Bile Acids Inhibit Mcl-1 Protein Turnover via an Epidermal Growth Factor Receptor/Raf-1-dependent Mechanism

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ABSTRACT

Bile acids have been implicated in biliary tract carcinogenesis, in part, by activating the epidermal growth factor receptor (EGFR). Overexpression of Mcl-1, a potent antiapoptotic protein of the Bcl-2 family, has also been reported in cholangiocarcinomas. Because receptor tyrosine kinases like EGFR may modulate antiapoptotic protein expression, we examined the hypothesis that bile acids modulate Mcl-1 expression levels via EGFR. Deoxycholate increased cellular Mcl-1 protein in a concentration-dependent manner. The deoxycholate-mediated increase of cellular Mcl-1 protein was blocked equally by EGFR tyrosine kinase inhibitors or an EGFR-neutralizing antibody. Although inhibition of mitogen-activated protein kinases did not attenuate the deoxycholate-associated increase in Mcl-1 protein, the Raf-1 inhibitor, BAY 37-9571, effectively blocked the cellular increase of this protein. Neither Mcl-1 transcriptional activity nor its mRNA stability was altered by deoxycholate treatment. However, Mcl-1 protein stability was increased by bile acid treatment, an effect duplicated by proteasome inhibition. Deoxycholate prolongation of Mcl-1 turnover was blocked by either EGFR inhibitors or the Raf-1 inhibitor. Whereas the deoxycholate-induced increase in Mcl-1 reduced Fas-mediated apoptosis, the Raf-1 inhibitor potentiated Fas apoptosis. Our results demonstrate that bile acids block Mcl-1 protein degradation via activation of an EGFR/Raf-1 cascade resulting in its cellular accumulation. Raf-1 inhibitors block this increase of Mcl-1 and render the cells more susceptible to apoptosis, a potential therapeutic strategy for cholangiocarcinomas.

INTRODUCTION

Bile acids may promote carcinogenesis by stimulating a variety of kinase signaling pathways, the best epidemiological and scientific evidence of which has been demonstrated in colorectal carcinogenesis (1). Given the similarities between colorectal carcinogenesis and the development of cholangiocarcinoma (e.g., epithelial cell cancers of the gastrointestinal tract where cells are physiologically exposed to bile acids), it is highly likely that bile acids may also participate in the genesis of cholangiocarcinoma. In fact, cholangiocarcinoma more frequently arises from the extrahepatic large bile ducts (2), which preferentially transport bile acids (3). We have recently demonstrated that bile acids stimulate EGFR activation in a human cholangiocarcinoma cell line (4). Intracellular signaling cascades emanating from receptor tyrosine kinases like EGFR would be expected to promote carcinogenesis by stimulating cellular replication and inhibiting apoptosis (5). Indeed, Green and Evan (6) have proposed that deregulation of cellular proliferation coupled to a suppression in apoptosis provides a platform essential for the development and progression of cancer. The mechanisms by which receptor tyrosine kinases stimulate cellular proliferation have been extensively studied (5). In contrast, the mechanisms by which they reduce cellular apoptosis remain incompletely understood. Insight into the mechanisms by which bile acid stimulation of EGFR suppresses apoptosis may, therefore, help provide strategies for the prevention and/or treatment of cholangiocarcinomas.

Enhanced expression of the antiapoptotic members of the Bcl-2 family has been implicated in carcinogenesis (7-9). The potent antiapoptotic Bcl-2 family protein, Mcl-1, is overexpressed in cholangiocarcinomas (10) and may provide these cancers with a potent apoptosis suppressing mechanism. Mcl-1, originally isolated from the differentiating human myeloid leukemia cell line (11), contains Bcl-2 family BH1 and BH2 domains (12). This protein is localized to mitochondria where it blocks cytochrome c release, a key mechanism necessary for the intrinsic apoptotic pathway (13, 14). Compared with other forms of antiapoptotic Bcl-2 family proteins, Mcl-1 is unique because of rapid and readily inducible changes in its expression after exposure to cytokines or growth factors (15). Unlike other Bcl-2 family members, this protein also has a very short half-life because of the presence of PEST sequences that facilitate efficient proteasome-mediated degradation (11). This information suggests that cholangiocarcinomas that overexpress Mcl-1 have developed mechanisms to either enhance this protein synthesis or block its degradation. However, information as to which process occurs is lacking.

