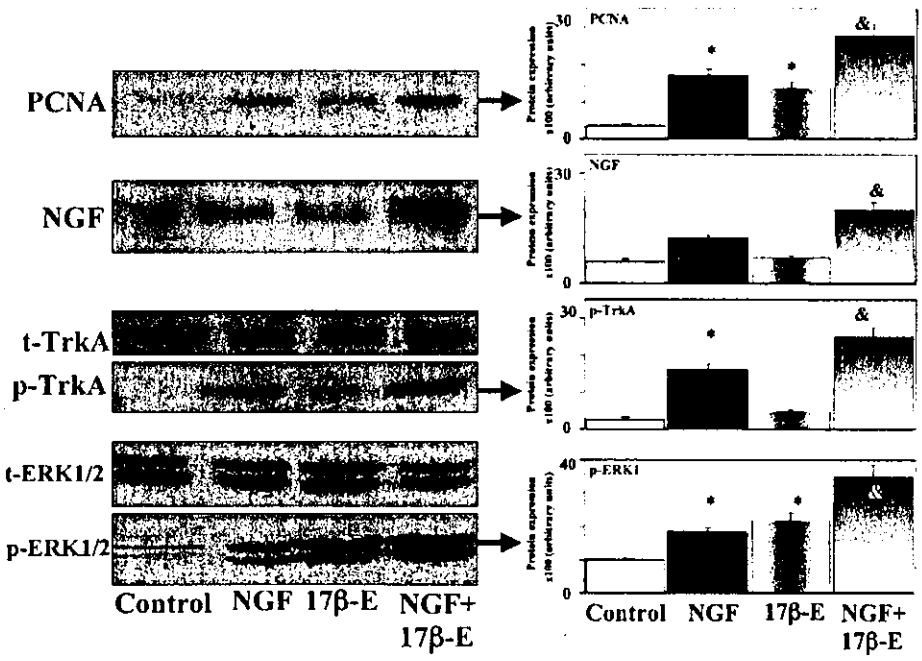

Figure 7. Additive effect of estrogens and NGF on cholangiocyte proliferation in vitro. Cholangiocytes isolated from normal rats (quiescent cells) were incubated (3 hours) with NGF (100 ng/mL) and/or 17β-estradiol (10 nmol/L), and PCNA, NGF, TrkA, and ERK1/2 protein expression was evaluated by Western blot. PCNA and p-ERK1/2 protein expression was higher in cholangiocytes incubated with NGF and/or 17β-estradiol in comparison with NGF or 17β-estradiol alone. 17β-estradiol showed no effect on the protein expression of NGF or p-TrkA but, when added together with NGF, induced an increase of NGF or p-TrkA protein expression in cholangiocytes that was higher in comparison with NGF or 17β-estradiol alone. The protein expression of total TrkA and total ERK was similar in the different experiments. $^{*}P < 0.05$ vs. controls; $^{\&}P < 0.05$ vs. NGF or 17β-estradiol alone.

rats; (3) cholangiocytes also express (RT-PCR) neurotrophin 4/5 and neurotrophin receptors TrkC, the low-affinity NGF receptor, and neurophilin 1; (4) NGF induces, in vitro, proliferation of normal cholangiocytes through the activation of both ERK and PI3-kinase pathways and exerts additive effects with estrogens; and (5) in BDL rats, the in vivo neutralization of NGF, through long-term administration of anti-NGF antibody, impairs the proliferative response of cholangiocytes to BDL, enhances apoptosis, and increases portal inflammation. Altogether, these findings indicate that NGF and its related receptors play an important role in modulating the physiopathology of the intrahepatic biliary epithelium and inflammatory processes in the course of extrahepatic cholestasis.

