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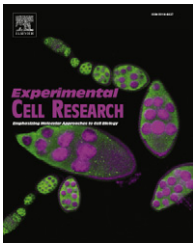

Research Article

Transforming growth factor-β inhibits cystogenesis in human autosomal dominant polycystic kidney epithelial cells

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited cause of kidney failure and characterized by the formation of multiple fluid-filled cysts in the kidneys. It is believed that environmental factors may play an important role in the disease progression. However, the molecular identity of autocrine/paracrine factors influencing cyst formation is largely unknown. In this study, we identified transforming growth factor-β2 (TGF-β2) secreted by normal human kidney (NHK) and ADPKD cells as an inhibitor of cystogenesis in 3D culture system using ADPKD cells from human kidneys. TGF-β2 was identified in conditioned media (CM) of NHK and ADPKD cells as a latent factor activated by heat *in vitro*. While all TGF-β isoforms recombinant proteins (TGF-β1, -β2, or -β3) displayed a similar inhibitory effect on cyst formation, TGF-β2 was the predominant isoform detected in CM. The involvement of TGF-β2 in the suppression of cyst formation was demonstrated by using a TGF-β2 specific blocking antibody and a TGF-β receptor I kinase inhibitor. TGF-β2 inhibited cyst formation by a mechanism other than activation of p38 mitogen-activated protein (MAP) kinase that mediated cell death in ADPKD cells. Further, we found that TGF-β2 modulated expression of various genes involved in cell–cell and cell–matrix interactions and extracellular matrix proteins that may play a role in the regulation of cystogenesis. Collectively, our results suggest that TGF-β2 secreted by renal epithelial cells may be an inhibitor of cystogenesis influencing the progression of ADPKD.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder characterized by the progressive formation of multiple cysts leading to massive kidney enlargement and loss of function. Pathological manifestations of ADPKD also include extra-renal cyst formation in the liver and pancreas, aneurysms and vascular abnormalities [1,2]. Renal cysts develop within existing tubules, which enlarge progressively, forming fluid-filled cavities lined by epithelial cells [3]. Progressive expansion of cysts is associated with multiple different features such as aberrant cell proliferation and apoptosis, defective polarity, modification of cell adhesion and cell contacts, dedifferentiation of epithelial cells, activation of trans-epithelial fluid secretion, extracellular matrix remodeling, renal inflammation and interstitial fibrosis [2,4,5]. ADPKD displays considerable genotypic and phenotypic variations that are thought to originate from the expression of modifying factors modulated by the genetic background and the presence of extracellular factors in the environment. As a result, ADPKD exhibits extensive

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Abbreviations: CM, conditioned media; HCM, heated conditioned media; NHCM, non-heated conditioned media; NHK, normal human kidney

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intrafamilial variance in humans and variability ranging from neonatal death to adequate adult kidney function [6].

Almost all known cases of ADPKD derive from mutations in *PKD1* or *PKD2* genes that encode for polycystin-1 and -2 [6]. Studies using metanephric organ cultures demonstrate that cAMP signaling has a synergic effect with activity of genes causing cyst formation [7,8]. Also, multiple signaling pathways are perturbed in ADPKD cells that may contribute to cyst formation and growth. Identification of aberrant active signaling molecules may lead to potential therapeutic targets for human ADPKD [4,7,9,10]. Animal models of ADPKD reveal that *PKD1* inactivation during kidney development results in rapid, massive cystic kidney disease and lethality. However, postnatal *PKD1* inactivation induces focal renal cysts and progressive kidney disease, which indicate that *PKD1* inactivation and cellular context can modulate the development of ADPKD and paracrine mechanisms likely have an important role in cystogenesis [11–13].

In the present study, we examined the effect of endogenous factors secreted by tubular epithelial cells on cyst formation. For this purpose, we used conditioned media (CM) from monolayers of normal human kidney (NHK) and ADPKD epithelial cells from human kidneys in primary cultures. The effect of CM was tested in a model of cyst formation using human ADPKD cells in 3D cultures that has been previously characterized in our laboratory [14,15].

