Activation of the MEK5/ERK5 Cascade Is Responsible for Biliary Dysgenesis in a Rat Model of Caroli’s Disease

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Polycystic kidney (PCK) rats exhibit a multiorgan cyst pathologic similar to human autosomal recessive polycystic kidney disease, and are proposed as an animal model of Caroli’s disease with congenital hepatic fibrosis (CHF). This study investigated the expression and function of selected components of the mitogen activated protein kinase (MAPK) pathway in cultured intrahepatic biliary epithelial cells (BECs) of PCK rats. Compared to the proliferative activity of cultured BECs of control rats, those of the PCK rats were hyperresponsive to epidermal growth factor (EGF). The increase in BEC proliferation was accompanied by overexpression of MAPK/extracellular signal-regulated protein kinase (ERK) kinase 5 (MEK5), and subsequent phosphorylation of ERK5 in vitro. The increased proliferative activity was significantly inhibited by the transfection of short interfering RNA against MEK5 mRNA. An EGF receptor tyrosine kinase inhibitor, gefitinib (“Iressa”, ZD1839), also significantly inhibited the abnormal growth of cultured BECs of PCK rats. By contrast, treatment with PD98059 and U0126, inhibitors for MEK1/2, was less effective. These results suggest that the activation of the MEK5-ERK5 cascade plays a pivotal role in the biliary dysgenesis of PCK rats, and also provide insights into the pathogenesis of Caroli’s disease with CHF. As the MEK5-ERK5 interaction is highly specific, it may represent a potential target of therapy. (Am J Pathol 2005, 166:49–60)
an imbalance between cell proliferation and apoptosis might be responsible for the aberrant proliferation of BECs of PCK rats in vivo.

Epithelial cell proliferation and differentiation are modulated by various cytokines and growth factors. Epidermal growth factor (EGF) is a major participant in cellular events including proliferation and differentiation. By binding to a prototype transmembrane tyrosine kinase receptor (EGF receptor, EGFR), EGF activates the mitogen activated protein kinases (MAPKs), which relay signals from the cell membrane to the nucleus. The MAPK pathway consists of three protein kinases that act sequentially within a pathway: a MAPK kinase kinase, a MAPK/ERK kinase (MEK), and a MAPK (ERK). The first and best studied is the MAPK pathway which consists of Raf-1 or B-Raf, MEK1/2, and ERK1/2. Recently, the novel MEK5-ERK5 pathway has been implicated in the regulation of cellular proliferation by acting with EGF. That is, EGF activates ERK5, and this activation requires the direct and specific upstream activator, MEK5.22

In murine ARPKD models, abnormal expression of EGF and EGFR has been implicated in the pathogenesis of cysts in the kidneys.16,23–25 ERK1/2 has been shown to be activated in renal epithelial cells within cysts in a rat model of autosomal dominant PKD (ADPKD).26 In addition, Nauta et al27 demonstrated that BECs obtained from mice with ARPKD were hyperresponsive to EGF. These findings suggest that activation of the MAPK pathway is critically involved in the development of cystic changes in the liver of PCK rats. To date, however, few studies have examined the pathogenesis associated with the extrahepatic cystic organs in PKD, including the liver.

Using the PCK rat as a model of Caroli’s disease with CHF, the involvement of the MAPK pathway in the development of biliary dysgenesis was examined in this study.

Materials and Methods

Animals

Crlj:CD (control) and PCK rats were purchased from Charles River Japan (Sagamihara, Japan), and a colony of PCK rats was maintained at the Laboratory Animal Institute of Kanazawa University School of Medicine, Kanazawa, Japan. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Takara-machi Campus of Kanazawa University.