The expression of neurotrophins and their receptors in human and rodent liver cells has been investigated in previous studies with different results. Shibayama et al.,[24] for example, failed to show any reactivity to Trk antibodies in human liver. Other studies, by RT-PCR or immunohistochemistry on human or rat liver sections, found abundant neurotrophin-3 transcription and trace levels of TrkB transcription as well as immunohistochemical positivity for neurotrophin 3 in all parenchymal liver cells.[25,26] Finally, Lomen-Hoerth and Shooter[27] showed a low level of low-affinity NGF receptor transcription in rat liver by ribonuclease protection assay but were not able to show any TrkA or TrkB transcription. As far as cholangiocytes are concerned, proliferative peribiliary glands of patients with hepatolithiasis showed an immunoreactivity for NGF.[28] In addition, a study by Cassiman et al.[29] showed immunoreactivity for neurotrophin 4/5 and TrkB but not for NGF in intrahepatic bile ducts of human (normal and cirrhotic liver) and rat (normal and galactosamine-treated) liver sections. No study has yet been performed in pure preparations of isolated cholangiocytes, and the role of neurotrophins in the modulation of cholangiocyte functions is virtually unknown.

In the present study, in pure preparations of cholangiocytes isolated from normal and BDL rats, we explored the expression of different neurotrophins and neurotrophin receptors by nonquantitative RT-PCR. Thereafter, our study was focused on NGF and the NGF preferred receptor TrkA, whose expression was significantly increased (Western blot) in cholangiocytes proliferating after BDL in comparison with normal quiescent cells. In contrast to RT-PCR and Western blot, immunohistochemistry for NGF and TrkA was negative in normal cholangiocytes. This discrepancy, which likely depends on the high sensitivity of RT-PCR and the amount of proteins loaded for immunoblotting, indicates a low expression of NGF and TrkA in normal cells and their scarce availability for the antibodies used for immunohistochemistry. After BDL, in agreement with the increased protein mass detected by Western blot, proliferating cholangiocytes showed immunohistochemical positivity for NGF and TrkA, which predominates in the

