CXCR2 agonists in ADPKD liver cyst fluids promote cell proliferation

Claudia R. Amura,1 Kelley S. Brodsky,1 Berenice Gitomer,2 Kim McFann,2 Gwendal Lazennec,3 Matthew T. Nichols,1 Alkesh Jani,2 Robert W. Schrier,2 and R. Brian Doctor1

1Division of Gastroenterology and 2Division of Renal Diseases and Hypertension, Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado; and 3Institut National de la Santé et de la Recherche Médicale U844, Montpellier, France

Submitted 2 October 2007; accepted in final form 10 January 2008

Am J Physiol Cell Physiol 294: C786–C796, 2008. First published January 16, 2008; doi:10.1152/ajpcell.00457.2007.—Autosomal dominant polycystic kidney disease (ADPKD) is a highly prevalent genetic disease occurring in 1 in 800 individuals. The most prominent clinical feature of ADPKD is the development of numerous, fluid-filled cysts within the kidneys and liver. Cytokines and growth factors secreted by the cyst-lining epithelia are positioned to initiate autocrine/paracrine signaling and promote cyst growth. Comparative analyses of human kidney and liver cyst fluids revealed disparate cytokine/growth factor profiles. CXCR2 agonists, including IL-8, epithelial neutrophil-activating peptide (ENA-78), growth-related oncogene-α (GRO-α), are potent proliferative agents that were found at high levels in liver but not kidney cyst fluids. Liver cysts are lined by epithelial cells derived from the intrahepatic bile duct (i.e., cholangiocytes). In polarized pkd2(WS25/−/−) mouse liver cyst epithelial monolayers, CXCR2 agonists were released both apically and basally, indicating that they may act both on the endothelial and epithelial cells within or lining the cyst wall. IL-8 and human liver cyst fluid induced cell proliferation of HMEC-1 cells, a human microvascular endothelial cell line, and Mz-ChA1 cells, a human cholangiocyte cell model. IL-8 expression can be regulated by specific stresses. Hypoxia and mechanical stretch, two likely stressors acting on the liver cyst epithelia, significantly increased IL-8 secretion and promoter activity. AP-1, c/EBP, and NF-κB were required but not sufficient to drive the stress-induced increase in IL-8 transcription. An upstream element between −272 and −1,481 bp allowed for the stress-induced increase in IL-8 transcription. These studies support the hypothesis that CXCR2 signaling promotes ADPKD liver cyst growth.

interleukin-8; cholangiocytes; endothelium; cytokines; growth factors; autosomal dominant polycystic kidney disease

While cystogenesis is clearly linked to the PKD1 or PKD2 mutations, the growth of liver cysts is impacted by extragenetic factors (39). It is hypothesized that autocrine/paracrine signaling by factors secreted by the cyst-lining epithelial cells controls the growth rate of kidney and liver cysts. A number of studies have implicated or demonstrated the presence of cytokines and growth factors within human liver cyst fluid. The present study directly compared and contrasted the cytokine/growth factor profiles in liver and kidney cyst fluids and found that the profiles were markedly different. Among the factors that were specifically elevated within human liver cyst fluids were agonists for CXCR2 receptors, including IL-8, epithelial neutrophil-activating peptide (ENA-78), and growth-related oncogene-α (GRO-α). CXCR2 agonists were of specific interest since they can not only attract inflammatory cells into diseased tissues (28) but can also promote angiogenesis in the absence of preceding inflammation (1) and induce proliferation of epithelial cells (44). Angiogenesis and proliferation of the cyst-lining epithelial cells are considered essential elements for cyst growth. Consequently, the present studies evaluated the potential for CXCR2 agonists within liver cyst fluid to directly promote the proliferation of both endothelial and epithelial cells.

Finally, the expression of cytokines and growth factors, including IL-8, is generally low under basal conditions and increased in response to specific stresses experienced by cells. Hypoxia and cell stretching are two stressors that are likely to be imposed onto the liver cyst epithelial cells. The lining epithelial cells in human ADPKD liver cyst wall display morphologic features of ischemia (10, 30), and ischemia induces the expression of a number of cytokines and growth factors, including IL-8 (36). In vivo human studies show that ADPKD liver cyst-lining epithelial cells retain a strong, regulated secretory capacity that increases intraluminal cyst pressure and, by inference, increase cell stretching (12). In pulmonary epithelial cells, cell stretching induced an increase in IL-8 (41). Further, secretion-induced stretching of epithelial cysts in three-dimensional matrices increased the rate of cell proliferation (38). Consequently, the impact of hypoxia and cell stretching on IL-8 expression was evaluated. In total, the present in vitro studies demonstrate the potential for CXCR2 agonists to contribute to the in vivo growth of ADPKD liver cysts and highlight the CXCR2 signaling pathway as a potential therapeutic target for the treatment of ADPKD liver cyst disease.
MATERIALS AND METHODS

Reagents

Unless otherwise noted, all cell culture media and reagents were purchased from Gibco-Invitrogen (Grand Island, NY). Fetal Bovine Serum (FBS) was purchased from Hyclone-GB Perbio (Logan, UT). All tissue culture plastic materials were purchased from Beckton-Dickinson (Lincoln Park, NJ). Mouse monoclonal anti-proliferating nuclear antigen (PCNA) antibody was purchased from BD-Pharmin- gen (San Jose, CA) and anti-human actin was from Sigma Chemical (St. Louis, MO). SB-225002 (CXC2 inhibitor) was purchased from Calbiochem (La Jolla, CA).