Antibodies

The antibodies used were anti-cytokeratin (CK)-7 (clone OV-TL 12/30, mouse monoclonal, Dako, Glostrup, Denmark), anti-γ-glutamyl transpeptidase (γ-GTP) (clone 5B9, mouse monoclonal, Cosmo-Bio, Tokyo, Japan), anti-vimentin (clone V9, mouse monoclonal, Dako), anti-EGF (sc-1343, goat polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-EGFR (sc-03, rabbit polyclonal, Santa Cruz), anti-mast cell tryptase (AA1, mouse monoclonal, Dako), anti-MEK1/2 (sc-436, rabbit polyclonal, Santa Cruz), anti-phosphospecific (p)-MEK1/2 (sc-7995R, rabbit polyclonal, Santa Cruz), anti-MEK5 (KAP-MA003, rabbit polyclonal, Stressgen, San Diego, CA), anti-ERK1/2 (KAP-MA001, rabbit polyclonal, Stressgen), anti-p-ERK1/2 (44–680, rabbit polyclonal, Bio-source International, Camarillo, CA), anti-ERK5 (KAS-MA002, rabbit polyclonal, Stressgen), anti-p-ERK5 (E7153, rabbit polyclonal, Sigma, St. Louis, MO), and anti-Ki-67 protein (MIB-5, mouse monoclonal, Immunotech, Marseille, France).

Cell Culture of Rat Intrahepatic BECs

Eight-week-old male rats were used. The isolation and passage culture of intrahepatic BECs were performed as described previously.28 Briefly, the livers were first perfused with Hanks’ balanced salt solution without (Ca2+1) and (Mg2+1) [HBSS(−)] via the portal vein trunk, and then with Dulbecco’s modified Eagle’s medium and Ham F-12 (DMEM/F-12) containing 0.04% collagenase (collagenase S-1, Nitta Zeratin, Osaka, Japan) and 0.22% dispase (Life Technologies, Grand Island, NE) for 20 minutes. After removal of the digested hepatic parenchyma, several tissue fragments were cut from parts of the intrahepatic large bile ducts, and then floated for 48 hours in DMEM/F-12 medium (standard medium) containing 10% Nu-Serum V culture supplement (Becton Dickinson, Bedford, UK), forskolin (2.1 μg/ml, Wako Pure Chemical Industries, Osaka, Japan), EGF (20 ng/ml, Up-state Biotechnology, New York, NY), and antibiotics in a 5% CO2 incubator. The tissue fragments were then placed as an explant on type I collagen gel (Cellmatrix Type I-A, Nitta Zeratin), and cultured in a 5% CO2 incubator.

BECs on collagen gel proliferated and spread from these explants. Areas of this sheet composed entirely of intrahepatic BECs were cut out and placed on other collagen gels for subculturing. Subcultured BECs on gels were fed with standard medium in a 5% CO2 incubator, and passaged every 3 weeks. After four passages, the cells were scraped off and placed in DMEM/F-2 medium containing 0.04% collagenase and 0.22% dispase. After centrifugation, the pellets were added to HBSS(−) and cells were set on collagen-1-coated plastic bottles for 3 weeks until confluent. Then, cultured cells were scraped off and stored for the following experiments.

Characterization of Cultured BECs

BECs cultured on collagen gel for 72 hours were fixed in 10% neutral-buffered formalin, and cut into several slices which were embedded in paraffin. Then, several sections, 3-μm thick, were cut and deparaffinized, and stained with HE and alcian blue at pH 2.5 for acid glyco-protein.

The BECs cultured on collagen-coated cover-glass (Sumitomo Bakelite, Tokyo, Japan) for 2 days were fixed in cold acetone for 10 minutes. After pretreatment with blocking reagent (Dako), primary antibodies against
Table 1. Sequences of the Primers and PCR Conditions Used in this Study

<table>
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<tr>
<th>Gene</th>
<th>Sequences (5’-3’)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>PCR cycles</th>
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<tr>
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<tr>
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<td>910</td>
<td>60</td>
<td>25</td>
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Abbreviations: MEK5, mitogen-activated protein/extracellular signal-regulated kinase 5; TK, thymidine kinase; TGFR-β, transforming growth factor-β; TGFR1, TGFR-β receptor type 1; EGF, epidermal growth factor; EGFR, EGF receptor; bFGF, basic fibroblast growth factor; GPx3, glutathione peroxidase.

