Fibroblast Growth Factor 19 Activates the Unfolded Protein Response and Mitogen-Activated Protein Kinase Phosphorylation in H-69 Cholangiocyte Cells

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Abstract

Objective: Cholangiocytes are injured in many cholestatic diseases that can progress to cirrhosis, liver cancer and need for liver transplantation. Recent studies demonstrate that the hormone Fibroblast Growth Factor 19 (FGF19) is produced in the ileum and regulates hepatic gene expression. However, the role of FGF19 in cholangiocytes remains largely unknown. The purpose of this study was to elucidate the effect of FGF19 on cholangiocyte gene and protein signaling.

Methods: Human cholangiocyte-derived H-69 cells were cultured and treated with varying concentrations of FGF19 (0–50 ng/ml) for 24 hours. Expression of the mitogen-activated protein kinase MAPK proteins JNK1/2, ERK1/2, and p38, and several Unfolded Protein Response (UPR) proteins were studied using Western blot analysis. Gene expression of UPR pathways was analyzed using real-time polymerase chain reaction (RT-PCR).

Results: FGF19 treatment increased BiP and CHOP protein expression in a concentration-dependent manner. Gene expression of BiP increased from 1.02 ± 0.24 to 2.16 ± 0.62 (vehicle vs. FGF19 25 ng/ml, respectively, p < 0.01) and CHOP expression increased from 1.05 ± 0.36 to 2.42 ± 0.56 (vehicle vs. FGF19 50 ng/ml, respectively, p ≤ 0.01). The UPR protein phosphorylated-eIF2α displayed a bimodal pattern of protein expression, with concentrations of 2.5–10 ng/ml of FGF19 maximally reducing expression and 50 ng/ml maximally increasing expression. Protein expression of phosphorylated JNK1/2, ERK1, and p38 also displayed a similar bimodal pattern of expression with a reduced expression at 2.5 ng/ml FGF19 and a return to baseline at 25 ng/ml.

Conclusion: These findings indicate that FGF19 treatment of H-69 cells selectively activates the UPR and MAPK pathways. We believe that FGF19 may have a role in the pathogenesis of human cholangiopathies.

Keywords: FGF19; Unfolded protein response; MAPK

Abbreviations

FGF: Fibroblast Growth Factor; UPR: Unfolded Protein Response; MAPK: Mitogen-Activated Protein Kinase; FXR: Farsenoid-X Receptor; FGFR4: FGF Receptor 4; CYP7A1: Cholesterol 7 Alpha-hydroxylase; HCC: Hepatocellular Carcinoma; ASBT: Apical Sodium-Dependent Bile Salt Transporter; ERK: Extracellular-Signal-Regulated Kinase; JNK: c-Jun N-terminal Kinase; BiP: Binding Immunoglobulin Protein; CHOP: CCAAT/Enhancer Binding Protein (C/EBP) Homologous Protein; eIF2α: Eukaryotic Initiation Factor 2 Alpha; ER: Endoplasmic Reticulum; PSC: Primary Sclerosing Cholangitis; PBC: Primary Biliary Cirrhosis

Introduction

Cholangiocytes, the epithelial cells that line the bile ducts, are affected in a wide variety of cholangiopathies such as Primary Sclerosing Cholangitis (PSC) and primary biliary cirrhosis (PBC) [1,2]. The etiologies of these diseases are unknown and their pathogeneses remain poorly understood. These cholestatic diseases often result in inflammation, cirrhosis, and the need for liver transplantation [1-4]. There are currently no effective treatments to alter the natural history of PSC. Ursodeoxycholic acid is the sole treatment for PBC and there have been no new approved therapies for PBC over the past 3 decades. In addition, ursodeoxycholic acid slows, but does not halt disease progression in PBC. Therefore, a greater understanding of the various processes that regulate cholangiocyte biology may be integral to the development of new therapies for these chronic liver diseases.