Materials and methods

Cells and 3D cultures

NHK cells are derived from tubules in the cortex and ADPKD epithelial cells from cyst walls of normal and ADPKD kidneys, respectively. The procedure for isolation and extensive characterization of these cells was published previously [15]. The primary cell cultures were propagated in growth media composed with MEMα (Invitrogen, Carlsbad, CA) supplemented with 10 ng/ml epidermal growth factor, 5 μg/ml hydrocortisone (Sigma, Saint Louis, MO), 6.25 μg/ml insulin, 6.25 μg/ml transferin, 6.25 ng/ml selenous acid (BioWhittaker, Walkersville, ME), 10% (v/v) heat inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1% antibiotic/antimycotic solution (Invitrogen) as previously described [15]. Treatment of ADPKD cells in monolayer cultures was performed in 35 mm 6 well plates. Cells were in incubated in growth media supplemented without or with 25 ng/ml prostaglandin E2 (PGE_2, Sigma) and SB203580 (Calbiochem, Gibbstown, NJ) as indicated in Fig. 4. DAPI (4′-6 diamino-2 phenylindole) staining was performed as previously described [15]. Cell cultures in 3D were established by suspension of ADPKD cells (0.5×10^5 cells/well) in a 4 °C solution containing complete MEMα and a 1:1 (v/v) mixture of liquid rat tail collagen I and Matrigel (Becton Dickinson, Franklin Lakes, NJ) on cell culture inserts [14,15]. The formation of cysts in 3D cultures of ADPKD cells was induced in growth media by 25 ng/ml PGE_2 as previously established [14]. Media and treatments (including CMs) were changed every 2–3 days and cultures were maintained during a period of 11–17 days in different experiments. Cell cultures were photographed in different field of views at magnification ×40 and cysts (with surfaces determined above a cut-off of at least 5 mm^2) were counted using a SPOT 4.6 (Diagnostic Instruments, Sterling Heights, MI) or Image-Pro 6.3 (Media Cybernetics, Bethesda, MD) software and calculations were performed using Prism 3.00 software (GraphPad, San Diego, CA). The procedures for cells from human kidneys were in accordance with the ethical standards of the Institutional Review Board of The University of Oklahoma Health Sciences Center that approved our study protocols.

Preparation of conditioned media

NHK and ADPKD cells were grown to confluence in 75 cm^2 flasks. Cells were washed with PBS and incubated in 12 ml of MEMα medium serum-free, supplement-free, antibiotic-free medium for 3 days. The conditioned-serum free media was removed and centrifuged to separate supernatant from free-floating cells and debris and frozen for storage. Heat activation of CM was performed by incubation for 30 min at 80 °C, stopped on ice and followed by storage at 4 °C until use. For western blot analysis, concentration of CM was achieved on centrifugal devices with Macrosep and Nanosep 30 K omega membranes (Pall, Port Washington, NY) and sterilized with 0.2 μm HT Tuffryn membranes (low protein binding, Pall) that have minimal effect on loss of the inhibitory activity on cyst formation. The effect of addition of 25% (v/v) CM to growth medium in 3D cultures was compared to 25% (v/v) non-conditioned MEMα medium without additive (defined as serum-free medium, SFM) used as control. Throughout our study, we used CM from NHK cells unless compared to CM from ADPKD cells.

TGF-β activity

Recombinant transforming growth factor (TGF)-β1, -β2 and -β3, rabbit anti-TGF-β2 (AB-12-NA) and mouse anti-TGF-β3 (MAB243) were obtained from R&D Systems (Minneapolis, MN). TGF-β RI kinase inhibitor (#616451) was purchased from Calbiochem. Immunoassay kit for TGF-β1 was purchased from Assay designs-Enzo Life Sciences (Farmingdale, NY). This assay measures the active form of mature TGF-β1 released from the latency-associated peptide.

Western blot analysis

To analyze TGF-β isoform expression, CM samples were approximately concentrated ×200, and solubilized in non-reducing sample buffer (62.5 mM Tris–HCl, pH 6.8, 2%SDS, 3 M urea, 20% glycerol) at 65 °C for 20 min. Phosphorylation of p38 and p38 expression was analyzed following treatment of ADPKD cells in monolayers as indicated in figure legend. Cells were solubilized in sample buffer supplemented with 1% mercaptoethanol, sonicated and heated at 65 °C. Proteins were subjected to SDS-PAGE 8–10% and immunoblot analysis was performed with primary antibodies [see previous section for TGF-β antibodies used at 1 μg/ml, phospho-p38 MAPK (Thr180/Tyr182) antibody#9211 (1:2000, Cell signaling, Danvers, MA), p38 MAPK (1:200, Santa Cruz, Santa Cruz, CA)], and anti-rabbit or anti-mouse secondary antibodies coupled to horseradish peroxidase (1:20000, Jackson, West Grove, PA). Detection was performed with an enhanced chemiluminescence detection system (Pierce, Rock Grove, IL).

Real time PCR analysis

mRNA was extracted from 3D gels using the TRIzol method (Invitrogen). mRNA analysis was performed using reverse transcription (RT) real-time PCR analysis and SYBR Green (Applied Biosystems, Foster City, CA) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as previously described