CK-7 (1:100), γ-GTP (1:50), and vimentin (1:50) were applied. Then, fluorescein isothiocyanate-labeled secondary polyclonal antibody against mouse IgG (Srotec, Raleigh, NC) was applied, and the sections were counterstained with propidium iodide (Vector Laboratories, Burlingame, CA). They were examined under a confocal laser microscope (LSM 410, Carl Zeiss, Gottingen, Germany). CK-7 and γ-GTP are known as biliary epithelial cell markers, while vimentin is a mesenchymal cell marker.

cDNA Microarray Analysis

BECs cultured in the presence of EGF (20 ng/ml) at the subconfluent stage were used, and a cDNA microarray assay was performed once. Total RNA was isolated from BECs using a RNA extraction kit (RNaseasy Mini Kits, Qiagen). Digestion with DNase was performed using a RNase-Free DNase Set (Qiagen). The RNA was dissolved in RNase-free water. The purity and integrity of the RNA were examined spectrometrically, and the concentration was adjusted to 10 µg/µl.

Using 19 µl of RNA sample, cDNA synthesis was performed with the use of an Atlas Glass Fluorescent Labeling Kit (Clontech Company, Tokyo, Japan) according to the manufacturer’s instructions. The cDNA obtained was labeled with the Cy3 dye using the Atlas Glass Fluorescent Labeling Kit. Cy3 dye (Cy3 Mono-Reactive Dye Pack) was purchased from Amersham Biosciences (Tokyo, Japan). Cy3 fluorescent-labeled cDNA samples were then hybridized with the Atlas Glass Rat 1.0 Microarray (Clontech), which consisted of 1081 unique probes arrayed onto glass slides, according to the manufacturer’s instructions.

The fluorescence generated by Cy3 immobilized at the target sequence on the slides was measured using a scanning system, GenePix 4000B (InterMedical, Co., Nagoya, Japan). The fluorescent intensity of each plot was analyzed with image processing software (GenePix Pro 3.0.6, InterMedical). The intensity was corrected by the expression of reference genes (housekeeping genes) and by subtracting background signals. When the fluorescent signals of an individual gene were increased over 2.0-fold and decreased by more than half, the gene was determined to be overexpressed and underexpressed, respectively.

Quantitative and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA (1 µg) extracted from the liver and cultured BECs was used to synthesize cDNA with reverse transcriptase (Reverse Transcriptase XL, Takara Biochemicals, Ohtsu, Japan). The sequences of the primers and conditions for PCR used are shown in Table 1. Amplification was performed in a total volume of 25 µl containing 1 µl of cDNA, 0.2 mmol/L dNTPs, 1 µmol/L each of 5’- and 3’-primers, and 2.5 U of TaqDNA polymerase (Takara EX Taq, Takara Biochemicals). For each reaction, an initial denaturation cycle of 94°C for 3 minutes and a final cycle of 72°C for 10 minutes were incorporated. The PCR products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. Semiquantitative analysis of the gel images was performed using the public domain NIH image software in the exponential range of each PCR amplification. Fold difference compared with β-actin expression was calculated.

For the determination of MEK5 and EGFR mRNA expression, real-time quantitative RT-PCR was performed using pre-made MEK5 (FAM), EGFR (FAM), and β-actin (FAM)-specific primers and the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Warrington, UK). RT-PCR was carried out with the TaqMan Universal RT-PCR Master Mix (PE Applied Biosystems)
using 5 μl of cDNA in a 25-μl final reaction mixture. Cycling conditions were incubation at 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fold difference compared with β-actin expression was calculated.

Western Blot Analysis

Proteins were extracted from cultured BECs using T-PER Tissue Protein Extraction Reagent (Pierce Chemical Company, Rockford, IL), and total protein was measured spectrometrically. First, 100 μg of the protein was subjected to 10% SDS-polyacrylamide electrophoresis, and then electrothoretically transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibodies against MEK1/2 (1:1000), p-MEK1/2 (1:100), MEK5 (1:100), ERK1/2 (1:1000), p-ERK1/2 (1:200), ERK5 (1:500), and p-ERK5 (1:100). The protein expression was detected using secondary antibody conjugated to peroxidase-labeled polymer, EnVision+ system (Dako). 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen.