ADPKD Liver and Kidney Cyst Fluids

The collection of all fluids and tissues from human patients was done in accordance with a protocol reviewed and approved by the Colorado Multiple Institution Review Board. All patients were fully informed and consented to participate in these studies. Fluids from liver and kidney cysts were obtained by laparoscopic fenestration procedures and needle aspiration. Fluids from 7 to 20 individual cysts per liver were collected from 10 patients; fluids from 3 to 18 individual cysts per kidney were collected from 11 patients. In some cases, cyst fluids from the same patient were pooled. All cyst fluid samples were stored at −80°C until assayed. Removal of information that would permit identification of the donor of the cyst fluid samples has disallowed the demographic data on the liver and kidney cyst fluids to be compared and contrasted.

pkd2(WS25/−) Mice

C57BL/6 pkd2(WS25/−) mice were developed by Stefan Somlo (Yale University) and pkd2(+/−) and pkd2(WS25/+) breeding pairs were provided for this project. Pkd2(WS25/−) mice closely model the human condition by having one copy of pkd2 knocked out and having a second, recombinant-sensitive allele (i.e., WS25) that undergoes high rates of recombination to yield knockouts of the second copy of the gene in somatic cells during the life span of the animals. By 4 mo of age, all pkd2(WS25/−) mice develop discernible kidney and liver cysts (45). Over the subsequent 4 to 8 mo, these cysts grow and emerge from the tissues. Mice were genotyped by Southern blotting as previously reported (4). All experimental protocols were approved by the University of Colorado Animal Care and Use Committee.

Cell Culture Protocols

Three different cell culture models were employed in these studies. Primary cultures of pkd2(WS25/−) mouse liver cyst epithelial cells were used to evaluate the polarity of cytokine release. HMEC-1 cells, a human microvascular endothelial cell line (2), were used to model the “cystic” cholangiocytes that line the liver cyst wall. Mz-ChA1 cells, a human microvascular endothelial cell line, were used to model the “cystic” cholangiocytes that line the liver cyst wall.

pkd2(WS25/−) liver cyst epithelial cells. Liver cyst epithelial cells were isolated from pkd2(WS25/−) mice as previously described (11). Briefly, liver cyst wall tissue was finely minced, suspended in growth media [DMEM/F12, 5% FBS, 2 mM glutamine, 1% nonessential amino acids, 1% lipid concentrate, 1% vitamin solution, 393 ng/ml dexamethasone (Sigma), 25 ng/ml epidermal growth factor (EGF; Upstate Biologics, Lake Placid, NY), 30 μg/ml bovine pituitary extract (Upstate Biologics), 3.4 μg/ml triiodothyronine, 1% insulin-transferrin-Se, 0.4 μg/ml forskolin (Sigma), and 50 μg/ml soybean trypsin inhibitor; pH 7.5], and placed above a 2-mm slab of rat tail collagen. Epithelial cells were allowed to grow out onto the slab. Cells were passaged by digesting the collagen slab [1 mg/ml collagenase (Sigma) and 2 mg/ml dispase; 60 min at 37°C], disrupting the monolayer into small epithelial “islands” and reseeding with these islands onto fresh collagen slabs. Cells were maintained at 37°C under a humidified atmosphere with 5% CO2.

HMEC-1 cells. Human HMEC-1 cells (a kind gift from Dr. Sean Colgan, University of Colorado Health Sciences Center, Denver) were cultured in MCDB-131 (Invitrogen) supplemented with 10% FBS, 10 mg/ml EGF (Upstate Biotechnologies, Lake Placid, NY), and 1 μg/ml hydrocortisone (Sigma). Cells were harvested with 0.25% trypsin-EDTA and were passaged once a week. Cells were maintained at 37°C under a humidified atmosphere with 5% CO2.

Mz-ChA1 cells. Human Mz-ChA1 cells were cultured in CMRL medium-1066 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Cells were harvested with 0.25% trypsin-EDTA and passaged once a week. Cells were maintained at 37°C under a humidified atmosphere with 5% CO2.

Cytokine Array and Specific ELISA Analysis

The comparative presence of 120 distinct cytokines and growth factors in human liver and kidney cyst fluids was evaluated by cytokine array analysis, as described by the manufacturer (Transignal Mouse Cytokine Antibody Arrays, RayBio, Redwood City, CA). Briefly, 1.5 ml human cyst fluid was incubated with Human Cytokine Antibody Arrays VI and VII supports, and the captured factors were labeled with a biotinylated antibody cocktail and detected by chemiluminescence with a UVP Photodocumentation System (Upland, CA). Densitometry of each signal was quantified with NIH Image software; values were normalized to internal controls present on each blot. The pattern of growth factors and cytokines that accumulated in mouse liver cyst fluids or were secreted in culture by pkd2(WS25/−) liver cyst epithelial cells was similarly evaluated using the mouse cytokine array 2 (RayBio). Quantitation of human IL-8 and EGF and mouse KC was performed by ELISA, as described by the manufacturers (R&D Biosysystems, Minneapolis, MN; and ElisaTech, Aurora, CO). Absorbance (405 nm) was measured with a microplate reader (Dynatech Laboratories, Chantilly, VA), and cytokine levels were correlated to internal standards included in each plate.

