Bile Salts Control the Antimicrobial Peptide Cathelicidin Through Nuclear Receptors in the Human Biliary Epithelium

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Backgrounds & Aims: **Under normal conditions, the biliary tract is a microbial-free environment. The absence of microorganisms has been attributed to various defense mechanisms that include the physicochemical and signaling actions of bile salts. Here, we hypothesized that bile salts may stimulate the expression of a major antimicrobial peptide, cathelicidin, through nuclear receptors in the biliary epithelium.** ***Methods:*** **The expression of cathelicidin was analyzed in human liver samples by immunostaining and reverse-transcription quantitative polymerase chain reaction. The regulation of cathelicidin expression by the endogenous bile salt, chenodeoxycholic acid, and by the therapeutic bile salt, ursodeoxycholic acid (UDCA), was assessed in human biliary epithelial cells in which endogenous nuclear receptor expression was blunted by siRNA or dominant-negative strategies.** ***Results:*** **In the human liver, biliary epithelial cells show intense immunoreactivity for cathelicidin and for the vitamin D receptor. In cultured biliary epithelial cells, chenodeoxycholic acid and UDCA induce cathelicidin expression through 2 different nuclear receptors: the farnesoid X receptor and the vitamin D receptor, respectively. Importantly, vitamin D further increases the induction of cathelicidin expression by both bile salts. In a prototypical inflammatory biliary disease (ie, primary biliary cirrhosis), we document that hepatic expressions of the vitamin D receptor and of cathelicidin significantly increased with UDCA therapy.** ***Conclusions:*** **Our results indicate that bile salts may contribute to biliary tract sterility by controlling epithelial cell innate immunity. They further suggest that in inflammatory biliary diseases, which involve bacterial factors, a strategy systematically combining UDCA with vitamin D would increase therapeutic efficacy.**

The biliary tract, despite being exposed to bacteria and bacterial products derived from the intestine, is a sterile milieu. Bile flow, mucous secretion, IgA, and bile salts are multiple defense factors that account for this pathogen-free environment.[1] In disease states such as in primary biliary cirrhosis (PBC), sterility may be disrupted, as evidenced by the presence of endotoxins in biliary epithelial cells.[2] In PBC patients, administration of the therapeutic bile salt, ursodeoxycholic acid (UDCA), causes a decrease in intracellular endotoxin accumulation and in circulating anti-endotoxin antibodies.[3,4] These observations and other lines of evidence suggest that bile salts may be central in the antibacterial defense mechanisms of the biliary tract.

First, bile salts are amphipathic molecules that bear direct bacteriolytic properties.[5] Bile salts increase mucus thickening and bile flow, and thereby have the potential to reduce epithelial bacterial colonization.[6] Bile salt–induced bile flow is driven by the stimulation of chloride secretion in biliary epithelial cells through calcium and cyclic adenosine monophosphate–dependent signaling pathways.[7] Recently, we have shown that bile salts may control biliary epithelial secretory functions through nuclear receptor activation.[8] This property may be relevant to epithelial innate defenses because nuclear receptors prone to bile salt activation, such as the farnesoid X receptor (FXR) or the vitamin D nuclear receptor (VDR),[9–11] have been reported to control antibacterial activity in cells of myeloid and epithelial lineages.[12–14]

Activation of VDR results in the induction of cathelicidin expression,[13,14] an antimicrobial peptide known to be protective in vivo against bacterial infection.[15–17] Of particular interest, cathelicidin exerts microbicidal activity against *Escherichia coli*,[18] a bacteria suspected to either cause or exacerbate PBC.[2] From these data, we hypothesized that bile salts may control innate immunity in biliary epithelial cells through VDR-induced cathelicidin expression.

Abbreviations used in this paper: CDCA, chenodeoxycholic acid; ERK, extracellular regulated kinase; FXR, farnesoid X receptor; hBD-2, human β 2 defensins; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; VDR, vitamin D nuclear receptor; VDRE, VDR response element.

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Methods

Reagents

Dulbecco's modified Eagle medium and fetal bovine serum were purchased from Invitrogen (Cergy Pontoise, France). $1\alpha,25(OH)_2D_3$ was provided by Sigma (Saint-Quentin Fallavier, France). GW4064 was purchased from Tocris (Bristol, UK). Sodium chenodeoxycholate (CDCA) and sodium ursodeoxycholate (UDCA) (99% pure), as well as PD98059 were obtained from Calbiochem (Meudon, France). Ribonuclease inhibitor RNazine was purchased from Promega (Charbonnières, France), Moloney murine leukemia virus reverse transcriptase was from Invitrogen, and Taq DNA polymerase was from Perkin–Elmer (Les Ulis, France).