WST1 Proliferation Assay and MAPK Pathway Inhibition Studies

The proliferative activity of BECs was assessed using a WST1 assay according to the manufacturer’s instructions (Roche, Mannheim, Germany). The WST-1 assay is a formazan-based colorimetric assay which has been frequently used for the determination of in vitro cell proliferative activity.28 In addition, to confirm that the proliferative activity assessed by the WST-1 assay can be regarded as an indicator of the actual cell number, an equal number of BECs obtained from control and PCK rats was seeded on collagen gels in 35-mm dishes. After a 48- and a 96-hour incubation with the standard medium containing 20 ng/ml of EGF, they were scraped off as a whole, and digested in the collagenase dissolution medium in test tubes. They were centrifuged, and the pellets were collected. After 0.05% crystal violet solution was added, the cells were lysed with a mixer, and the nuclei were counted using a hemocytometer. For comparison, the WST1 assay was performed as described below in a 96-well format.

A total of 1250 cells per well were used in a 96-well collagen-coated plate. After 24-hour preincubation with the standard medium, the medium was exchanged for the signal-containing medium, the medium was exchanged for the standard medium, the medium was exchanged for the signal-containing medium, the proliferation of Ki-67-positive cells was assessed. That is, more than 500 BECs were surveyed and the percentage of Ki-67-positive BECs was defined as the Ki-67 labeling index in each rat.

Immunohistochemistry

Immunohistochemistry was performed with primary antibodies against EGF, EGFR, p-ERK1/2, p-ERK5, and Ki-67. Formalin-fixed, paraffin-embedded liver sections, 4-μm thick, were prepared from rats at 3 weeks, 2 months, and 10 months of age. After deparaffinization, antigen retrieval was performed for p-ERK1/2, p-ERK5, and Ki-67 protein by microwaving in 10 mmol/L citrate buffer, pH 6.0. After the blocking of the endogenous peroxidase, the sections were incubated overnight at 4°C with individual primary antibodies: anti-EGF (1:400), anti-EGFR (1:50), anti-p-ERK1/2 (1:200), anti-p-ERK5 (1:100), and anti-Ki-67 protein (1:50). Then, the sections were incubated with secondary antibodies conjugated to peroxidase-labeled polymer, EnVision+ system (Dako) or Histofine Simple Stain MAX PO(G) (Nichirei, Tokyo, Japan). Color development was performed using 3,3’-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin.

Ki-67-positive cells were quantitatively assessed. That is, more than 500 BECs were surveyed and the percentage of Ki-67-positive BECs was defined as the Ki-67 labeling index in each rat.

Double-immunostaining for EGF and mast cell tryptase was conducted as follows; the deparaffinized sections were incubated overnight at 4°C with anti-mast cell tryptase (1:50), and then incubated using the EnVision+ system (Dako). Color development was performed using the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories). Then, the detection of EGF was performed as above. Similarly, double-immunostaining for EGFR and Ki-67 protein was performed using primary antibodies against EGFR and Ki-67 protein, and color development was performed using the benzidine reaction for EGFR and Vector blue reaction for Ki-67 protein.

Control sections were evaluated by substitution of the primary antibodies with nonimmunized serum, which resulted in no signal detection. For the determination of EGF expression, additional control sections were incubated with the primary antibody absorbed with the respective peptide antigen.

Statistics

The means and SEM were calculated for all parameters determined. Statistical significance was evaluated by us-
ing analysis of variance or Mann-Whitney’s U-test. \( P < 0.05 \) was accepted as statistically significant.

**Results**

**Characteristics of Cultured BECs**

The dilatation of bile ducts spread throughout the liver of PCK rats, involving both intrahepatic large and small bile ducts. Since the dilatation of intrahepatic large bile ducts has been regarded as an essential feature of Caroli’s disease, BECs were isolated from intrahepatic large bile ducts, and cultured for the analysis. Isolated and cultured intrahepatic BECs from the livers of both control and PCK rats proliferated and spread as an epithelial cell sheet on the collagen gel (Figure 1A). Histologically, the cultured BECs after four passages, which were used for the subsequent experiments, showed a monolayer of cuboidal epithelial cells on the collagen gel. An oval or round nucleus was located in the middle of the cytoplasm of BECs, and acid glycoprotein was clearly detected on the apical cell surface (data not shown). CK-7 and \( \gamma \)-GTP were spread diffusely in the cytoplasm of cultured BECs (Figure 1, B and C), while vimentin was absent. These histological and phenotypic features were similar in the cultured BECs of control and PCK rats.