Cell Proliferation Assays

The proliferation rates of the human cell culture models were measured by Alamar Blue assay, as described by the manufacturer (Biosource/Invitrogen, Carlsbad, CA). Briefly, cells were seeded at subconfluent density into six-well plates and allowed to attach overnight in their respective growth media. Cells were then incubated for 24 h in serum-free media. Cells maintained in serum-free media served as a baseline (0%) control, and media containing 10% FBS served as a positive (100%) control. In experimental groups, cells grown in serum-free media were treated with experimental agents for 24 h, and the comparative levels of proliferation were measured. These agents included 0.01 to 1,000 ng/ml recombinant human IL-8 (R&D Biosysystems), 10% human liver cyst fluid, or 10% human renal cyst fluid. Subsequently, serum-free media containing 10% Alamar Blue was added to the cells for 2 h, the media evaluated for absorbance at 540 nm and 600 nm and the reduction of Alamar Blue calculated. The proliferative effect of liver cyst fluid was also confirmed by Western blotting for PCNA (BD-Pharmingen, Palo Alto, CA) and actin (Sigma), with actin serving as a loading control. Cell proteins were solubilized in 5× PAGE buffer (5% sodium dodecyl sulfate, 25% sucrose, 50 mM Tris, 5 mM ethylenediamine tetracetic acid, 0.2% dithiothreitol, and complete protease inhibitor II Ameer- sham, pH 8.0). The proteins were separated by gel electrophoresis, transferred onto nitrocellulose, and probed by Western blotting. PCNA and actin bands were captured digitally using a UVP Photodocumentation System. Densitometric analysis was performed using NIH Image software.
Modeling of Cellular Stressors

Evidence indicates that liver cyst epithelial cells experience hypoxic conditions and undergo cell stretching. To model the effect of these stressors on IL-8 production, Mz-ChA1 cells were subjected to either hypoxia or cell stretching, and IL-8 secretion and IL-8 promoter activity were measured. In IL-8 promoter activity studies, cells were first cotransfected with IL-8-luciferase chimeric and renilla-luciferase constructs. The effect of epithelial cell stretching was performed by mechanical deformation of cells on six-well silicone elastomer-bottom culture plates using a Flexercel Stress Unit (Flexcell, McKeensport, PA). Mz-ChA1 cells (2 x 10⁵ cells/well) were seeded onto the elastomer plates, allowed to attach overnight, and then subjected to a stretching protocol. This protocol subjected cells to specific elongation over a 25-h time period. This included 5%, 7.5%, 10%, 15%, and 20% stretch with each increasing step taken after 5 h.

The cells experienced stretching at 20 cyles/min. Previously shown to induce hypoxic gene response (15, 19), cellular hypoxia was achieved by incubation of cells (1 x 10⁵/well in 12-well plates) in a 1% O₂-5% CO₂ environment for 8 h. Following the experimental period, the media were collected, and IL-8 secretion was measured by ELISA (see above). Cell proliferation was measured by Alamar Blue assay (see above). IL-8 promoter activity was measured as described below.

IL-8 Promoter Activity Assays

Chimeric constructs of the 5’ region of the IL-8 promoter were previously ligated to a firefly luciferase reporter and used to evaluate IL-8 promoter activity (14). The constructs included a full-length construct (-1.482 bp up to +44 bp), a series of 5’ truncations (-272/+44, -98/+44, –50/+44), and an empty vector control. In addition, constructs with mutations in the known activator protein-1 (AP-1), cellular enhancer-binding protein (c/EBP), and NF-κB sites that disrupt binding were used (14). Finally, a single base (A/T) substitution in the -251 bp was generated by using Quick Change Mutagenesis Kit, as directed by the manufacturer (Clontech, Palo Alto, CA). Plasmids were transfected into Mz-ChA1 cells using Lipofectamine 2000 (Life Technologies/Invitrogen, Gaithersburg, MD), as directed by the manufacturer. As a control for transfection efficiency, a plasmid containing a renilla-luciferase reporter gene under the TK promoter was cotransfected into the cells (5). Following transfection, cells were seeded either in 12-well plates at 1 x 10⁵ cells/well for hypoxia experiments or in collagen-coated, elastomer-based six-well plates at 2 x 10⁵ cells/well for stretch experiments. After 18-24 h, media were changed to serum-free media, and cells were incubated for an additional 24 h. Subsequently, cells were subjected to stress as indicated, and cell extracts were then prepared in 500 μl Luciferase Passive Lysis Buffer (Promega, Madison, WI). Both firefly and renilla luciferase activities were assayed in 60-μl lysate aliquots using the Dual Luciferase Kit (Promega) and detected in a 96-well plate luminometer (Luminoskan Ascent, Thermo Electron; Franklin, MA). Firefly luciferase was normalized to renilla-luciferase activity in each group.

Statistical Analysis

Values in the text and table are presented as means ± SE. All statistics were evaluated by GraphPad Instat (San Diego, CA) analysis software. In studies comparing two groups, the data was analyzed either by Student t-test of unpaired samples (Fig. 2 and Table 1) or of paired samples (Fig. 6). P < 0.05 was considered statistically significant. In studies comparing groups of three or more samples (Figs. 4, 5, 7, 8, and 9), the means were subjected to an analysis of variance. A Tukey multiple-comparisons test was performed to identify which groups were significantly different (P < 0.05). In the presentation of the analysis of variance, groups or bars without a shared letter are significantly different from each other.

