BMB
reports

Mxi1 influences cyst formation in three-dimensional cell culture

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Cyst formation is a major characteristic of ADPKD and is caused by the abnormal proliferation of epithelial cells. Renal cyst formation disrupts renal function and induces diverse complications. The mechanism of cyst formation is unclear. mIMCD-3 cells were established to develop simple epithelial cell cysts in 3-D culture. We confirmed previously that Mxi1 plays a role in cyst formation in Mxi1-deficient mice. Cysts in Mxi1 transfectanted cells were showed by collagen or mebiol gels in 3-D cell culture system. Causative genes of ADPKD were measured by q RT-PCR. Herein, Mxi1 transfectants rarely formed a simple epithelial cyst and induced cell death. Overexpression of Mxi1 resulted in a decrease in the PKD1, PKD2 and c-myc mRNA relating to the pathway of cyst formation. These data indicate that Mxi1 influences cyst formation of mIMCD-3 cells in 3-D culture and that Mxi1 may control the mechanism of renal cyst formation. [BMB reports 2012; 45(3): 189-193]

INTRODUCTION

Polycystic kidney disease (PKD) is one of the most common genetic diseases worldwide. It is a genetically heterogeneous disorder with a gene frequency of 1 in 200-1,000 in the general population, and is the cause of 8-10% of all end-stage renal disease. The disease is named for the large number of epithelial-lined cysts that grow from the nephrons and collecting ducts of affected kidneys (1).

Cyst formation is believed to require an overproliferation of renal epithelial cells from the walls of kidney tubules (2). Evidence to support this hypothesis has come from microdissection of cystic kidneys, which reveals that cyst size is due to an increase in the number of epithelial cells lining the cyst and not to the stretching of the cyst wall (3).

The multibranched inner medullary collection ducts of the kidney are highly ordered and terminally differentiated structures consisting of polarized epithelial cells derived embryologically from the ureteric bud. The collecting system of the kidney is formed by iterative branching morphogenesis of the ureteric bud. Although this process has been studied in whole animal, organ, and cell culture models, the molecular cues for its control are still poorly understood (4).

Especially, three-dimensional (3-D) cell culture is similar to the in vivo system in cell shape and cellular environments, and is a powerful cystogenetic method to study the epithelial architecture that is specifically involved with molecular signals. Presently, we exploited 3-D culture to investigate genes related to cyst formation.

Mxi1 is a tumor-suppressor gene that has essential roles in cellular growth control and in the induction and maintenance of the differentiated state. In addition, the Mxi1 gene is an antagonist of c-myc, a well-known oncogene that is also linked with cyst formation diseases including ADPKD. Additionally, cultured epithelial cells from ADPKD cysts display enhanced rates of proliferation, and genes associated with increased proliferation such as c-myc have been found to be overexpressed in cystic epithelium (5). In our previous study, we showed that lack of Mxi1 gene expression leads to cyst formation in Mxi1-deficient mice (6). Presently, we established a mIMCD-3 cell with stably overexpressed Mxi1 to investigate whether Mxi1 contributes to cyst formation in 3-D cell culture.

RESULTS

In vitro cyst formation of Mxi1 transfectants

The Mxi1 gene is an antagonist of c-myc and has been implicated in cyst formation in the kidneys of Mxi1 knockout mice (6). Presently, we established stably Mxi1 overexpressed mIMCD-3 cells. The expression vector pCMV-Tag2B with Mxi1 was transfected into mIMCD-3 cells, and stable transfectants were selected based on G-418 resistance. The clone was examined for the expression of Mxi1 by semi-quantitative RT-PCR and immunoblot analysis (Fig. 1A, B). GADPH and β-actin amplification were used as a constitutive control. Cells transfected with pCMV-Tag2B/Mxi1 showed strong expression

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http://dx.doi.org/10.5483/BMBRep.2012.45.3.189

Received 8 September 2010, Revised 16 September 2010, Accepted 28 September 2010

Keyword: Cyst formation, mIMCD-3 cell, Mxi1, 3-D culture

and non-transfected cells (control cells) showed weak expression of Mxi1.