**cDNA Microarray Analysis of Cultured BECs**

The overall differences in gene expression between cultured BECs of control and PCK rats were examined using a cDNA microarray analysis. The vast majority of the 1081 genes examined were expressed to a similar degree in both rats. In the PCK rat, the expression of 18 genes was enhanced by at least twofold, and the expression of three genes was reduced by half or less compared with the control. The 21 differentially expressed genes are listed in Table 2. Several of these genes were associated with cell proliferation, apoptosis, and/or fibrotic processes. These included the transcripts encoding MEK5, thymidine kinase (TK), junD, transforming growth factor (TGF)-\( \beta \), TGF-\( \beta \) receptor type 1 (TGFR1), basic fibroblast growth factor (bFGF), and plasma glutathione peroxidase (GPx3) (Figure 2A). Quantitative (Figure 2B) and semi-quantitative (Figure 2C) RT-PCR analysis were further added to confirm the differentially expressed genes of interest.

Among the products of the 21 differentially expressed genes, MEK5 is a component of the MAPK pathway, which is typically involved in epithelial cell proliferation by acting with EGF. In the following experiments, therefore, we focused on selected components of the MAPK pathway.

**Expression of EGF and EGFR**

EGF was immunohistochemically localized in the mononuclear cells around the relatively large bile ducts in control rats and around dilated bile ducts of all sizes in PCK rats (Figure 3, A and B). There was a tendency for more EGF-positive cells to accumulate around dilated large bile ducts rather than small ones in the PCK rat. EGF-positive cells were not seen in the small portal tracts of the control rat. The signals were diminished when the sections were incubated with the primary antibody absorbed with the respective peptide antigen, suggesting...
the specificity of positive signals for EGF. Furthermore, RT-PCR disclosed that EGF mRNA was similarly detected in the liver at 3 weeks, 2 months, and 10 months of age and also similarly in the control and PCK rats (Figure 4, A and B). Double-immunostaining demonstrated that EGF-positive cells were also positive for mast cell tryptase, that is, mast cells, in both rats (Figure 3B, inset).

The expression of EGFR in the liver sections was seen on almost all BECs as well as hepatocytes in both rats (Figure 3, C and D). The subcellular localization of EGFR on BECs was mostly basolateral (Figure 3, C and D, insets), and positive EGFR signals were also focally seen on apical surfaces. Hepatocytes showed cell membrane expression presenting a honeycomb appearance in both rats. Consistent with the results of immunohistochemistry, EGFR mRNA was similarly detected in liver tissue of control and PCK rats at different ages by RT-PCR (Figure 4, A and B), and was also detected in cultured BECs of control and PCK rats to the same degree as in the quantitative RT-PCR analysis (Figure 2B). Thus, the degree and localization of immunohistochemical expression of EGFR and EGFR in BECs and hepatocytes were similar at the different ages (3 weeks, 2 months, and 10 months) of both rats. Double immunostaining of EGFR and Ki-67 expression disclosed that while almost all BECs of both rats expressed EGFR, a few and a considerable number of these BECs were positive for Ki-67 in control and PCK rats, respectively (see below).

**Proliferative Activity of Cultured BECs and Effects of EGF**

As for the relation between the WST-1 assay and cell proliferative activity, the counted cell number was identical to the value measured with the WST-1 assay in both control and PCK rats at 48 and 96 hours after the seeding of cultured BECs (Figure 5, A and B), confirming that the measured value obtained using WST-1 reagent is directly proportional to the cell number, and thus, the proliferative activity assessed by WST-1 assay can be regarded as an indicator of the actual cell number in this study.

