PKD2, which encodes polycystin-1 and polycystin-2 proteins (PC-1 and PC-2), respectively[5, 6]. ADPKD is characterized by the formation of myriads fluid-filled cysts, which may arise as focal outpouches from any segment of the nephron or collecting duct (CD). The less common autosomal recessive polycystic kidney disease (ARPKD), results from mutations in the *PKHD1* gene (6p12), which encodes for a large multi-domain integral membrane protein (fibrocystin/polyductin, FPC). ARPKD is characterized by cysts specifically derived from focal dilatations of the CD[7]. Both the PC-1 or PC-2 and the FPC are developmentally regulated proteins that are expressed at high levels in the ureteric bud epithelium in the normal developing kidney, where they are localized to the basal areas of the cell membrane that are in focal contact with the ECM[8, 9]. As the cysts expand, they compress and destroy the surrounding functional parenchyma, ultimately leading to end-stage renal failure.

Nuclear factor-kappa B (NF-κB) is an important regulator of cell survival, proliferation, and differentiation, and is frequently involved in malignant transformation. It is normally sequestered in the cytoplasm by its interaction with IκB, which is the NF-κB inhibitor. Following the appropriate stimulus that activates IκB kinases (IKKs), degradation of IκB exposes the nuclear localization signal of NF-κB. NF-κB then is translocated to the nucleus and activates the transcription of its target genes. The transcription factor NF-κB is a ubiquitously expressed and highly regulated dimeric molecule that plays a pivotal role in modulating the expression of genes responsible for inflammation, cell proliferation, and suppression of apoptosis[10].

Several reports showed that NF-κB may have a central role in pro-apoptotic FC1-depleted cells[11, 12]. The proapoptotic caspase-3 level increases in the kidney of Han:SPRD rats with PKD[13]. In this study, to better investigat the cellular mechanisms responsible for the formation of renal cysts in PKD, we first designed highly appropriate experiments to set up an *in vitro* model of tubulogenesis in mCCD cells. The experimental induction of tubule-to-cyst conversion *in vitro* was carried out in conditions that approximate the *in vivo* conditions as closely as possible. To assess whether the activation of the NF-κB signaling pathway can induce the enlargement of established tubular structures, mCCD cells were treatment with TNF-α, a canonic activator of the NF-κB pathway. The results demonstrated that the preformed tubules progressively enlarged and cystlike structures formed upon treatment with TNF-α. Consistently, caspase-3 inhibition reduces tubular apoptosis and proliferation and prevents tubule enlargement and cyst formation. These results further support the notion that NF-κB signaling plays a key role in renal cystogenesis. NF-κB signaling at least in part reduces cyst formation and disease progression in PKD by inhibiting caspase-3 activity and reducing tubular cell apoptosis and proliferation. Therefore, targeting the NF-κB and caspase pathways to inhibit proliferation and apoptosis in polycystic cells may be a promising approach for developing novel therapies for PKD.

MATERIAL AND METHODS

Reagents

TNF-α and z-DQMD-FMK were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The pharmaceuticals were reconstituted in sterile dimethyl sulphoxide (DMSO, Sigma -Aldrich), and DMSO was used as the vehicle-only control. Antibodies against NF-κB-p65, IκBα, phospho-IκBα (Ser32), and anti-PCNA antibody (sc-7907, 1:50) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA).

Cell culture and treatment

Mouse kidney cortical collecting ducts microdissected cells (mCCDcl1 cells) were maintained in DMEM/F-12 (1:1, Life Technologies Inc.) with Glutamax. All growth and experimental incubations of cell cultures took place in a tissue culture incubator with a humidified atmosphere at 37°C in 5% CO2, and 95% air. mCCD cells were grown in collagen/matrigel gels for 6 days to allow initial tubule formation, then treated with TNF-α (75 ng/ml) for 4 days. Treatments were performed to assess whether activation of the NF-κB pathway could induce the enlargement of established tubular structures. For caspase-3 inhibition, the cells were treated with 25 μM caspase-3 inhibitor z-DQMD-FMK or solvent only (DMSO) with a final DMSO content of 0.1%. Under the assay conditions, these activators were not cytotoxic as indicated by the MTT assay.