Liver and Kidney Cyst Fluids Contain a Distinct Profile of Cytokines and Growth Factors

Cytokine array analyses found that the profiles of cytokines and growth factors in ADPKD liver and kidney cyst fluids were markedly distinct (Fig. 1). A densitometric analysis of all the factors detected in the individual arrays for liver (n = 5) and kidney (n = 6) cyst fluids is presented in Table 1. These values were normalized to the internal control samples included in the arrays. The arrays showed that some factors (e.g., angiogenin) were present in both liver and kidney cyst fluids. Hepatocyte growth factor (HGF) was found sporadically, being detected in one of the five liver cyst fluid samples and one of the six kidney cyst fluid samples tested. Other factors were specifically elevated in either kidney cyst fluids or liver cyst fluids. For example, all kidney cyst fluid samples tested contained leptin and macrophage inflammatory protein-1α, but these factors were not observed in any of the liver cyst fluid samples. Conversely, liver cyst fluids had readily detected levels of IL-8, but only two of the kidney cyst fluid samples tested had detectable levels of IL-8, and IL-8 was present at weakly detectable or undetectable levels in the other four samples. While the signals were not as robust as those for IL-8, ENA-78 and GRO were also present in liver but not kidney cyst fluids.

IL-8, ENA-78, and GRO-α are each agonists for CXCR2 chemokine receptors, which suggests that they may act in a coordinate fashion to impact cyst growth. Consequently, their concentrations in liver and kidney cyst fluids were measured directly by ELISA (Fig. 2). The concentration of IL-8 was significantly higher in liver cyst fluids (4.13 ± 0.24 ng/ml; n = 67; *P < 0.05) than in kidney cyst fluids (0.53 ± 0.07 ng/ml;
and GRO-H9251/H11022 (1,000 nA) on semipermeable membrane supports formed high resistance monolayers (>1,000 Ω·cm²). The transepithelial resistance of these monolayers was grown to confluence on semipermeable membrane supports.

### Table 1. Densitometric analysis of cytokines detected in liver and kidney cyst fluids

<table>
<thead>
<tr>
<th>Construct</th>
<th>Liver Cysts (n = 5)</th>
<th>Kidney Cysts (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>IL-8</td>
<td>0.56±0.21</td>
<td>0.12±0.10*</td>
</tr>
<tr>
<td>ENA-78</td>
<td>0.08±0.01</td>
<td>0.00±0.00*</td>
</tr>
<tr>
<td>GRO</td>
<td>0.04±0.01</td>
<td>0.00±0.00*</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.06±0.01</td>
<td>0.00±0.00*</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>0.53±0.06</td>
<td>0.65±0.22</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.06±0.03</td>
<td>0.04±0.04</td>
</tr>
<tr>
<td>HGF</td>
<td>0.34±0.34</td>
<td>0.20±0.20</td>
</tr>
<tr>
<td>EGF</td>
<td>0.00±0.00</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.35±0.09</td>
<td>0.52±0.19</td>
</tr>
<tr>
<td>Acrp30</td>
<td>0.32±0.01</td>
<td>0.87±0.41</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.00±0.00</td>
<td>0.61±0.32†</td>
</tr>
<tr>
<td>MIP-1</td>
<td>0.00±0.00</td>
<td>0.61±0.32†</td>
</tr>
</tbody>
</table>

Values are means ± SE. ENA-78, epithelial cell-derived neutrophil attractant-78; GRO, growth-regulated oncogene/melanoma growth stimulatory activity; HGF, hepatocyte growth factor; MCP-1, macrophage chemotactic protein 1; Acrp30, adipocyte complement-related protein of 30 kDa; MIP-1, macrophage inflammatory protein 1. *Concentrations detected in liver cyst fluids are greater than in kidney cyst fluids (P < 0.05). †Concentrations detected in liver cyst fluids are less than in kidney cyst fluids (P < 0.05).

$n = 30$). Similarly, the concentrations of ENA-78 (liver: 1.18 ± 0.08 ng/ml, $n = 53$; kidney: 0.05 ± 0.00 ng/ml, $n = 24$) and GRO-α (liver: 2.10 ± 0.15 ng/ml, $n = 53$; kidney: 0.03 ± 0.00 ng/ml, $n = 24$) were higher in liver cyst fluids. As a comparative control, ELISA analyses of the same cyst fluid samples for EGF found that the levels were below detection levels in both liver (0.00 ± 0.00 ng/ml) and kidney (0.01 ± 0.01 ng/ml) samples (data not shown).

**CXCR2 Agonists Are Secreted Across Apical and Basolateral Membranes**

The presence of CXCR2 agonists in liver cyst fluid indicates that these factors are secreted across the apical membrane of the cyst-lining epithelial cells. This would position these agonists to interact with receptors on the apical membrane of the cyst-lining epithelial cells. To determine whether factors could also be secreted across the basolateral membrane, where they could interact with receptors on the basolateral membrane of the epithelial cells or with endothelial cells of the cyst wall vasculature, cytokine profiles were evaluated from the apical and basal chambers of pkd2(WS25/) mice grown on semipermeable supports form high resistance monolayers (>1,000 Ω·cm²). Cytokine array analysis of media collected after 72 h of incubation showed a parallel pattern of cytokine and growth factor release into both the apical and basal media. Boxes show internal array controls.