Morphogenesis of Mxi1 transfectants in collagen gel

To investigate the effect of Mxi1 on cyst formation, Mxi1 and control transfectants were cultured in the collagen gel. The control plasmid transfectants behaved very much like the wild-type mIMCD-3 cells. Morphologically, the control transfectants also developed cysts when cultured in collagen gel within 9-15 days (Fig. 1C). In contrast, the majority of Mxi1 transfectants developed into cell aggregates and did not form the definitive cysts at the same stage (Fig. 1C). Both control and Mxi1 transfectants showed round cell aggregates and apoptosis of lumen at the initial stage. At the later stage, control transfectants exhibited cell aggregates that grew larger and developed simple round cysts. However, at the same stage, Mxi1 transfectants displayed disrupted cyst morphology and apoptotic cells aggregated in the lining of the cells, consistent with Mxi1 attenuation of development of cyst formation.

Cyst number of control and Mxi1-overexpressed mIMCD-3 cells in 3-D cell culture

To investigate the effect of proliferation, we counted the number of cysts during Mxi1 overexpressed mIMCD-3 cell in 3-D culture. In the mebiol gel, the number of cysts in control cells was 2.5-fold greater at day 5 and 4-fold greater at day 12 than Mxi1 transfectants. In the collagen gel, the number of cysts in control cells was 1.5-fold higher at day 7 and 4-fold higher at day 17 than Mxi1 transfectants (Fig. 2).

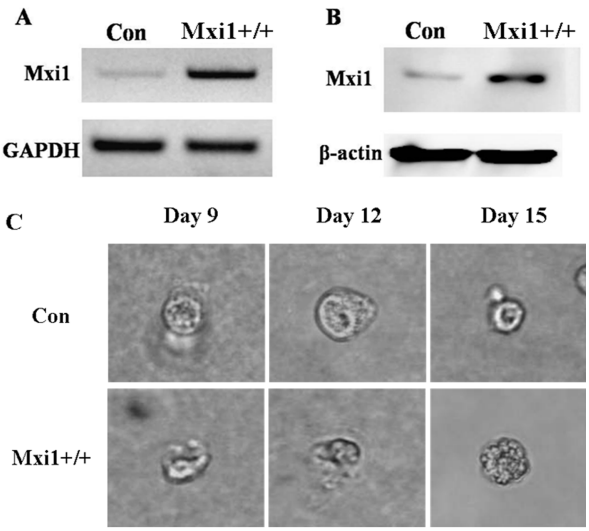

Fig. 1. Morphology of control and stably Mxi1-overexpressed mIMCD-3 cells in 3-D cell culture. (A) RT-PCR of Mxi1 and GADPH in control cells and Mxi1 cells over-expressing the Mxi1 gene. (B) Immunoblot of Mxi1 in control cells and Mxi1 cells over-expressing the Mxi1 gene. β-actin was used as a loading control. (C) Phase-contrast micrographs with magnification of ×400. Upper panel shows a control cell and lower panel shows a Mxi1 over-expressed cell. Each cell was cultured in a collagen gel for 9, 12 and 15 days.

Effect of cell death on Mxi1-overexpressed mIMCD-3 cell in 3-D cell culture

Based on the observation that Mxi1 transfectants did not completely develop simple epithelial cysts, we performed an apoptosis assay with Annexin V and 7-AAD staining using flow cytometry (Fig. 3). We cultured control and Mxi1 transfectants in a 2-D (floor state) as well as 3-D system and compared the percentages of apoptotic cells in the two culture systems. Control and Mxi1 transfectants similarly induced 34-36% apoptosis in 2-D cell culture. In collagen gel, Mxi1 transfectants displayed increased apoptosis (12%) compared to control cells. These results are consistent with the suggestion that Mxi1 regulates the cyst growth of mIMCD-3 cell, with Mxi1 being involved in cyst formation.

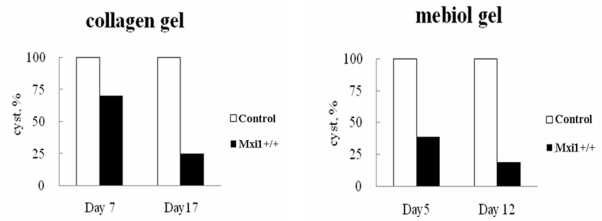

Fig. 2. Effect of cyst number on Mxi1 over-expressing cells in 3-D cell culture. Control and Mxi1 over-expressing cells were cultured in collagen and mebiol gels. The graph shows comparisons of the percentage of cyst number in the early and later stages.