Adult 4/5 kidney tubular cell line was purchased ATCC (Manassas, VA). The cells were cultured in DMEM/F12 medium (Gibco) supplemented with 10% inactivated FBS (Hyclone), 2 mM L-glutamine, 0.5 mM HEPES, in a chamber supplied with 5% CO_2 at 37 ºC. In order to establish stable FC1-silenced and control kidney tubular 4/5 cell lines, FC1 shRNA and control constructs were transfected into subconfluent kidney tubular 4/5 cells. FC1 shRNA (against PKHD1 exon V sequence: 5′-AAGCAGTCCAAATCCAGGACC-3′) and control small hairpin RNA (shRNA) (Wuhan Genesil Biotech, China) were cloned as described previously [Zheng et al., 2009]. After transfection for 24 h, DMEM containing 5% FCS and

1 mg/ml G418 was used to select G418-resistant clones. After culturing G418-selective cells for 14 days, the G418-resistant cells were plated in six-well dishes at 10^3 cells per well for the expansion. a single colony was picked and transferred into a new 24-well culture plate (Costar).

Establishment of collecting duct-derived mCCD cell

The mCCD cell line was established from an individual multicellular colony of mCCDcl1 cells formed in a collagen gel culture as described previously[14]. Briefly, mCCDcl1 cells were lysed with a type I collagen solution (Sigma) to obtain a concentration of 250 cells /ml. A small fragment of collagen gel containing the colony was removed under sterile conditions and dissolved by incubation with 4 mg/ml *Clostridium histolyticum* collagenase (Sigma). The cells were then seeded into six-well plates and allowed to confluence. The resulting cells were examined under a phase-contrast microscope for the definition of three-dimensional structures and a inverted photomicroscope for measurement of the lumen diameter.

Microscopy

For definition of three-dimensional structures, the cells were mixed with an extracellular matrix solution composed of 80% type I collagen and 20% growth factor-reduced Matrigel (Sigma). The cultures were incubated for six days to allow initial formation of tubelike structures. After 11 days of culture, a three-dimensional colony endowed with branching tubular outgrowths was identified using a phase-contrast photomicroscope. A cyst was defined as an ovoid structure in which the ratio between the major and minor axis of the lumen was less than two, and structures exhibiting an intermediate shape were defined as enlarged tubules.

Lumen diameter was measured using an inverted photomicroscope with an on-screen caliper. In tubules and ovoid cysts, maximum lumen diameter was taken as the largest width of the cavity along a line traced perpendicularly to the long axis of the structure. In spherical cysts, this value corresponds to the internal diameter of the cyst.

Proliferation Assay

Cells were grown to 70% confluency before supplementation with 10 ng/ml HGF with or without the addition of 75 ng/ml TNF-a. Proliferating cell nuclear antigen (PCNA) staining was performed using an anti-PCNA antibody. The number of PCNA-positive cells per tubule was counted using a Nikon Eclipse E400 microscope. To avoid confusion between noncystic tubules and small cysts as well as potential changes in tubular cells lining massive cysts, PCNA-positive tubular cells were counted in "medium sized cysts" of ≈250 μm diameter. For each sample, at least 15 areas in the cortex were randomly selected.

Detection of apoptosis

The TUNEL method was used to detect in situ DNA strand breaks. TACS 2 TdT-blue label in situ apoptosis detection kit (Trevigen, Gaithersburg, MD) was used following the manufacturer's instructions. Briefly, the cells were incubated with proteinase K for one hour at 37°C for permeabilization. Endogenous peroxidase activity was quenched by incubating the tissue sections with 3% hydrogen peroxide in methanol for 5 minutes. After incubating with streptavidin-horseradish peroxidase, the apoptotic cells were detected with Blue Label. The number of apoptotic cells per tubule was counted using a microscope equipped with a digital camera connected to imaging software. At least 15 areas per sample in the cortex were randomly selected.