The proliferative activity of the cultured BECs of both rats increased dose-dependently in response to EGF (Figure 5C). Compared to the proliferative activity of BECs of control rat, a significant increase in that of PCK rats was observed on the stimulation with EGF at concentrations of 20 and 200 ng/ml. In the subsequent experiments in vitro, the concentration of EGF in the culture medium was adjusted to 20 ng/ml.

**Expression of Selected Components of the MAPK Pathway**

The expression of MAPK kinases (MEK1/2, p-MEK1/2, and MEK5), and MAPks (ERK1/2, p-ERK1/2, ERK5, and p-ERK5) in the BECs was examined by Western blot analysis. Under the stimulation with EGF, overexpression of MEK5 was observed in cultured BECs of the PCK rat (Figure 6), which corresponded with its gene expression.
Accordingly, the expression of p-ERK5 was increased in the PCK rat. Although the expression of MEK1/2, ERK1/2, and ERK5 was similarly detected in cultured BECs of both rats, the expression of p-MEK1/2 and p-ERK1/2 was faint or invisible (Figure 6, data not shown for p-MEK1/2). Immunohistochemically, no positive signals for p-ERK1/2 were observed in interlobular bile ducts of control rats, while a few positive nuclear signals were seen in the bile duct epithelium of PCK rats (Figure 7, A).
and B). Positive signals for p-ERK5 were spread diffusely in the nuclei of BECs and hepatocytes in both control and PCK rats, and the signal intensity in BECs was remarkably higher in the PCK rat (Figure 7, C and D). This increased expression of p-ERK5 in BECs of the PCK rat was observed at the different ages of the rats. The Ki-67 labeling index of BECs was continuously higher in the PCK rat at the different ages, with a value of 10.5%, 8.2%, and 13.5% at 3 weeks, 2 months, and 10 months of age, respectively, when compared to the index of the control rats (2.1%, 0.5%, and 0.3% at 3 weeks, 2 months, and 10 months of age), suggesting that p-ERK5 overexpression was involved in cell proliferative activity in the PCK rat.

**In Vitro MAPK Pathway Inhibitor Experiments**

Gefitinib, an EGFR tyrosine kinase inhibitor, significantly inhibited the proliferative activity of BECs of both rats in a dose-dependent fashion (Figure 8A). Both PD98059 and U0126, inhibitors for MEK1/2, were less effective in the inhibition of BECs from both rats (Figure 8, B and C). The EGF-induced proliferative activity of BECs of the PCK rat was significantly inhibited by the transfection of siRNA against MEK5 mRNA (Figure 8D). Western blot analysis confirmed that siRNA against MEK5 mRNA blocked the protein expression in the cultured BECs of PCK rats (Figure 8E).

![Figure 6](image1.png)

**Figure 6.** Western blot analysis of the selective components of the MAPK pathway in cultured BECs of control and PCK rats. BECs were cultured in the presence of EGF (20 ng/ml), and 100 μg of total protein extracted from BECs was used. Overexpression of MEK5 and of p-ERK5 was observed in cultured BECs of PCK rats in comparison with control rats. There were no differences in the expression of MEK1/2, ERK1/2, and ERK5, and p-ERK1/2 was not detected in either of the rats.

![Figure 7](image2.png)

**Figure 7.** Immunohistochemical analysis of the expression of p-ERK1/2 (A and B) and p-ERK5 (C and D) in liver sections of 3-week-old rats. No positive signals for p-ERK1/2 were observed in the portal area of control rats (A), while a few positive nuclear signals were seen in dilated bile duct epithelium of the PCK rat (B, arrowheads). Positive signals for p-ERK5 were diffusely seen in the nuclei of control (C) and PCK (D) rats, and the signal intensity in BECs was greater in PCK rats (D) than in control rats (C). Higher magnifications of bile ducts are given in the insets (A, C, and D). Arrows indicate interlobular bile ducts. *, denotes bile duct lumen. Original magnification: ×200 (A, C, and D), ×400 (B), insets, ×400.
dent apoptosis. The overexpression of transcripts encoding TGF-β is known to induce fibrogenesis in the liver. The overexpression of the transcript encoding TGF-β3 as well as one of the other fibrogenic growth factors, bFGF, in the PCK rat indicates that these growth factors are involved in the progressive portal fibrogenesis. Maser et al reported that various organs including the kidney of mice with ARPKD expressed extracellular GPx, an antioxidant enzyme, and the enzymatic activity of GPx was reduced in kidneys and blood plasma of ARPKD mice. It is considered that GPx reduces excessive oxidative stress and protects tissues from the deleterious effects of reactive oxygen species. Recent findings suggest that intracellular oxidants are involved in the induction of apoptosis. The reduced expression of GPx3 mRNA in the BECs of the PCK rat may lead to excessive oxidative stress, promoting apoptosis. Although the discussion concerning the results of the cDNA microarray analysis remains speculative, the imbalanced cell kinetics including enhanced apoptosis in the BECs of the PCK rat may be related to the dilatation of intrahepatic bile ducts.