EGFR signaling increases Mcl-1 expression in a human esophageal carcinoma cell line, protecting these cells from staurosporine-induced apoptosis (16). Therefore, it is highly plausible that bile acid activation of EGFR in human cholangiocytes and/or cholangiocarcinomas (4) may also lead to Mcl-1 induction, contributing to the development and/or promotion of cholangiocarcinomas. However, the effect of bile acids on Mcl-1 expression has not been examined. Therefore, the overall objective of this study was to examine the effect of bile acids on Mcl-1 expression in a human cholangiocarcinoma cell line. Our specific aims were to address the following key questions: (a) do bile acids induce Mcl-1 protein expression and, if so, is the expression dependent upon EGFR signaling; and (b) what are the cellular mechanisms regulating Mcl-1 protein expression by EGFR? Collectively, the results of the current study demonstrate that bile acids inhibit Mcl-1 protein degradation, thereby, increasing total cellular Mcl-1 protein levels. This prolongation of the Mcl-1 protein half-life is dependent upon activation of an EGFR/Raf-1 cascade. These observations suggest that bile acid-induced increases of Mcl-1 protein may participate in cholangiocyte carcinogenesis by inhibiting apoptosis.

MATERIALS AND METHODS

Cell Culture and Bile Acid Treatment. KMBC cells, a human cholangiocarcinoma cell line (7), were grown in DMEM supplemented with 10% fetal bovine serum, 100,000 units/liter penicillin, 100 mg/liter streptomycin, and 100 mg/liter gentamicin. Unless otherwise specified, cells were serum-starved for 24 h before bile acid treatment to avoid the confounding variable
of serum-induced signaling. We used DC, which is a secondary, hydrophobic bile acid present in bile (Sigma Chemicals Co., St. Louis, MO).

**Immunoblot Analysis.** Cells were lysed for 20 min on ice with lysis buffer (50 mM Tris-HCl (pH 7.4); 1% NP40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 μg/ml aprotinin, leupeptin, pepstatin; 1 mM Na3VO4; and 1 mM NaF) and centrifuged at 14,000 × g for 10 min at 4°C. Samples were resolved by 7.5 or 12% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies at a dilution of 1:1,000. Peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA) were incubated at a dilution of 1:1000 to 1:5000. Bound antibodies were visualized using chemiluminescent substrate (enhanced chemiluminescence; Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT film. The following primary antibodies were used: mouse antiphosphotyrosine clone 4G10 from mouse anti-EGF receptor clone 4G10 from Invitrogen. Mcl-1 mRNA was quantitated using real-time PCR technology (Fig. 1A). Cellular Mcl-1 protein levels increased over time after DC treatment and reached a maximum after 24 h of incubation (Fig. 1B). These data directly demonstrate that bile acids such as DC modulate Mcl-1 expression in cholangiocytes.

**RESULTS**

**Does DC Modulate Mcl-1 Expression in a Human Cholangiocyte-derived Cell Line?** KMBC cells were cultured in the presence and absence of DC and cellular Mcl-1 protein assessed by immunoblot analysis. Examination of whole cell lysates revealed that DC, in a concentration-dependent manner, increased Mcl-1 protein expression (Fig. 1A). Cellular Mcl-1 protein levels increased over time after DC treatment and reached a maximum after 24 h of incubation (Fig. 1B). These data directly demonstrate that bile acids such as DC modulate Mcl-1 expression in cholangiocytes.