We followed the manufacturer's protocol when using the annexin V-FITC kit from Promega (Madison, WI, USA). Apoptotic cells were quantified by measuring the percentage of Annexin V-positive cells. Briefly, cells were washed in cold phosphate-buffered saline (PBS) three times, followed by re-suspension in cold binding buffer, and Annexin V-FITC plus propidium iodide (PI) was added. The percentage of apoptotic cells was calculated as following: apoptotic cells (%) = (total number of apoptotic cells /total number of cells counted) ×100.

Immunoblot analysis

Cells were seeded on plastic plates and grown to 70% confluency before supplementation with 10 ng/ml HGF with or without the addition of 75 ng/ml TNF-a. Cell debris were removed by centrifugation of the cell lysates. After clarification, equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Millipore, Billerica, MA). The membrane was blocked and incubated with specific antibodies overnight at 4ºC in Tris-buffered saline with 0.1% Tween-20 and 5% nonfat dry milk as indicated in each experiment, followed by incubation with an appropriate anti-mouse or anti-rabbit immunoglobulin secondary antibody.

Caspase-3 activity assay

Caspase-3 activity was determined using the CaspACE colorimetric assay Kit (Promega, Madison, WI) as recommended by the manufacturer's instructions. Briefly, after treatment with or without the addition of 25 μM z-DQMD-FMK, cells were collected and resuspended in cell lysis buffer. After lysis, cell extracts (50 μg protein) were incubated with reaction buffer containing dithiothreitol and a chromogenic caspase-3 substrate, DEVD-*p*-nitroanilide (DEVD-pNA). Caspase-3 activity was determined by measuring the cleavage of the colorimetric substrate, which is indicated by the increase in an absorbance at 405 nm. Absorbance of each sample was determined by subtraction of the mean absorbance of the blank from that of the sample.

Electrophoretic mobility shift assay

After cultured cells were harvested and washed, nuclear proteins were extracted with NE-PER^{TM} nuclear and cytoplasmic extraction reagents (Pierce Chemical Co, IL) in accordance with the manufacturer's instructions. The nuclear extracts were subjected to assessment of NF-κB-DNA binding using the LightShift^{TM} EMSA kit (Pierce) with a biotin end-labeled NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′).

Statistical analyses

Results are expressed as means ± SE of the mean. For lumen diameter, quantitations are expressed as mean lumen diameter per structure ± SE, and statistical significance was determined using Student's unpaired *t*-test. A value of $P < 0.05$ was used to assert statistical significance.

RESULT AND DISSCUSS

mCCD cells form tubular structures

We first determined whether mCCD cells have the ability to form tubelike structures in a three-dimensional extracellular matrix. We observed that mCCD cells proliferated and formed branched rodlike structures within 3-4 days when suspended in collagen/matrigel gels. Over 6-8 days, the rods progressively developed an axial cavity, resulting in the generation of tubular structures (Figure 1).

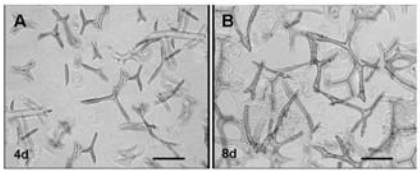

Figure 1 : mCCD cells form well-organized tubular structures. A. Four days after embedding in collagen/Matrigel gels, mCCD cells have formed branching rodlike structures. Bar = 200 µm. B. The initial rods of cells progressively elongate and develop an axial cavity in a 8-day culture. Bar = 200 µm.

Activation of NF- κ B induce tubule enlargement and cyst formation

To analyze the activation state of the NF-κB pathway in the mCCD cells, we firstly examined IκBα phosphorylation, NF-κB p65 nuclear translocation, and NF-κB-DNA binding by using canonic activator of the NF-κB pathway. As shown in Figure 2A, TNF-α treatment increased IκBα phosphorylation and led to degradation of IκBα. Densitometric analysis of immunoblot studies showed a decrease in IκBα levels and an increase in nuclear p65 expression after treatment. The translocation of NF-κB p65 into the nuclear fraction was increased after a TNF-α pretreatment. We observed a shifted band in untreated cells, thereby indicating basal NF-κB activity. Pretreated cells enhanced NF-κB activity as evidenced by a significant increase in the intensity of the shifted band observed in these cells (Figure 2B).