Immunohistochemical Staining

Liver tissue sections (4 μm) were incubated with an anticathelicidin antibody (Hycult Biotechnology, Le Perray-en-Yvelines, France) at a dilution of 1:150 or an anti-VDR antibody (Santa Cruz Biotechnology, Le Perray en Yvelines, France) at a dilution of 1:75. After microwave antigen retrieval (750 W for 15 minutes followed by 150 W for 15 minutes in 10 mmol/L citrate buffer; pH 6), immunolabeling was performed using a SuperSensitive Link-Label Immunohistochemistry detection system (Biogenex, San Ramon, CA) according to the manufacturer's protocol. Peroxidase activity was detected using 3-amino-9-ethyl carbazole as the substrate and Mayer's hematoxylin for counterstaining. Human tissue specimens were collected using informed consent in accordance with Inserm ethical guidelines.

Cell Culture

The human biliary epithelial cell line Mz-ChA-1[19] was cultured in Dulbecco's modified Eagle medium, supplemented with 1 g/L glucose, 10 mmol/L HEPES, and 10% fetal bovine serum, under 95% air and 5% CO_2 at 37°C. The culture medium was renewed every 48 hours.

Preparation of Cell Extracts

Whole-cell extracts, nuclear extracts, and cytosolic extracts were prepared as follows. For whole-cell extracts, Mz-ChA-1 cells were lysed in a buffer composed of 50 mmol/L Tris (pH 7.4), 450 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% NP-40. For preparation of cytoplasmic and nuclear extracts, we performed cell fractionation with the NE-PER kit (Pierce, Berbières, France) as recommended by the manufacturer with minor modifications. Briefly, cells were incubated for 10 minutes with accutase (PAA Laboratories, Les Mureaux, France) to detach cells from culture flasks. Cells then were collected by centrifugation at 1500 $\times$ *g* for 5 minutes. The cell pellet was resuspended in adequate volume of cytoplasmic extraction buffers. Cells then were centrifuged at maximal speed for 5 minutes. The cytoplasmic extract (supernatant) was collected and stored at −80°C. The pellet fraction was resuspended in nuclear extraction reagent and centrifuged at maximal speed for 10 minutes. The supernatant containing the nuclear extract was collected and stored at −80°C. Protein concentration was determined using the BCA Protein Assay (Pierce).

Immunoblot Analyses

Mz-ChA-1 cells were incubated either with CDCA (10–200 μmol/L) or UDCA (10–200 μmol/L) for 1–24 hours. In mitogen-activated protein kinase inhibition experiments, Mz-ChA-1 cells were preincubated with PD 98059 (50 μmol/L) for 1 hour before either CDCA (100 μmol/L), UDCA (100 μmol/L), or $1\alpha,25(OH)_2D_3$ (0.1 μmol/L) was added and maintained for the following 2 hours. Whole-cell protein extracts (20 μg) were subjected to electrophoresis through a 10% sodium dodecyl sulfate

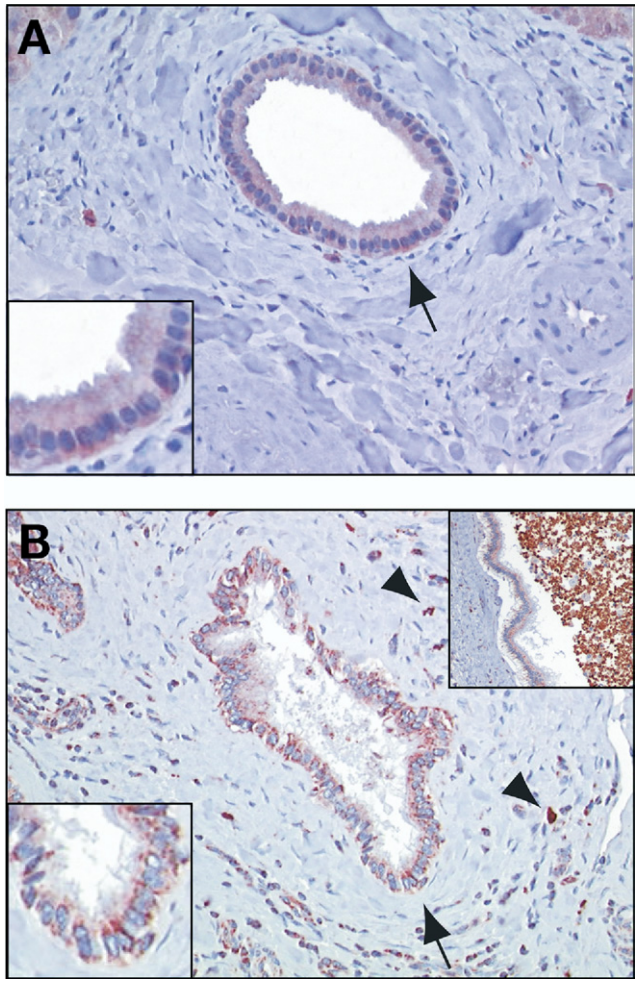

Figure 1. Cathelicidin expression in the human biliary epithelium. Representative immunostaining of cathelicidin in the biliary epithelium (*A*) within normal human liver and (*B*) in the liver of a patient with suppurative cholangitis. Immunostaining is localized in biliary epithelial cells (*arrows* and higher magnification within *insets in the lower left corners*) and in infiltrating inflammatory cells (*arrowheads*). *Inset in the upper right corner* of panel *B* illustrates positive staining in the biliary tract lumen.

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