**Is Bile Acid Activation of the EGFR Responsible for Mcl-1 Induction?** After treatment of KMBC cells with DC, both the EGFR kinase inhibitors, AG1478 and PD168393, and the EGFR neutralizing antibody, which competitively blocks ligand binding, inhibited tyrosine phosphorylation of a protein with a molecular weight of Mr 170,000–190,000 (Fig. 2A). We have previously confirmed, by immunoprecipitation experiments, that this tyrosine-phosphorylated protein in the same cell line was EGFR (4). Upon inhibition of bile acid-mediated EGFR activation by either the pharmacological inhibitor or the blocking antibody, Mcl-1 induction was also inhibited (Fig. 2B). These data suggest that bile acids enhance Mcl-1 protein expression via EGFR activation.

**Is Bile Acid Activation of EGFR-dependent Kinase Cascades Responsible for Enhanced Mcl-1 Protein Expression?** We have previously demonstrated that bile acid activation of the EGFR is functional in activating several MAPK signaling cascades, including p42/44, p38, MAPKs, and JNK (4). We next determined if bile acid activation of these kinases participates in enhanced Mcl-1 protein expression. The inhibitors of MAPK, U0126 for MEK (Fig. 3A) and SB203580 for p38 (Fig. 3B), and the inhibition of JNK activity by DN

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**Statistical Analysis.** All data represent at least three independent experiments using three separate isolations and are expressed as the mean ± SD. Differences between groups were compared using either two-tailed Student’s t tests or a Tukey-Kramer Multiple Comparisons Test.
TAK1 (Fig. 3C) did not block Mcl-1 induction by bile acid. Given that SB203580 also inhibits the phosphatidylinositol 3'-kinase/Akt pathway, it would appear this kinase cascade also does not modulate cellular Mcl-1 protein levels. However, BAY 37-9751, an inhibitor of Raf-1, which is a kinase also activated by EGFR activation (22), blocked Mcl-1 induction by bile acid (Fig. 4). Because the expression level of Mcl-1 has also recently been reported to be regulated by COX-2 (23, 24), we next determined if bile acid-mediated COX-2 induction in our cells is responsible for enhanced Mcl-1 cellular protein levels. However, neither inhibition of COX-2 induction by a p38 MAPK inhibitor nor inhibition of COX-2 activity by a specific COX-2 inhibitor, NS398, blocked the bile acid-stimulated increases in cellular Mcl-1 protein (data not shown). These data suggest that neither COX-2 induction nor its activity is necessary for bile acid-mediated increases of cellular Mcl-1 protein. Collectively, these data suggest that bile acid activation of the EGFR results in increases of cellular Mcl-1 protein via a Raf-1-associated mechanism.

**Do Bile Acids Alter Mcl-1 Transcription?** Bile acids did not increase the level of Mcl-1 mRNA as assessed by real-time PCR (Fig. 5A), suggesting that bile acids do not alter Mcl-1 transcription. However, the protein kinase C agonist, PMA, did increase Mcl-1 mRNA as previously reported (12), indicating that Mcl-1 is transcriptionally regulated in these cells. To further exclude transcriptional regulation of Mcl-1 by DC, a reporter gene assay was performed (Fig. 5B). This assay also failed to demonstrate any effect of DC on transcription from the Mcl-1 promoter. Because mRNA stabilization will also alter rates of protein expression, the effect of DC on Mcl-1 mRNA half-life was assessed by quantitating Mcl-1 mRNA over time in cells treated with the transcriptional inhibitor, actinomycin D. However, DC did not prolong the half-life of Mcl-1 mRNA (Fig. 5C). Taken together, these data suggest that bile acids modulate Mcl-1 protein levels at a posttranscriptional level.