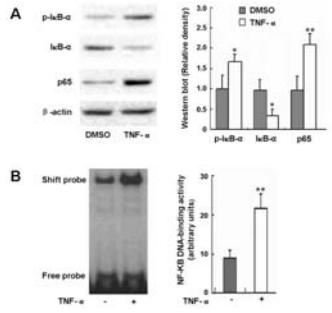

Figure 2: Analysis of NF-κB activity after TNF-α treated. A. The protein levels of p-IκB-α, IκB-α, and NF-κB-p65 in mCCD cells. The density of each band was quantified, and the ratio of bands was calculated and shown as relative levels (right panel). * $P < 0.05$ and ** $P < 0.01$ TNF-α vs. DMSO. B. EMSA assay for NF-κB activity. ** $P < 0.01$ TNF-α vs. DMSO.

Next, a quantitative analysis demonstrated that the former tubules underwent progressive lumen expansion. A significant ($P < 0.01$) effect was observed after 48 hours of treatment (Figure 3A). In untreated cultures, epithelial tubules maintained a cylindrical shape and exhibited a slight increase in lumen diameter in the same time period. The number, length and number of branches of tubular structures not led to different in control cells and in cells treated with TNF-α.

Effect of caspase-3 inhibition on the development of cystogenesis

Recent reports have shown that NF-κB inactivation is required for the suppression of caspase-3 activity and the reduction of cell death in FC1-depleted cells. Therefore, we examined the involvement of caspase-3 activation in mCCD cells. We analyzed cytoplasmic extracts from mCCD cells in the presence or absence of TNF-α for caspase-3 activation. The results show that caspase-3 activity was significantly increased in mCCD treatment with TNF-α (Figure 3B). To determine whether the effect of TNF-α induced tubule dilatation was mediated through the activation of caspase-3, we used a selective caspase-3 inhibitor z-DQMD-FMK (25 μM). As shown in Figure 3C, z-DQMD-FMK (25 μM) decreased TNF-α induced tubule enlargement in a dose-dependent manner.

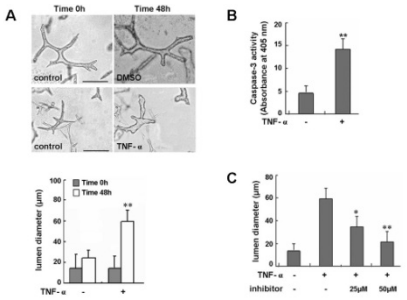

Figure 3: Activation of the NF-κB pathway induces cystic dilatation of existing tubules. A. mCCD cells were grown in collagen/Matrigel gels for 6 days and subsequently untreated or treated with TNF-α for a further 48 hours. Bar = 200 μm. ** *P* < 0.01 vs. control. B. Caspase-3 activity in mCCD clones cultured for further 48 hours with TNF-α. ** *P* < 0.01 vs. untreated cultures. C. Effect of caspase-3 inhibitor on TNF-α induces lumen enlargement. * *P* < 0.05 and ** *P* < 0.01 vs. treated with TNF-α alone.

To determine whether increased cell apoptosis could contribute to tubule enlargement, collagen/matrigel gels incubated in the presence or absence of TNF-α for 6 days were dissolved with collagenase. The number of TUNEL-positive apoptotic tubular cells per tubule in cystic tubules in the cortex was 0.31 ± 0.08 in untreated mCCD cells, and 0.15 ± 0.03 in z-DQMD-FMK-treated mCCD cells ($P < 0.05$). The number of TUNEL-positive cells per cyst in the cortex was 0.36 ± 0.07 in untreated cystlike cells, and 0.11 ± 0.04 in treated cells ($P < 0.05$) (Figure 4A). The FACS assay further confirming that caspase inhibition attenuates cellular apoptosis in the cystic tubules (data not shown).