**IL-8 and Human Liver Cyst Fluid Promote Endothelial Cell Proliferation**

Endothelial cell proliferation is an essential component of angiogenesis. As the liver cyst wall is directed to grow,
neovascularization is likely needed to support the new growth. The effect of CXCR2 agonists in liver cyst fluids on endothelial cell proliferation was evaluated by treating HMEC-1 cells with either IL-8 or human liver cyst fluids with or without a CXCR2 inhibitor (Fig. 4). Treatment with increasing doses of IL-8 for 24 h significantly enhanced the rate of proliferation of HMEC-1 cells (Fig. 4A). HMEC-1 cells had a significant proliferative response when treated with 0.1 ng/ml IL-8 (58 ± 4% of the 10% FBS response; n = 3) and a peak response with 100 ng/ml IL-8 (121 ± 6% of the 10% FBS response; n = 3).

Subsequently, the effect of CXCR2 signaling from constituents within human liver cyst fluid was tested (Fig. 4B). Treatment of HMEC-1 cells with 10% of human liver cyst fluid induced a significant proliferative response (52 ± 6% of the 10% FBS response; n = 4). The contribution of CXCR2 signaling to the responses to liver cyst fluid was evaluated by pretreating paired HMEC-1 cells with 50 nM SB-225002, a specific CXCR2 inhibitor. SB-225002 blunted the proliferative response of HMEC-1 cells to human liver cyst fluid (34 ± 8% of 10% FBS response; n = 4; *P < 0.05), which indicates that part of the proliferative response to liver cyst fluid was due to CXCR2 signaling. Higher concentrations of SB-225002 (e.g., 500 nM) resulted in greater inhibition of proliferation, but concerns about nonspecific effects begin to arise at these concentrations (data not shown). These results are consistent with the levels of CXCR2 agonists found in liver cyst fluid and support the hypothesis that CXCR2 signaling contributes to the development and maintenance of the vasculature within human liver cyst walls.

Interestingly, treatment of HMEC-1 cells with 10% kidney cyst fluid also induced cell proliferation (59 ± 8% of the 10% FBS response; n = 4). This indicates pro-growth factors are also present in kidney cyst fluids. Paired samples pretreated with SB-225002 before exposure to kidney cyst fluid showed a rate of proliferation that was 41 ± 13% of the 10% FBS response (n = 4). An analysis of variance failed to show that this level was significantly different from cells receiving kidney cyst fluid alone. Given the modest number of study repetitions, however, it cannot be ruled out that a more variable but significant effect of SB-225002 on proliferation induced by kidney cyst fluids is occurring. Such an effect would be in line with the lower and more variable levels of CXCR2 agonists present in kidney cyst fluids.

**IL-8 and Human Liver Cyst Fluid Promote Epithelial Cell Proliferation**

In a similar vein, it was important to determine whether IL-8 and CXCR2 signaling could promote the proliferation of liver cyst epithelial cells. In the absence of a primary human liver cyst cell line, Mz-ChA1 cells, a cell culture model of human cholangiocytes, was used to approximate the proliferative responses of cystic cholangiocytes. Importantly, CXCR2 is expressed at the apical domain of both normal and cystic cholangiocytes (30) and is expressed in Mz-ChA1 cells (data not shown). The proliferative responses to IL-8 were measured qualitatively using PCNA expression (Fig. 5A) and quantitatively using Alamar Blue reduction assay (Fig. 5B). Elevated PCNA expression was observed in cells treated with 10 ng/ml or more of IL-8. Similarly, Alamar Blue reduction showed a small but significant proliferative response to 1 ng/ml IL-8 (7 ± 3% of 10% FBS response; n = 4) and robust proliferative responses to IL-8 concentrations at or above 10 ng/ml IL-8 (10 ng/ml: 54 ± 4% of 10% FBS response; n = 4; 100 ng/ml: 73 ± 16% of 10% FBS response; n = 4). The proliferative response to 100 ng/ml IL-8 was completely inhibited in paired samples pretreated with 50 nM SB-225002 (n = 4; data not shown).

The capacity of CXCR2 agonists within human liver cyst fluids to promote cell proliferation was measured in Mz-ChA1 cholangiocytes (Fig. 5C). Treatment of Mz-ChA1 cells with 10% human liver cyst fluid induced a significant proliferative response (72 ± 4% of 10% FBS response; n = 5). Inhibition of CXCR2 signaling with 50 nM SB-225002 significantly reduced this proliferation response (32 ± 16% of 10% FBS
Analysis of PCNA expression confirmed these observations, with 10% human liver cyst fluid inducing a potent increase in the PCNA-to-actin ratio, whereas normal human bile, a negative control, failed to induce cell proliferation (data not shown). As with the endothelial cells, the incomplete inhibition of proliferation by SB-225002 suggests that other factors present within the liver cyst fluids also contribute to the proliferative response by these cells. Treatment with 10% kidney cyst fluid also induced Mz-ChA1 cell proliferation (60 ± 14% of FBS response; n = 6). This proliferative response, however, was not inhibited by pretreatment with SB-225002 (59 ± 4% of 10% FBS response; n = 4).