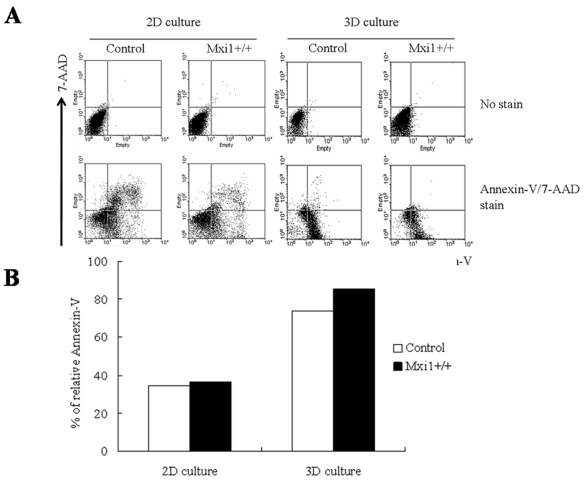

Fig. 3. Effect of apoptosis on Mxi1 over-expressing mIMCD-3 cells in 2-D and 3-D cell culture. (A) Control cell and Mxi1 transfectants were cultured in 2-D or 3-D culture for 7 days. Apoptosis was assayed using double staining with annexin-V and 7-AAD. (B) The graph represents the percentage of apoptotic cells in 2-D or 3-D culture.

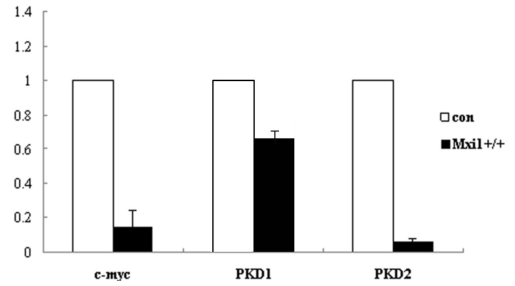

Fig. 4. Validation of genes involved in cyst formation in 2-D and 3-D cell culture. Control and Mxi1 overexpressed cells were cultured in 2-D and 3-D culture for 20 days. RNA was extracted and the expression pattern of genes known to be involved in cystogenesis in humans and mice was monitored by semi-quantitative PCR. The expression of c-myc, PKD1 and PKD2 decreased in Mxi1 overexpressed cells compared with control.

Monitoring expression pattern of genes related cystic disease in the Mxi1 overexpressed cell line

To deduce the molecular mechanisms of fundamental cyst formation in Mxi1 overexpressed cell lines, we monitored the expression pattern of genes known to be involved in cystogenesis in humans and mice. We performed real time PCR using cDNA from Mxi1 and control transfectants (Fig. 4). We cultured the control and Mxi1 transfectants in a 2-D system and compared the expression pattern of the PKD1, PKD2, and c-myc genes. Finally, we confirmed the down-regulation of PKD1 and PKD2 (the major genes responsible for ADPKD) and also confirmed that c-myc mRNA, which is a potent antagonist of Mxi1, decreased in Mxi1 overexpressed cells. c-myc mRNA was decreased 10-fold (0.15 $\pm$ 0.09), PKD1 mRNA was decreased 1.4-fold (0.77 $\pm$ 0.54), PKD2 mRNA was decreased 16-fold (0.07 $\pm$ 0.01) in Mxi1 over-expressed cell lines compared with control. These genes were expressed more in Mxi1-deficient mice than the wild type counterpart.