There have been a number of studies on the role of EGF and EGFR in the development of the renal cystic pathology. According to previous reports, EGF expression is generally diminished in rodent models of PKD, while EGFR in the kidneys is overexpressed in murine PKD, and human PKD. EGF inhibits developmentally regulated apoptosis in the kidney, and neonatal injections of exogenous EGF have been shown to inhibit cystic enlargement and/or renal dysfunction in infantile forms of PKD indicating the early protective effects of EGF and the involvement of apoptosis in these effects. However, continued administration of EGF during the postnatal period has been shown to accelerate the disease. Similarly, there have been studies showing that promotion of proliferation by members of the EGF family and/or excessive stimulation of EGFR also can increase cell proliferation and therefore exacerbate the progression of PKD. Diminished renal EGF may be an early contributor to PKD by inhibiting collecting duct differentiation, and leading to an up-regulation of EGFR that affects disease progression, but the precise role of EGF and EGFR in the renal cystic pathology and progression of PKD is still unclear.

This study showed that EGFR mRNA expression was observed to the same degree in the cultured BECs of both control and PCK rats by cDNA microarray and quantitative RT-PCR analysis. EGFR mRNA was also detectable in the liver tissue of control and PCK rats, and EGFR was similarly expressed immunohistochemically on the cell membrane of almost all BECs of both control and PCK rats. However, Ki-67 was detected in a few and a considerable number of these BECs in control and PCK rats, respectively, suggesting that another factor(s) in addition to EGFR expressed on BECs is important in the proliferation of BECs of the PCK rat. In this study, EGF mRNA was detected in the liver of control and PCK rats by RT-PCR and EGF was shown to be located in mast cells around the bile ducts by double-immunostaining. These EGF-expressing mast cells were rather dense around small and large bile ducts of PCK rats in compar-
ison with control rats in which EGF-positive mast cells were seen around the large bile ducts but not the small portal tracts, suggesting that these EGF-positive cells are involved in the proliferation of BECs and cystic dilatation of bile ducts in the PCK rat. Although mast cells are known for their role in allergic reactions, there are also nonimmune-related roles of mast cells that predominate in connective tissue. These nonimmune mast cells are a source of various growth factors, thereby participating in cell differentiation and the synthesis of extracellular matrix molecules. Recently, mast cells have been shown to express abundant EGF mRNA in the rat. The interaction of EGF and EGRF was shown in isolated BECs of rats, suggesting that mast cell-derived EGF and EGRF expressed on BECs is critically involved in the cystic bile duct enlargement in the PCK rat. In addition, EGF in the bile and plasma, which mainly originates from submammary glands and has been implicated in liver pathophysiology, may relate to the stimulation of BEC growth.