Hypoxia and Cell Stretch Stimulates IL-8 Synthesis by Cyst Epithelia

The expression of most cytokines and growth factors is moderated by external cues or “stresses” placed on the cells. Evidence indicates that the ADPKD liver cyst epithelium is subjected to cell stretching (11, 12) and hypoxia (30). In other cell types, cell stretching and hypoxia are potent inducers of IL-8 expression (19, 26). Consequently, the effect of cell stretching and hypoxia on IL-8 secretion by liver cyst epithelial cells was modeled by subjecting Mz-ChA1 cells to stretching or hypoxia (Fig. 6). Cell stretching induced an increase in IL-8 secretion (basal: 0.91 ± 0.07 ng/ml; stretch: 1.56 ± 0.14 ng/ml; n = 5). Similarly, hypoxia induced a significant increase in IL-8 release (basal: 0.54 ± 0.13 ng/ml; hypoxia: 0.89 ± 0.22 ng/ml; n = 5). While other stressors may also moderate IL-8 expression, these observations demonstrate that both hypoxia and cell stretching increase IL-8 synthesis and release.

Stress-Responsive Elements Regulating IL-8 Transcription

The transcription factors AP-1, c/EBP, and NF-kB have been shown in different model systems to be potent activators of IL-8 gene expression with defined binding sites within the IL-8 promoter (14). An IL-8 promoter-luciferase construct that included these three sites as well as an extended upstream region was used to explore the regions and sites responsible for the stress-induced expression of IL-8 in cholangiocytes (Fig. 7A and 8A). An initial analysis was performed using 5’ truncations of the full length -1,482/+44 IL-8-Luc promoter.
conditions, the promoter activity of the full-length IL-8 promoter was doubled in cells exposed to either hypoxia (217 ± 24%; n = 6; Fig. 7C) or cell stretching (220 ± 28%; n = 8; Fig. 7B). In the cell stretch study, the −272/+44 promoter had a diminished basal promoter activity (49 ± 7%; n = 8), and it failed to show a robust increase in promoter activity under stretched conditions (75 ± 18%; n = 8; Fig. 7B). The −272/+44 construct in the hypoxia study retained its basal activity (83 ± 19; n = 6; Fig. 7C), but it showed no significant increase in activity under hypoxic conditions (124 ± 20%; n = 6; Fig. 7C). Both the basal and stretch-induced promoter activities in the −98/+44 and −50/+44 truncation mutants were essentially abolished. In the hypoxia studies, the −98/+44 and −50/+44 promoter truncation mutants also presented minimal basal activities and no increase in promoter activities in response to hypoxia.

In complementary studies, the basal and stress-induced IL-8 promoter activities were measured in constructs with the AP-1, c/EBP, and NF-κB binding sites mutated to eliminate their binding and activation (Fig. 8). The three sites were mutated individually, in pairs, or in triplicate. Mutation of either the AP-1 site or the NF-κB site resulted in a profound decrease in the basal IL-8 promoter activity level. Neither hypoxia nor cell stretching significantly increased the IL-8 promoter activity of the mutated constructs. Mutation of the c/EBP site resulted in a more modest decrease in the basal promoter activity. The mutation of any two of the AP-1, c/EBP, or NF-κB binding sites or mutation of all three sites largely eliminated the basal IL-8 promoter activity, corroborating the essential participation of these transcription factors in allowing IL-8 gene expression. These results were confirmed using a smaller (−131/+11) IL-8 “core” promoter that had specific mutations within the AP-1, c/EBP, and NF-κB sites (data not shown) (20). With regards to the promoter activity in response to hypoxia and cell stretching, despite an intact sequence upstream of the AP-1, c/EBP, NF-κB binding sites, there was little or no increase in IL-8 promoter activity when any of the known transcription factor binding sites were mutated. Together the truncation and mutation studies confirm that the “core promoter”, including the AP-1, c/EBP, NF-κB sites, is essential for both the basal and stress-activated activities. Regions upstream of the core promoter, however, are also required for stress-dependent activation of the IL-8 promoter.

**IL-8 Promoter Polymorphism Modifies Stress-Induced IL-8 Expression**

Several single-nucleotide polymorphisms (SNPs) have been reported within the IL-8 gene. The IL-8 SNP at −251 bp (T>A; rs4073) has been well characterized and is associated with altered IL-8 expression, increased risk for gastric carcinoma, and increased respiratory infections (21, 35, 37). To assess whether this polymorphism moderates IL-8 promoter activity under basal or stressed conditions, a single base A>T substitution was introduced at −251 bp by site-directed mutagenesis in the full-length −1,481/+44 IL-8 promoter-luciferase vector and the comparative promoter activities were measured under basal and stress conditions (Fig. 9). Under
basal conditions, there was no difference in promoter activities between the −251A (100%; n = 5) and −251T IL-8 promoters (101 ± 9%; n = 5). In contrast, the stretch-dependent increase in IL-8 promoter activity measured in −251A construct (255 ± 25% of −251A basal activity; n = 5) was blunted in the −251T reporter construct (173 ± 2% of −251A basal activity; n = 5; Fig. 9B). A similar response was observed in preliminary hypoxia studies (data not shown). It will be of significant clinical interest to determine if individuals with a −251A SNP secrete and accumulate greater amounts of IL-8 in liver cyst fluids than individuals with a −251T SNP.