**Do Bile Acids Decrease Mcl-1 Protein Degradation?** Unlike other Bcl-2 family members, Mcl-1 contains a PEST sequence, which is responsible for its short half-life presumably because of continuous and efficient proteasome-mediated degradation (11). To test this concept, we examined the effect of a proteasome inhibitor on cellular Mcl-1 protein levels. The selective proteasome inhibitor, MG-132, increased Mcl-1 protein levels comparable with those observed with DC treatment (Fig. 6A). These data indicate that Mcl-1 can be regulated by posttranslational processes. Therefore, we next examined the effects of DC on Mcl-1 protein stability (Fig. 6B). In the presence of a protein translation inhibitor, cycloheximide, DC increased the half-life of the Mcl-1 protein, and this effect of DC was similar to that observed with the proteasome inhibitor (Fig. 6B). These findings support the hypothesis that bile acids modulate Mcl-1 expression at a posttranscriptional level, specifically by inhibiting Mcl-1 turnover. To integrate these observations with those above, we next determined if the bile acid-associated EGFR/Raf-1 cascade blocks Mcl-1 protein degradation. EGFR kinase inhibitors, which prevented EGFR activation by bile acids (Fig. 2A), also inhibited the bile acid-induced cellular Mcl-1 protein accumulation (Fig. 6C). The Raf-1 inhibitor also blocked Mcl-1 protein accumulation (Fig. 6C). These data, therefore, collectively suggest that bile acid activation of the EGFR/Raf-1 cascade increases cellular Mcl-1 protein by prolonging the protein half-life.

**Do Bile Acids Block Death Receptor-mediated Apoptosis?** Because DC treatment increases cellular Mcl-1 protein in KMBC cells, we next determined if Mcl-1 induction attenuates apoptotic signaling by Fas. Cells were treated with the Fas agonistic antibody to induce apoptosis (Fig. 7). Whereas pretreatment with DC attenuated Fas-induced apoptosis, the Raf-1 inhibitor potentiated Fas apoptosis (Fig. 7). These observations suggest that bile acid-induced increases in cellular Mcl-1 protein may suppress apoptosis by proapoptotic stimuli.
The principal findings of this study relate to the effect of bile acids on EGFR stimulation and Mcl-1 protein expression in a human cholangiocarcinoma cell line. The results demonstrate that (a) bile acids increase cellular Mcl-1 protein levels, thereby blocking proapoptotic stimuli; (b) bile acids increase cellular Mcl-1 protein levels by an EGFR/Raf-1 signaling cascade; and (c) the bile acid EGFR/Raf-1 signaling increases cellular Mcl-1 protein levels by inhibiting Mcl-1 degradation. The results provide new information regarding the antiapoptotic effects of bile acids in cholangiocytes and cholangiocarcinoma. Each of these findings will be discussed below.

We demonstrated that bile acids increase cellular Mcl-1 protein levels in cholangiocytes, and thereby suppress apoptosis by proapoptotic stimuli. Among the bile acids tested, unconjugated DC was more potent than the glyco- and tauro-conjugates in increasing Mcl-1 protein levels (data not shown). The potent Mcl-1 induction by deconjugated DC is in accordance with the previous observation that DC was the most active bile acid in triggering EGFR activation (4). This observation suggested that DC-mediated changes in cellular Mcl-1 protein levels are because of DC activation of the EGFR. Indeed, EGFR kinase inhibitors and a neutralizing antibody, which prevented EGFR activation by DC, inhibited the bile acid increases in cellular Mcl-1 protein. These findings indicate that bile acid activation of the EGFR is functional and is capable of modulating Mcl-1 protein expression. Bile acid enhancement of cellular Mcl-1 protein levels was sufficient to suppress apoptosis by Fas. As cholangiocarcinomas have been shown to overexpress Mcl-1, and suppression of apoptosis is a key feature of human malignancies (6), bile acid-induced modulation of Mcl-1 likely participates in the development and progression of these cancers.