DISCUSSION

mIMCD-3 cells are polarized, immortalized, mouse renal tubular epithelial cells that undergo cystogenesis with formation of a lumen in 3-D culture (7). This cell line has been widely used to study tubular kidney epithelial morphogenesis *in vitro* (8). Appropriately, we presently used mIMCD-3 cells to study the morphology of cyst formation and tubulogenesis in an *in vitro* system. In our histological experiments, we found that mIMCD-3 cells form a solid aggregate or cell mass in the early stage and developed a cyst cavity later. The cyst cavity became larger as the cyst grew and, once again, clear indications of apoptosis were observed inside of the cyst. Especially, mIMCD-3 cells formed tubule morphology at the later stage (16-20 days) (data not shown). To address cyst formation in ADPKD morphologically, we examined cystogenesis and tubulogenesis assay in an *in vitro* model. Three-dimensional tubulogenesis assays are a well-described model system used to recapitulate the events of branching morphogenesis (4). In a 3-D gel, mIMCD-3 cells can develop a closed lumen, which tends to be enlarged by proliferation and fluid secretion of the lining epithelium or perhaps other mechanisms. It is well established that mIMCD-3 cells can also undergo membrane remodeling to form lumens in response to 3-D gel overlay (9).

Mxi1 is a tumor-suppressor gene that has essential roles in cellular growth control and in the induction and maintenance of the differentiated state. Loss of Mxi1 function contributes to renal cystic disease, further implicating the Myc pathway in normal kidney development (10). From the earlier observations, we expected that cyst formation might be controlled if the Mxi1 gene was adversely overexpressed in cells that originally formed cysts. As expected, cysts did not form well in cells overexpressing the Mxi1 gene. We found that Mxi1 was active in the cyst formation process and confirmed that Mxi1 affected cell growth. When we counted the number of cysts on 3-D culture, the number in Mxi1 transfectants was much less than in control cells, and decreased as time passed. Furthermore, Mxi1 transfectants induced more apoptosis than control cells in 3-D cell culture. Mxi1 overexpression in 3-D culture increased the percentage of mIMCD-3 cell apoptosis and inhibited cyst formation.

We confirmed the down-regulation of PKD1 and PKD2, the major genes responsible for ADPKD in Mxi1 overexpressed cells, and confirmed that c-myc mRNA, which is a potent antagonist of Mxi1, decreases in Mxi1 overexpressing cells. The activity of these genes increased in Mxi1-deficient mice as compared to wild type mice. These results suggest that Mxi1 may be related directly or indirectly to cyst formation through the PKD1 and PKD2 pathways. We conclude that Mxi1 plays an important, as yet undefined, role in cyst formation. This study should spur efforts to clarify the mechanism of cyst formation and the signaling pathway between Mxi1 and other genes.

MATERIALS AND METHODS

Cell line and medium

Inner medullary collecting duct (mIMCD-3) cells obtained from a mouse renal epithelial cell line (American Tissue Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's medium and Ham's F12 medium (DMEM/F12) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere.

Preparation of collagen gel and 3-D cultured

Three-dimensional collagen I gels were made from rat tail collagen (Collaborative Biomedical Products, Bedford, MA, USA) by adding an equal volume of 10× reconstitution buffer (262 mM sodium bicarbonate, 20 mM HEPES) and enough culture medium to make a final gel concentration of 3.8 mg/ml. Immediately, cells were added to the solution at a concentration of 2.5×10^5 to 3×10^5cells/ml and the solution was

transferred into dishes and warmed to 37°C. After 24 h, when gels had formed, culture medium was added. The medium was changed every 2-4 days by replacing part of the volume. It was necessary to culture cells for 8-10 days before morphological changes were noted.

Preparation of mebiol gel and 3-D culture

A flask containing 10 ml of sterile mebiol gel (Mebiol, Yamanashi, Japan) was opened and 10 ml of culture medium were added. The gel was dissolved in the medium at 4-8°C over 3 days. Cell number was adjusted to 2×10^5cells/ml and mixed in the proportion of 1 : 1 of the liquid gel on ice without formation of air bubbles. 500 μl–1 ml of the gel-cell mixture was placed at a pre-warmed 6-well plate. The plate was left inside the CO_2 incubator until the gel solidified. The cells embedded in the gel were checked for their individual dispersal under phase contrast inverted microscopy (Olympus, Tokyo, Japan). Once the gel had solidified, 1-2 ml of complete culture medium was added to cover the solidified gel-cell mixture and the plate was incubated at 37°C in 5% CO_2 atmosphere.