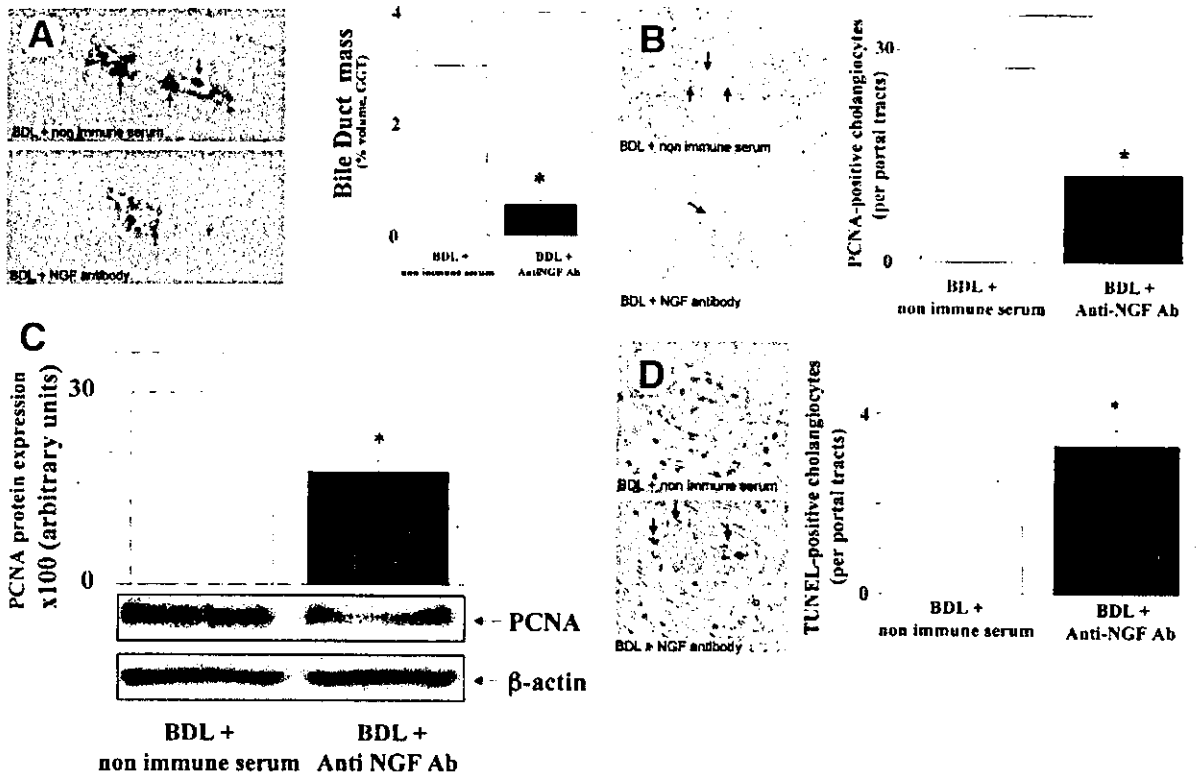

Figure 8. Effect of long-term administration of immunoneutralizing anti-NGF antibody on bile duct mass and proliferation of cholangiocytes in BDL rats. (*A*) BDL rats were treated for 7 days with an anti-NGF antibody capable of immunoneutralizing endogenous NGF ($n = 8$) or with nonimmune serum (controls, $n = 8$). Bile duct mass, evaluated by quantitative morphometry after γ-glutamyltransferase staining, was significantly decreased by anti-NGF antibody treatment in comparison with controls ($^*P < 0.01$). (*B*) Immunohistochemistry for PCNA (marker of proliferation) in the liver of BDL rats treated with immunoneutralizing anti-NGF antibody or nonimmune serum. In BDL rats, treatment with anti-NGF antibody induced a marked decrease ($^*P < 0.05$) in the number of PCNA-positive cholangiocytes in comparison with BDL rats treated with nonimmune serum. (*C*) Western blot analysis of PCNA in cholangiocytes isolated from BDL rats treated with immunoneutralizing anti-NGF antibody or nonimmune serum. In BDL rats, treatment with anti-NGF antibody induced a marked decrease ($^*P < 0.05$) in the protein expression of PCNA in comparison with BDL rats treated with nonimmune serum. β-actin was similar. (*D*) TUNEL staining in the liver of BDL rats treated with immunoneutralizing anti-NGF antibody or nonimmune serum. The number of TUNEL-positive cholangiocytes (per portal tract) after treatment with neutralizing anti-NGF antibody was significantly higher with respect to BDL controls treated with nonimmune serum ($^*P < 0.05$).

cytoplasm. Recent studies have shown that binding of NGF to its cognate receptor TrkA induces formation of signaling endosomes containing both the NGF and activated TrkA receptor, with the latter displaying very high dynamic trafficking between the cell surface and internal cell locations.[30,31] Therefore, the predominant cytoplasmic staining for TrkA in proliferating cholangiocytes likely represents the receptor endocellular trafficking. Nevertheless, the fact that a portion of TrkA is effectively located in basolateral plasma membranes of BDL cholangiocytes was shown by Western blot performed in plasma membrane subfractions. Other than overexpressing NGF, proliferating cholangiocytes also secrete this neurotrophin in higher amounts than nonproliferating cells, as indicated by measurement of NGF concentration in the supernatant of BDL and normal cholangiocyte suspensions. This finding, consistent with a 4-fold increase of NGF serum level in BDL rats, indicates an increased endogenous production and secretion of NGF by proliferating cholangiocytes and suggests a paracrine or autocrine action on TrkA receptors expressed by the same or contiguous cell. Indeed, the supernatant of BDL but not normal cholangiocytes induces proliferation of normal quiescent cholangiocytes, an effect partially blocked by anti-NGF antibodies (Figure 6). The effects of NGF are mediated by binding to its high-affinity receptor, TrkA, which induces TrkA dimerization and autophosphorylation.[11,12] Furthermore, the expression of TrkA together with low-affinity p75 receptor and neurophilin 1,[11,12] which are known to regulate the effect of NGF on cell proliferation, further suggests that the complex network of NGF receptors and modu-

lators, including neurotrophin 4/5, are actively operating in the regulation of cholangiocyte pathophysiology.