Numerous studies demonstrated that increased tubular cell proliferation is accompanied by increased tubular apoptosis in PKD. Our results show that in cystic tubules, the mean number of PCNA-positive cells per tubule in TNF-α stimulated culture was higher than untreated culture. But when tubule-containing cultures were incubated with TNF-α in the presence of the z-DQMD-FMK, the number of PCNA-positive cells per cyst was 0.9 ± 0.2 in untreated cystlike cells, and 0.11 ± 0.06 in z-DQMD-FMK-treated cystlike cells ($P < 0.05$) (Figure 4B), suggesting that caspase-3 activity plays a role in the increased tubular cell proliferation in PKD.

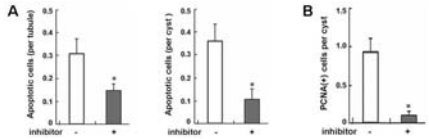

Figure 4: Tubular cells apoptosis and proliferation. A. The number of TUNEL-positive cells in mCCD cells. Bars show the mean ± SD of apoptotic cells, **P* < 0.05 vs. untreated cells. B. Tubular Cell Proliferation. Bars show the number of PCNA-positive cells per tubule in cystic tubules in the cortex. * *P* < 0.05 vs. untreated cells.

CONCLUSIONS

In PKD, cell apoptosis and cell proliferation are involved in pathological features of tubulomorphogenesis and cyst formation[15]. In the current study, we evaluated the correlation between NF-κB survival pathway and caspase-3 activity, and cell apoptosis and proliferation in mCCD cells,. We demonstrate that NF-κB signaling pathway is involved in the modulation of renal cyst formation at least in part by promoting caspase-3 activity.

Activation of the NF-κB signaling pathway has been implicated in the control of many critical cell responses. Many NF-κB activation pathways have been revealed, and all of them rely upon sequentially activated kinase cascades[16]. NF-κB was involved in renal fibrotic events[17], and we have previously demonstrated that there is an increase in the NF-κB level in HEK293 PKHD1shRNA cells[12]. Our results show that mCCD cells form tubular structures in three-dimensional culture. The *in vitro* findings showed that TNF-α induced tubule enlargement. The fact that NF-κB binding activity increased in pretreated

mCCD cells, in accordance with earlier studies, suggests that in FC1-deficient human cells there is an abnormally elevated activity of NF-κB.

Significant evidence indicates that caspase-3 is either partially or solely responsible for the proteolytic cleavage of many key proteins, facilitating cellular disassembly and serves as a marker for cells undergoing apoptosis[18, 19]. Despite the presence of apoptosis in most PKD models, the caspase signaling pathways have only recently been described. It has been described in detail that there is an increase in caspases activity and apoptosis in Han:SPRD rats with PKD[20]. The *in vivo* studies suggest that caspase or apoptosis inhibition attenuates cyst formation. In fact, following caspase-3 inhibited the tubule enlargement. These findings indicate that TNF-α induced tubule enlargement may require the activity of caspase-3. The finding was associated with a significant decrease in apoptosis both in the noncystic tubules and in the tubular cells surrounding the cyst of the mCCD cellular models.

Kidneys from patients and animal models of PKD have high levels of cellular proliferation as well as apoptosi. Our results suggest NF-κB could stimulate the proliferation of tubular cells derived from cysts of PKD. Similar to the effect on apoptosis, caspase-3 inhibition not only decreased apoptosis but also profoundly decreased tubular cell proliferation. Our *in vitro* findings are consistent with *in vivo* experiments performed in animal models of PKD. The results show that administration of caspase inhibitor decreases apoptosis and proliferation in cystic tubules and inhibits renal enlargement and cystogenesis.

Taken together, the results presented herein show that the NF-κB pathway may be implicated in promoting the pathological conditions of PKD in mCCD cells, an *in vitro* model of tubule-to-cyst conversion, in which both proliferation and apoptosis are typical features. Moreover, the discovery further confirms the key role of caspase-3 in cystogenesis and reduction of tubular cell apoptosis and proliferation. The exaggerated NF-κB and caspase activation in the cystic tubular cell models may afford a new area for therapeutic investigation of this disease.

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