Mxi-1 constructs and establishment of Mxi1 over-expressed mIMCD-3 cell line

The Mxi1 expression vector pCMV Tag2B (Stratagene, La Jolla, CA) was constructed by cloning Mxi1 cDNA isolated from mIMCD-3 cells. Mxi1 CDS constructs were made by PCR amplification with the upstream primer 5′GATCGGATCC (*Bam*HI) ATGCCGAGCCCCCGG3′ and the downstream primer 5′TCG ACTCGAG (*Xho*I) CTAGGACGCGAAGGA GG3′. Reverse transcription-polymerase chain reaction (RT-PCR) products were cut using *Bam*H1 and *Xho*1, gel purified and ligated to a similarly cut pCMV Tag2B vector. The mouse Mxi1 expression plasmid pCMV Tag2B/Mxi1 was transfected into mIMCD-3 cells with Lipofectamine LTX (Invitrogen, Carlsbad, CA), and was selected and maintained with 200 ug/ml of G418 to generate the stable subclones.

Phase contrast image acquisition

Phase contrast images of the mIMCD-3 cysts within the gels were acquired before and after experiments using an Olympus IX70 microscope equipped with 10× and 20× objectives. Images were collected using a cooled CCD camera (Hamamatsu, Shizuoka, Japan) and transferred directly to computer storage using the IPLab software (Scanalytics, Franklin Lakes, NJ).

Semi-quantitative RT-PCR

Total RNA was prepared by using a commercial kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Single-strand cDNA was synthesized by incubating 5μg total RNA with 200 units avian myeloblastosis virus, 100 nM oligo(dT)12-18, 1 mM dNTP mixture and 40 units RNase inhibitor at 42°C for 1 h in a final volume of 25 μl. Reaction was terminated by incubating at 70°C for 15 min. The initial amount of mRNA and the reaction conditions were optimized to obtain linearity for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). RT-PCR cycling conditions for GAPDH and Mxi1 were 10 min at 95°C, 25 cycles of 50 s at 94°C, 50 s at 57°C, 50 s at 72°C and 5 min at 72°C. The amplified products were separated on a 1% agarose gel.

Real time PCR

Total RNA was extracted using a RNeasy Mini kit (Qiagen) according to the instructions of the manufacturer, and treated with DNase at 37°C for 40 min to protect against genomic DNA contamination. RNA was cleaned using an RNeasy kit (Qiagen). Total RNA synthesized cDNA used the QuantiTect® reverse transcription kit (Qiagen). Quantitative- PCR reactions were determined using the real-time PCR SYBR green kit (Qiagen). The primers used were: 18s rRNA, sense 5′-GTAAC CCGTTGCACCCCATT-3′, antisense 5′-CCATCCAATCGGTA GTAGCG-3′; c-myc, sense 5′-GCTTCCCACCCCGCCCCTGT C-3′, antisense 5′-CCACCGCCGCCGTCATCGTCTT-3′; PKD1, sense 5′-GGCCCAACTCAACATCACCGTAAA-3′, antisense 5′-TGTCACAGAGCACCCAGGAGTAAT-3′, PKD2, sense 5′-CTC GCCGTCTCCTCCGCTTTCGTC-3′, antisense 5′-GG CGCCAC TCCACATCCATCTCTA-3′.

Immunoblotting

Expression of Mxi1 in wild-type mIMCD-3 and Mxi1 transfectants was determined by immunoblotting. In brief, cell homogenate protein (40 ug) from specific samples was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically blotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was incubated with mouse antihuman Mxi1 monoclonal antibody and β-actin antibody (BD Biosciences, Franklin Lakes, NJ), and detected with horseradish peroxidase conjugated goat antimouse antibody. The immunocomplexes were detected by enhanced chemiluminescence.

Cell viability and apoptosis analysis

Viable cells were identified using the trypan blue dye exclusion method and counted in a hemocytometer. Apoptotic cell death was analyzed by Annexin V and 7-AAD staining using a FACScan flow cytometer (BD Biosciences). The flow cytometric data were analyzed using Cell Quest software (BD Biosciences).

Acknowledgements

This work was supported by the Sookmyung Women's University Research Grant (2010).

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