DISCUSSION

Multiple Factors May Influence the Clinical Progression of ADPKD

ADPKD has been definitively linked to mutations in the PKD1 and PKD2 genes. The clinical presentation of ADPKD, however, is markedly heterogeneous and is impacted by both genetic and extragenetic factors. For example, the onset of disease occurs, on average, 15 y earlier in individuals with PKD1 mutations versus those with PKD2 mutations. Furthermore, ADPKD is considered a molecular recessive disease that arises from a germ line mutation in one copy of the gene and a somatic mutation that later inactivates the second PKD allele. The rate, timing, and intragenic site of the somatic mutation can impact cystogenesis (18, 34). Despite different rates of cystogenesis in individuals with PKD1 versus PKD2 mutations, the rates of cyst growth in these individuals are similar (9, 17). This suggests that cystogenesis is largely determined by the primary mutation but that cyst growth is likely moder-
ated by extragenetic factors. Significant intrafamilial variability in disease severity also indicates that extragenetic factors influence the presentation of the disease (31). Some of the extragenetic factors that modify disease severity have begun to be identified by genetic linkage analyses (13). Discovering and defining the extragenetic factors responsible for driving cyst growth is specifically important for liver cyst growth since it is the total volume (i.e., cyst growth) and not the number of liver cysts (i.e., cystogenesis) that dictates the clinical presentation of ADPKD liver cyst disease.

**Growth Factor and Cytokine Profiles in Liver and Kidney Cyst Fluids are Distinct**

Cytokines and growth factors secreted by the cyst lining epithelial cells have long been predicted to promote cyst growth. Various pro-growth factors, including IL-1, IL-2, TNF-α, and EGF, have been reported to accumulate in kidney cyst fluids. Various pro-growth factors, including IL-1, IL-2, TNF-α, and EGF, have been reported to accumulate in kidney cyst fluids (16, 29, 40). Factors such as IL-8, ENA-78, IL-6, and VEGF, have been discovered more recently in ADPKD liver cyst fluids (30). It remained unclear, however, if the cytokine/growth factor profile was largely determined by changes that occurred within all epithelial cells in which *PKD1* or *PKD2* was mutated or if the profile was largely independent of the primary mutation and dictated by the individual epithelial cell type forming the cyst. Paired cytokine array analyses and ELISA studies documented that, despite some overlap, the profiles were distinct and several factors were specific either to the liver or kidney cyst fluids. (Fig. 1 and Table 1). De-identification of the samples did not allow demographic differences in the liver and kidney cyst fluid donors to be evaluated. However, with liver cyst disease being far more prevalent in women than men and, conversely, kidney cyst disease is more prevalent in men than women, differences in the sex of the donors may influence the differences in the factors found in the liver and kidney cyst fluids. Regardless, the distinct cytokine and growth factor profiles indicate distinct mechanisms likely regulate the development and growth of the liver and kidney cysts and highlight the need to specifically study the molecular mechanisms underlying ADPKD liver cyst formation and growth.

**CXCR2 Agonists are Concentrated in Liver Cyst Fluids**

A striking difference between the liver and kidney cyst fluids was the higher concentrations of CXCR2 agonists, including IL-8, GRO-α, and ENA-78, in the liver cyst fluids (Figs. 1 and 2). All three factors were present at levels near or above their dissociation constant for CXCR2 binding (24, 27). Studied in the greatest detail, IL-8 is a potent proinflammatory chemokine, induces angiogenesis, promotes tumor invasion and metastasis, and stimulates epithelial cell proliferation (3, 7, 22, 23, 33, 43). In epithelial cell models, IL-8 has been shown to contribute to the induction of transepithelial migration of neutrophils (8). Preliminary studies in liver cysts have not consistently found evidence of neutrophil infiltration into liver cyst fluids (data not shown), but additional studies are required to assess whether CXCR2 agonists induce neutrophil migration or an inflammatory response into the liver cyst wall.

The secretion of IL-8 across both the apical and basolateral membranes, as seen in Fig. 3, positions CXCR2 agonists to signal angiogenesis within the liver cyst wall and proliferation of the liver cyst epithelial cells, CXCR2 is expressed both on human endothelial cells (1, 25) and on human liver cyst epithelial cells (30). In vitro proliferation assays demonstrated that exogenous IL-8 and liver cyst fluids containing CXCR2 agonists induced proliferation of both endothelial cells and cholangiocytes (Figs. 4 and 5). If CXCR2 is similarly activated in the native epithelial and endothelial cells within liver cyst walls, this pathway may contribute to in vivo liver cyst growth. It should be noted that other factors are present in the cyst fluids and likely act in conjunction with CXCR2 agonists to moderate the cellular proliferation and growth response of liver cysts. This is highlighted by the observation that the robust proliferative response elicited by liver cyst fluid was not fully blocked by pretreatment with SB-225002, a potent CXCR2 receptor inhibitor (Figs. 4 and 5). VEGF is an example of another factor that is present in liver cyst fluids and likely contributes to liver cyst growth. VEGF is present in mouse and human liver cyst fluids. Inhibition of VEGF signaling blunted the in vitro proliferative response of mouse liver cyst epithelial cells following treatment with mouse liver cyst fluid and impeded in vivo liver cyst growth (26). Interestingly, cooperation between the CXCR2 and VEGF receptor 2 signaling pathways for cell activation has been demonstrated (32). In the future, it will be of significant interest to investigate the potential interplay and synergism between the different signaling pathways that are activated in response to the complex mixture of cytokines and growth factors present within the cyst fluids.