Fibroblast growth factor 19 (FGF19) is secreted in the ileum in response to stimulation by the nuclear bile acid farsenoid-X receptor in order to regulate hepatic metabolism and transport functions [5-8]. FGF19 expression has been observed in the small intestine, cholestatic livers, and gallbladder; and has an important role in both gallbladder filling and the enterohepatic circulation of bile salts [9-11]. FGF19 binds to a complex of FGF receptor 4 (FGFR4)-Klotho in the liver, which results in the activation of mitogen-activated protein kinase (MAPK) pathways and reduced transcription of cholesterol 7 α-hydroxylase (CYP7A1), the gene encoding for the rate-limiting step of the bile acid synthesis pathway [6,7,12-16]. FGF19 has also been shown to metabolically regulate hepatic protein and glycogen synthesis in the liver [17,18]. In addition, FGF19 increases metabolic rate and improves glucose homeostasis in diabetic mice [19-22]. Therefore, studies indicate that FGF19 has broad metabolic and protective roles in hepatocytes. In addition, hepatocyte proliferation is also increased in the presence of FGF19 [23-25]. In contrast to hepatocytes, the effect of FGF19 signaling on cholangiocytes remains poorly understood.

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Cholangiocytes are routinely exposed to high concentrations of bile salts. The Apical Sodium-Dependent Bile Salt Transporter (ASBT) is primarily responsible for regulating the transport of bile acids across cholangiocytes and its up-regulation in chronic cholestasis may have a role in causing bile-acid-induced liver injury [26]. FGF19, in the presence of βKlotho, has been shown to inhibit ASBT in human cholangiocytes [27]. FGF19 is also highly expressed in the livers of patients with extrahepatic cholestasis, which is an adaptation to protect the liver from the toxicity of bile salts [11].

The Unfolded Protein Response (UPR) is a protective cellular response to ER stress, and it is present in all eukaryotic cells. In the presence of ER stress, there can be an accumulation of cellular misfolded and unfolded proteins. The UPR acts to reduce the amount of unfolded cellular proteins, and if excess ER stress still persists, it induces apoptosis [28,29]. Although much is known about UPR activation in hepatocytes, little is known about its regulation in cholangiocytes. In addition, there is cross-talk signaling between the UPR and MAPK signaling pathways [30-33]. In the present study, we examined employed H-69 cells, a human-derived cholangiocyte cell line, to help elucidate the effects of FGF19 on cholangiocyte UPR and MAPK expression [34-38]. These findings may have important implications not only for cholangiocyte biology, but also for enhancing our understanding of the pathogenesis of cholangiopathies.

Materials and Methods

Cell culture

The H-69 biliary cell line [39] was kindly provided by Dr. Cara Mack (Denver, CO). The cell line was cultured in DMEM and DMEM/Ham’s F12 (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum, penicillin/streptomycin, 1.8×10⁻⁴ M adenine (Sigma-Aldrich, St. Louis, MO), 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 5.5×10⁻⁶ M epinephrine (Sigma-Aldrich, St. Louis, MO), 2×10⁻⁹ M triiodothyronine (Sigma-Aldrich, St. Louis, MO), 1.64×10⁻⁶ M epidermal growth factor (Sigma-Aldrich, St. Louis, MO), and 1.1×10⁻⁶ M hydrocortisone (Sigma-Aldrich, St. Louis, MO). Cells were maintained at 37°C in 10% CO₂. HepG2 cells (ATCC, Manassas, VA) were also cultured in DMEM with 10% fetal bovine serum and maintained at 37°C in 5% CO₂. Preliminary experiments optimized 24-hour treatment duration; therefore, treatments were carried out for 24 hours in serum-free supplemented DMEM. Human FGF19 was purchased from Sigma-Aldrich (St. Louis, MO) and was used in final concentrations of 0, 2.5, 5, 10, 25 and 50 ng/ml.

Analysis of gene expression by real-time quantitative PCR

Total RNA was obtained using TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA were prepared using a SuperScript First Strand kit (Invitrogen, Carlsbad, CA) for reverse transcription-PCR. Real-time quantitative PCR was performed using 2 μl of cDNA from each sample in a 25 μl reaction mixture containing Quantitect SYBR Green PCR Mastermix (Qiagen, Valencia, CA) and the primers specific for the gene of interest. Human ubiquitin C was utilized as a housekeeping gene. The primer sequences are shown in Supplementary Table 1.

Table 1: Gene expression for other UPR genes Relative expression, mean ± S.D. of n=4 is shown. aP<0.05 versus control. bP<0.01 versus control.