Because bile acid activation of EGFR leads to subsequent kinase activation, including p42/44 and p38 MAPKs and JNK (4), the effect of bile acid/EGFR activation of these kinases on cellular Mcl-1 protein levels was examined. Inhibition of MAPKs did not block the bile acid-stimulated increases in Mcl-1 protein levels. In contrast, Raf-1 inhibition effectively prevented the increases in cellular Mcl-1 levels. Raf-1 can be activated by EGFR signaling and is most commonly known as an upstream kinase that regulates MEK leading to
leukemia specimens (8), and Mcl-1 overexpression has, indeed, recently been reported in cholangiocarcinomas (10). Collectively, our current study demonstrates that bile acids increase cellular Mcl-1 protein levels by blocking its degradation via activation of an EGFR/Raf-1 cascade in a human cholangiocarcinoma cell line. Along with the activation of EGFR/MAPK/COX-2 cascade by bile acids (4), this bile acid-stimulated increases of cellular Mcl-1 protein likely also participate in cholangiocyte carcinogenesis. From a therapeutic perspective, the data suggest that targeting either EGFR/Raf-1 cascades or Mcl-1 could be useful in the treatment of cholangiocarcinomas. The data correlating the sensitivity of these cells to Fas with Mcl-1 expression support this latter concept.

ACKNOWLEDGMENTS

We thank Sara Erickson for her secretarial assistance. We also thank Neil Gibson for providing BAY 37-9751.

REFERENCES


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Fig. 7. Bile acids inhibit Fas-mediated apoptosis in a human cholangiocyte-derived cell line. KMBC cells were incubated in the presence and absence of DC (100 μM) and BAY 24 h, and then, the apoptotic anti-Fas antibody (APO-1-3, 100 nM/ml, FAS), preincubated with protein A, was added for an additional 24 h. Apoptosis was quantitated, using 4',6-diamidino-2-phenyldihydrochloride and fluorescent microscopy. All experiments were performed in 10% FBS-containing media. Data were expressed as mean ± SD from three independent experiments.

p42/44 MAPK activation (22). However, in addition to MEK regulation, Raf-1 may have other signaling regulatory functions (25). Thus, Raf-1 functions, independent of MAPK activation, likely participate in Mcl-1 induction by bile acid activation of EGFR signaling. Maintenance of cellular protein levels is complex and includes the interplay of transcriptional, translational, and posttranslational regulatory processes. As assessed by both real-time PCR and a reporter gene assay, bile acids did not enhance Mcl-1 mRNA expression or promoter activation. Mcl-1 mRNA half-life was also not altered by bile acid treatment. These data implicated a transcriptional or posttranslational process as explanation for bile acid increases of Mcl-1. Indeed, bile acids via an EGFR/Raf-1 mechanism inhibited Mcl-1 protein turnover. This effect of bile acids could also be duplicated by using a proteasome inhibitor. Most studies to date have suggested that receptor tyrosine kinases such as an EGFR transmit survival signals via a phosphatidylinositol 3'-kinase/Akt pathway. This pathway is thought to promote cell survival by modulating phosphorylation of Bad or caspase 9 or by activating nuclear factor kBa (26, 27). Although STAT3-mediated enhancement of Mcl-1 gene transcription has been well characterized, indicating this protein can be transcriptionally regulated (28), the current data implicate an additional process by which receptor tyrosine kinases enhance cell survival, namely, inhibiting the turnover of survival proteins such as Mcl-1.

The proteasome inhibitor, MG-132, also increased cellular Mcl-1 protein levels, suggesting that Mcl-1 is rapidly degraded by the ubiquitin proteasome pathways. The ubiquitin pathway consists of ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-ligase enzymes (29, 30). Whereas a single E1 activates ubiquitin and E2 proteins share a significant homology, the E3s specificity of the ubiquitin system is determined either by substrate-specific E3s or by ancillary proteins associating with a substrate (29). Bile acids may result in Mcl-1 phosphorylation, preventing recognition of Mcl-1 by the ubiquitin system. Indeed, Mcl-1 is a phospho-protein and phosphorylation alters its physiological activity (32). Alternatively, because most deubiquitinating enzymes are now considered to function in the proofreading and reversal of protein ubiquitination (30), bile acids may activate these enzymes via kinase cascades, leading to deubiquitination and stabilization of Mcl-1.

An enlarging body of literatures implicates Mcl-1 in carcinogenesis. For example, transgenic mice overexpressing Mcl-1 exhibit a high incidence of B-cell lymphoma (33). In man, elevated expression of Mcl-1 was demonstrated at the time of leukemic relapse in clinical


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