**IL-8 Expression was Influenced by Cellular Stress and Genetic Polymorphisms**

With concentrations poised around the *Kₐ* for CXCR2, increases in IL-8 expression are predicted to increase the magnitude of CXCR2 signaling. The expression of many cytokines and growth factors is regulated by different stresses experienced by cells. In other cell types, cell stretching and hypoxia (15, 26) both induce IL-8 expression. Evidence suggests that human cystic cholangiocytes in liver cyst walls experience both stretching and hypoxia.

Cell stretching would be predicted to occur when fluid is secreted into the enclosed lumen of the liver cysts or the diaphragm presses down on the superficial liver cysts. In vitro studies using *pkd2*(*WS25/−*) mouse liver cyst epithelial cells show that cystic cholangiocytes retain their secretory responses to both elevated extracellular ATP or cAMP, (11). Interestingly, ATP concentrations are elevated in a subset of both mouse and human liver cyst fluid samples. In vivo human ADPKD liver cysts secrete fluid into the cyst lumens in response to secretin, a potent cAMP-dependent choleretic hormone (12). Alternatively, ultrasonography of in vivo *pkd2*(*WS25/−*) mouse liver cysts showed that the downward movement of the diaphragm physically distorted the liver cysts, and manometer readings of pressure within liver cysts detected a positive luminal pressure with chest compressions (data not shown). In the present study, applying a respiratory pattern of cell stretching resulted in an increased expression of IL-8 from Mz-ChA1 cells (Fig. 6). This increase in expression was accompanied by increases in IL-8 promoter activity (Figs. 7 and 8). Paralleling studies in other cell types (14), AP-1, NF-κB, and, to a lesser extent, c/EBP binding sites were
required for activating the IL-8 promoter in Mz-ChA1 cells (Figs. 7 and 8). These sites, however, were not responsible for activating the IL-8 promoter in response to cell stretching. Instead, a region lying between −252 bp and −1,481 bp, well upstream of the AP-1, c/EBP, and NF-κB binding sites, enabled cell stretching to increase IL-8 promoter activity. Full characterization of the specific cis-sites and trans-factors responsible for the stress response of the IL-8 promoter may provide a therapeutic target to intercede in IL-8 expression within liver cyst lining epithelial cells.

The walls of large human ADPKD liver cysts also display features of hypoxic stress. The cyst-lining epithelial cells have hypoxic characteristics, including microvillar elongation, loss of microvillar structure/blebbing, and loss of microvillar density (10, 30), and large human liver cyst walls were relatively avascular (average vessel to epithelium distance was >100 μm; data not shown). As with cell stretching, hypoxia induced Mz-ChA1 cells to secrete greater concentrations of IL-8 than paired normoxic cells (Fig. 6) and was paralleled by increased IL-8 promoter activity (Figs. 7 and 8). These observations indicate that cell stretching and hypoxia can increase the synthesis and release of IL-8. These elevated levels of IL-8 would likely promote increases in the proliferation rates of the liver cyst epithelial and endothelial cells.

In addition to the environmental stressors, an individual’s genetic background can also influence IL-8 expression. The IL-8 gene has several SNPs that can influence IL-8 expression. Among the best characterized, the −251T>A polymorphism correlates with increased IL-8 expression, greater tumorigenic capacity, and a poorer prognosis in a number of specific tumor types (21, 35, 37). In Mz-ChA1 cells, constructs with the −251A>T IL-8 polymorphism had no effect on the basal IL-8 promoter activity but blunted the increase in IL-8 promoter activity that is induced by cell stretching (Fig. 9). With IL-8 having been implicated in the pathogenesis of different diseases, it will be of significant interest to evaluate the role of IL-8 in the ADPKD liver cyst disease by comparing and contrasting the concentrations of IL-8 accumulated in liver cyst fluids and the severity of liver cyst disease in ADPKD patients with the −251A versus the −251T polymorphisms in their IL-8 promoter. More broadly, strategies to intercede in the induction of IL-8 expression, whether from cellular stresses or genetic polymorphisms, should reduce IL-8 signaling and any consequent growth within ADPKD liver cysts.

In summary, the present studies support a principal role for IL-8 and CXCR2 signaling in impacting the progression of ADPKD liver cyst disease. Signaling from IL-8 and other CXCR2 agonists that amasses in ADPKD liver cyst fluid was capable of driving proliferation of both epithelial and endothelial cells. With liver cyst expansion being the primary factor dictating the clinical manifestations of ADPKD liver cyst disease, the present studies support the investment in in vivo studies to directly determine the contribution of CXCR2 signaling in promoting ADPKD liver cyst growth.

ACKNOWLEDGMENTS

The atmospheric chamber was kindly provided by Dr. Sean Colgan (Gastroenterology Division, University of Colorado Health Sciences Center (UCDSCH)). The Flexercell Stress Unit (Flexcell, McKeesport, PA) was graciously provided by Dr. Raphael Nemenoff (Renal Division, Department of Medicine (DOM), UCDSHC). The renilla luciferase reporter gene plasmid was graciously given by Dr. Lynn Heasley (Renal Division, DOM, UCDSHC). The “core” IL-8 promoter-luciferase constructs, including mutations for AP-1, NF-κB and c/EBP binding sites, were a kind contribution from Dr. Michael Kracht (Medical School Hannover, Germany). The abdominal ultrasonographic evaluation in mice was kindly performed by Dr. Kelly Ambler (Cardiology Division, DOM, UCDSHC).

GRANTS

This research was supported by grants to R. B. Doctor from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-034039) and the Polycystic Kidney Foundation (109a2).

REFERENCES