Nauta et al demonstrated that BECs from BALB/c-bpk mice showed increased sensitivity to the proliferative effect of EGF. In other reports, renal cyst epithelial cells were hypersensitive to EGF-induced mitogenesis in some but not all, cases. Nagao et al showed that activation of the MAPK pathway, including B-Raf and ERK1/2, in renal cyst epithelial cells might be responsible for the development of cysts in ADPKD. In our experiments, BECs of the PCK rat were hyperresponsive to EGF, and this effect was mediated primarily by the overexpression of MEK5, which subsequently led to the increased phosphorylation of ERK5, rather than by the activation of the MAPK pathway consisting of MEK1/2 and ERK1/2. The findings obtained in this study that p-ERK5 was overexpressed and cell proliferative activities assessed by Ki-67 labeling were increased in a considerable number of BECs of PCK rats at different ages, when compared to control rats, support this scenario. We also demonstrated that the enhanced proliferative activity of BECs of the PCK rat was significantly inhibited by the transfection of siRNA against MEK5 mRNA. Kato et al demonstrated that the MEK5-ERK5 pathway is critically involved in the mitogenic activation by EGF, and also that ERK5 is required for EGF-induced cell proliferation and progression through the cell cycle from G1 to S phase. In addition, overexpression of the transcript encoding TK, a rate-limiting enzyme for DNA synthesis, observed in this study, may be associated with the overgrowth of BECs of the PCK rat. We consider that the activation of the MEK5-ERK5 cascade plays a pivotal role in the biliary dysgenesis of the PCK rat.

Gefitinib, an EGFR tyrosine kinase inhibitor, was originally developed as an anticancer agent. In this study, we used gefitinib to inhibit the overgrowth of BECs of the PCK rat. As expected, this agent had prominent inhibitory effects on the growth of BECs. It has been demonstrated that treatment of BALB/c-bpk mice with a tyrosine kinase inhibitor (EKI-775) inhibited renal enlargement, the development of renal dysfunction, and hepatic pathology, suggesting the possibility of using tyrosine kinase inhibitors as therapeutic agents for ARPKD.

In normal BECs, gefitinib had an inhibitory effect on cell proliferative activity, but inhibitors for MEK1/2 (PD98059 and U0126) and siRNA for MEK5 mRNA did not, suggesting that the proliferative effect of EGF on normal BECs is mediated neither by MEK1/2 nor by MEK5. In renal epithelial cells, it has been shown that activation of EGFR led to cell proliferation, and this proliferative effect was mediated by phosphoinositide-3-kinase (PI3K) and subsequent phosphorylation of Akt, rather than by phosphorylation of ERK1/2. One possible mechanism is that cell proliferation in response to EGF is mediated by the PI3K pathway in normal BECs.

Proliferating bile duct epithelial cells have been shown to be a major source of connective tissue growth factor, and thus proliferating BECs can be a contributor to liver fibrogenesis. Recently, Mehta et al demonstrated that overexpression of MEK5 led to the upregulation of the transcript encoding matrix metalloproteinase-9, an extracellular matrix degrading protease, in human prostatic cancer, and the overexpression was positively associated with tumor progression. Their data indicate the involvement of MEK5 in the kinetics of extracellular matrix molecules. Inhibition of the MEK5-ERK5 pathway by the administration of gefitinib may be effective for the inhibition of cyst development in the PCK rat, and this inhibition may also lead to an improvement in the progressive portal fibrosis. Therefore, further study in vivo is warranted.

Recently, Masyuk et al have showed that fibrocystin, a protein product of PKHD1, is expressed in cholangiocyte cilia and the disruption of PKHD1 results in abnormalities in ciliary morphology. Although the precise role of fibrocystin is yet to be determined and the causal relation between fibrocystin and overexpression of MEK5 remains unknown, the present study provided evidence to support an important role of activation of the MEK5-ERK5 pathway in the pathogenesis of cysts in the PCK rat, and identify key aspects of dysregulated BEC growth. The data obtained here also provide insights into the pathogenesis of biliary dysgenesis and hepatic fibrosis in Caroli’s disease with CHF. As the MEK5-ERK5 interaction is highly specific, it may represent a potential target of therapy for Caroli’s disease.

Acknowledgments

We thank AstraZeneca, Macclesfield, UK for kindly providing the Gefitinib (“Iressa”), a trademark of the AstraZeneca group of companies.

References

42. Nakashima K, Gattone II VH, Sweeney WE, Avner ED: Renal dysfunction but not cystic change is ameliorated by neonatal epidermal growth factor in bpk mice. Pediatr Nephrol 2001, 16:45–50
44. Richards WG, Sweeney WE, Yoder BK, Wilkinson JE, Woychik RP,