<table>
<thead>
<tr>
<th>Gene</th>
<th>0 ng/ml</th>
<th>2.5 ng/ml</th>
<th>5 ng/ml</th>
<th>10 ng/ml</th>
<th>25 ng/ml</th>
<th>50 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBIP</td>
<td>1.02 ± 0.24</td>
<td>1.46 ± 0.08</td>
<td>1.30 ± 0.25</td>
<td>1.95 ± 0.21</td>
<td>2.16 ± 0.62</td>
<td>2.28 ± 0.92</td>
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<tr>
<td>hCHOP</td>
<td>1.05 ± 0.36</td>
<td>1.39 ± 0.56</td>
<td>1.58 ± 0.42</td>
<td>1.47 ± 1.16</td>
<td>1.76 ± 1.16</td>
<td>2.42 ± 0.56</td>
</tr>
<tr>
<td>hXbp1s</td>
<td>1.02 ± 0.25</td>
<td>0.75 ± 0.12</td>
<td>0.65 ± 0.19</td>
<td>0.91 ± 0.23</td>
<td>0.81 ± 0.35</td>
<td>0.85 ± 0.33</td>
</tr>
<tr>
<td>hEDEM1</td>
<td>1.01 ± 0.17</td>
<td>0.98 ± 0.43</td>
<td>1.19 ± 0.19</td>
<td>0.99 ± 0.23</td>
<td>1.00 ± 0.12</td>
<td>1.33 ± 0.20</td>
</tr>
<tr>
<td>hATF4</td>
<td>1.05 ± 0.35</td>
<td>1.74 ± 1.33</td>
<td>3.15 ± 1.22</td>
<td>1.40 ± 1.12</td>
<td>2.61 ± 1.74</td>
<td>4.69 ± 1.37</td>
</tr>
<tr>
<td>hATF6</td>
<td>1.01 ± 0.20</td>
<td>1.07 ± 0.37</td>
<td>1.55 ± 0.37</td>
<td>1.16 ± 0.53</td>
<td>1.59 ± 0.89</td>
<td>2.33 ± 0.34</td>
</tr>
<tr>
<td>hGADD34</td>
<td>1.00 ± 0.02</td>
<td>0.93 ± 0.07</td>
<td>0.95 ± 0.08</td>
<td>0.98 ± 0.11</td>
<td>0.87 ± 0.05</td>
<td>1.13 ± 0.10</td>
</tr>
</tbody>
</table>

Analysis of protein expression by western blotting

Protein was extracted from H-69 cells using a mixture of T-Per (Thermo Scientific, Hanover Park, IL), protease mixture inhibitor (Thermo Scientific, Hanover Park, IL), and Halt phosphatase inhibitor (Thermo Scientific, Hanover Park, IL). The Bradford assay was utilized to determine protein concentrations of the homogenates in Coomassie Blue reagent (Pierce, Rockford, IL). 3.75 μg of sample protein were separated through electrophoresis using 10% or 12% SDS-polyacrylamide gels. Protein samples represent either pooled samples of four separate samples or single samples. Protein detection was performed using polyclonal rabbit antibodies to total SAPK/JNK (1:1000, Cell Signaling Technology, Danvers, MA), phospho-SAPK/JNK (1:1000, Cell Signaling Technology, Danvers, MA), total p44/p42 MAPK (ERK1/2) (1:1000, Cell Signaling Technology, Danvers, MA), phospho-p44/p42 MAPK (ERK1/2) (1:1000, Cell Signaling Technology, Danvers, MA), p38 MAPK (1:1000, Cell Signaling Technology, Danvers, MA), phospho-p38 MAPK (1:1000, Cell Signaling Technology, Danvers, MA), β-actin (1:1000, Cell Signaling Technology, Danvers, MA), C/EBP homologous transcription factor (CHOP) (1:500, Cell Signaling Technology, Danvers, MA), and a monoclonal mouse antibody to β-actin (1:5000, Sigma-Aldrich, St. Louis, MO). The bound antibody was detected using goat-anti rabbit or goat anti-mouse polyclonal HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and an ECL Western blotting substrate (Pierce, Rockford, IL) was used. Western blotting of HepG2 ERK1/2 activation was used as a positive control for FGF19 response to validate our experiments. β-actin was used as a loading control in all western blots. Digital densitometry was performed using NIH Image (http://rsb.info.nih.gov/nih-image/) and all data were normalized for β-actin expression (protein expression/β-actin expression).

Statistical analysis

Data analysis was performed using Student’s t-test to compare data between 2 groups. Results are stated as mean ± standard deviation. Data were deemed statistically significant if p < 0.05.
Results

FGF19 treatment causes an up-regulation of BiP in a dose-dependent manner

When treated with FGF19, BiP protein and gene expression was found to increase in a concentration-dependent manner in H-69 cholangiocyte cells (Figure 1A). Figure 1B demonstrates that BiP mRNA was also significantly increased as a result of FGF19 treatments, with a greater than a two-fold increase at doses greater than 25 ng/ml of FGF19 (1.02 ± 0.24 vs. 2.16 ± 0.62, for 0 and 25 ng/ml, respectively, \( p \leq 0.01 \)) (Table 1).

CHOP is up-regulated following FGF19 treatment

CHOP protein and gene expression in H-69 cells is also up-regulated by FGF19 treatment (Figure 2). CHOP mRNA is significantly up-regulated only at 50 ng/ml FGF19, with nearly a 2.5-fold increase over controls (1.05 ± 0.36 vs. 2.42 ± 0.56; for 0 and 50 ng/ml, respectively, \( p < 0.01 \)) (Table 1). However, lower concentrations of FGF19 also display a trend towards a concentration-dependent increase.

P-eIF2α expression is altered in a bimodal manner due to FGF19 supplementation

The phosphorylated form of eIF2α (P-eIF2α) is the active form of the protein and treating H-69 with FGF19 altered the expression of cellular phosphorylated eIF2α. P-eIF2α expression displayed a bimodal pattern of activation with 2.5-10 ng/ml FGF19 maximally reducing and 50 ng/ml maximally increasing expression in the H-69 cell line. Figure 3A shows the effect of varying (0-50 ng/ml) concentrations of FGF19 in pooled samples (n=4 for each sample). Figure 3B reveals protein expression of the individual samples for concentrations of either 0, 2.5, or 25 ng/ml. Densitometry reveals a 37% decrease in expression by 2.5 ng/ml of FGF19 (1.14 ± 0.20 vs 0.73 ± 0.10; for 0 and 25 ng/ml, respectively, \( p=0.002 \)) and a return to baseline at 25 ng/ml (p<0.005 vs 2.5 ng/ml).

Figure 2: FGF19 supplementation leads to up-regulation of CHOP in a concentration-dependent manner. H-69 protein expression of CHOP was analyzed through Western blotting (A) and mRNA expression was analyzed via Real-time qPCR (B) Representative Western blots of pooled samples (n=4). β-actin was used as a loading control. Relative mRNA expression, mean ± SD, * \( p \leq 0.05 \) and ** \( p \leq 0.01 \) versus control.

Figure 3: P-eIF2α activation in H-69 cells following FGF19 treatment. (A) Western blotting was performed on H-69 homogenate to detect expression of phosphorylated (P-) and total (T-) eIF2α after a 24 h incubation period with varying concentrations of FGF19. Data represent pooled samples with n=4. β-actin was used as a loading control (B) Western blot of the unpooled samples after treatment with 0, 2.5, or 25 ng/ml of FGF19

FGF19 activates MAPK proteins in a bimodal pattern

Mitogen-activated protein kinases have previously been shown to be activated by FGF19 in HepG2 cells \([13,15,16]\). Therefore, we investigated whether the MAPK pathways are activated in human cholangiocyte cell lines. Using pooled samples (n=4 for each sample), Figure 4A demonstrates that H-69 cells treated with FGF19 of varying concentrations appear to display a bimodal pattern for the expression of phosphorylated ERK. Figure 4B reveals P-ERK1/2 protein expression of the individual samples for concentrations of 0, 2.5, or 25 ng/ml. Densitometry reveals that there was not a significant reduction in P-ERK1/2, although the 44kD P-ERK1 was diminished. Expression of the 42kD P-ERK2 was not statistically changed.
We further observed the expression of P-JNK1/2 and P-p38 in H-69 cholangiocyte cells. Using pooled samples (n=4), treatment with 2.5 ng/ml FGF19 reduced the expression of both phosphorylated P-JNK and P-p38, with a return to baseline at 25 ng/ml (Figure 5A). Figure 5B reveals protein expression of the individual samples for concentrations of either 0, 2.5, or 25 ng/ml of FGF19.

Discussion

FGF19 is an important regulator of bile salt synthesis through its regulation of hepatic CYP7A1 expression. It is produced in the ileum in response to intestinal bile salt stimulation and is then secreted into the portal circulation, where it can be taken up into the liver and regulate hepatic genes by binding to FGFR4. FGF19 is also produced in the human liver in response to extrahepatic cholestasis, but is not expressed in healthy livers [11]. FGF19 also has important metabolic roles involving glucose homeostasis and hepatic lipid metabolism. Little is known, however, about the role of FGF19 signaling in cholangiocytes. Therefore, our study sought to elucidate the role of FGF19 signaling in H-69 human cholangiocyte cells. We employed H-69 cells, which are a human-derived cholangiocyte cell line that has been widely used to study cholangiocyte biology [34-38].

Our findings indicate that FGF19 activates the UPR pathways in cholangiocytes as evident by the increased expression of both BiP and CHOP. We observed that BiP and CHOP were activated by higher concentrations of FGF19. In addition, phosphorylated eIF2α was also increased in response to FGF19, but it displayed a bimodal pattern of activation. BiP (along with unfolded proteins) is a major upstream regulator of UPR pathways, and our data demonstrates that CHOP is a major downstream target of FGF19 signaling in H-69 cholangiocyte cells. Previous studies on FGF19 in the liver have shown that it stimulates the MAPK/ERK1/2 pathway [16]. However, no prior study reports its effect on UPR pathways in cholangiocyte cells. UPR pathways are activated in response to endoplasmic reticulum stress, which leads to an accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum. ER stress and UPR activation are important in several liver diseases, including viral hepatitis, alpha 1-antitrypsin deficiency, alcohol-induced liver injury, and fatty liver disease [40]. The activation of this pathway through FGF19 signaling in H-69 cholangiocyte cells may also serve an important role in the pathogenesis of, or as a compensatory response to, cholestatic liver disease.

The FGF19 concentrations used in our study were similar or lower than FGF19 concentrations that have been used in several cell culture systems [41-45]. Human serum FGF19 concentrations have been reported to be in the picomolar range, and sulfated glycosaminoglycans such as heparin sulfate, heparin, and chondroitin sulfates, may be required for its signaling via FGFR4 in the presence of beta-Klotho [46,47]. Thus, the concentrations used in this study may be higher than in vivo serum levels. Of note, circulating levels of FGF19 are elevated in response to extrahepatic cholestasis [11]. In addition, the tissue concentrations near cholangiocytes remain unknown, and FGF19 concentrations used in this study were typical for many FGF19 cell culture systems. FGF19 concentrations in cholestasis may range from 10-fold to 250-fold higher than serum FGF19 concentrations in humans [48]. Thus, the higher concentrations of FGF19 in our study may represent tissue levels in cholestatic liver disease where FGF19 levels are elevated, and the activation of the UPR pathway through BiP, CHOP, and eIF2α may serve as a protective measure in response to environmental stress to cholangiocyte cells.
In intestinal epithelial cells, the UPR gene ATF4 induces the production of FGF19 [45]. Intestinal cells secrete FGF19, and FGF19 enters the portal circulation and acts as a hormone in the liver [49,50]. FGF19 acts in hepatocytes to regulate hepatic lipid and glucose metabolism, as well as transport function [51,52]. In fact, in diseases of metabolic syndrome (obesity, diabetes, dyslipidemia) and fatty liver, hepatocytes activate the UPR to in response to the ER stress. However, little is known about the effects of FGF19 and ER stress on cholangiocytes. FGF19 acts predominantly as a protective hormone, and in fact, is necessary for the liver to regenerate normally [53,54]. Thus, it may prove protective for cholangiocyte injury.

MAPK pathways have been extensively studied in multiple tissues and have been shown to be important for a range of cellular effects including cellular injury and repair, cell proliferation and malignant transformation. In addition, there appears to be signaling cross-talk between the UPR and MAPK pathways [30-33]. We demonstrate that the three main branches of the MAPK pathway are up-regulated in a bimodal manner in response to FGF19. The activation of MAPK proteins in response to FGF19 has previously been reported in other tissues, and our study confirms that this pathway is also activated in H-69 cholangiocyte cells. However, the bimodal pattern of activation is a novel finding. We hypothesize that low levels of FGF19 may be necessary for the normal functioning of cholangiocytes. Furthermore, elevated levels of FGF19 may be a marker for cholestasis, and the up-regulation of the MAPK in this case could also serve as a protective measure. The combined activation of both MAPK and UPR pathways could be a response to inflammation, a pro-apoptotic effect in the setting of cholestasis or may represent aberrant signaling during pathologic cholestatic states.

These data indicate that FGF19 can regulate UPR and MAPK signaling pathways. Additional research on the impact of FGF19 signaling in vivo would further elucidate its protective effects and its therapeutic potential. These findings may have important implications not only for cholangiocyte biology, but also for enhancing our understanding of the pathogenesis of cholangiopathies.

Acknowledgements

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References