Next, we directly evaluated the effect of NGF on cholangiocyte proliferation in vitro and the effect of NGF immunoneutralization on the proliferative response of the intrahepatic biliary epithelium to BDL in vivo. The in vitro exposure of normally quiescent cholangiocytes to NGF induces a marked increase in the protein expression of PCNA, and this is indicative of stimulated cell proliferation. In association with enhanced PCNA protein expression, a significant and rapid (5-minute) increase in ERK and Akt phosphorylation occurs in cholangiocytes incubated with NGF, whereas phosphorylation of JNK and p38 occurs later (30–60 minutes). This indicates that the ERK and PI3-kinase are the intracellular signaling transduction pathways, which are immediately activated by NGF in cholangiocytes. Consistently, NGF-induced cholangiocyte proliferation (i.e., PCNA expression) was significantly decreased by the MEK inhibitor[32] UO125 and completely abolished by the PI3-kinase inhibitor wortmannin,[21] with both inhibitors able to completely abolish phosphorylation of their respective target proteins in cholangiocytes. A number of previous studies have shown that in cells expressing the TrkA receptor, NGF modulates growth and differentiation by acting on the Ras/Raf/ERK as well as on the PI3/Akt kinase cascade, with the latter activated via a Ras-dependent mechanism as well as through the recruitment of the adaptor proteins Shc, Grb-2, and Gab-1.[11,12,33,34] In nerve tissues, survival, apoptosis, differentiation, and response to injury are all controlled by the modulation of these signaling pathways by neurotrophins.[1,12,33–35]

We have also shown that NGF exerts additive effects with estrogens[36] on modulating cholangiocyte proliferation and that this effect is played at the level of the ERK pathway, as p-ERK1/2 protein expression was synergistically enhanced by NGF plus 17β-estradiol. However, the interaction between estrogens and NGF in modulating cholangiocyte proliferation involves multiple mechanisms, because 17β-estradiol also augmented the effect of NGF on phosphorylation of TrkA receptors and on NGF expression in cholangiocytes. In keeping with the above, in other cell types it has been hypothesized that estrogens and NGF may act synergistically by cross-talk between receptors or at the level of common intracellular pathways.[22,23]

We next showed that the in vivo immunoneutralization of endogenous NGF in BDL rats was followed by a depression of cholangiocyte proliferation and enhancement of apoptosis, resulting in decreased bile duct mass in response to BDL. The effective immunoneutralization of the endogenous NGF by the in vivo administration of the anti-NGF antibody we used in our study has been documented in a number of previous studies[19,20] with the same doses and modalities of administration. The anti-NGF antibody should link NGF in the extracellular compartment, hampering the NGF binding to TrkA in the basolateral plasma membranes. Moreover, the immunohistochemical evaluation of NGF and TrkA receptors showed no marked changes, and therefore it is unlikely that the decreased bile duct mass and cholangiocyte proliferation are the consequence of an inhibitory effect of anti-NGF on the hepatic expression of NGF and TrkA receptor. In the BDL model of experimental cholestasis, we also showed an anti-inflammatory effect of endogenous NGF. In fact, portal inflammation associated with BDL significantly worsened when BDL rats were treated with anti-NGF antibody. Similar effects of anti-NGF antibodies have been previously described during experimental colitis, arthritis, or pancreatitis, suggesting a general anti-inflammatory role of NGF[20,37,38] that may modulate the responsiveness of immune-competent cells in inflammation.[37,38]

Because stellate cells also produce NGF and express neurotrophin receptors, including TrkA,[31] our findings may suggest a cross-talk between cholangiocytes and stellate cells, where NGF and the related receptors are involved. This could explain why, in different experimental models of hepatic regeneration and of cholestasis, proliferation of cholangiocytes in reactive bile ductules and activation, proliferation, and migration of stellate cells are associated events.[39,40]

In conclusion, we provide evidence that NGF plays a role in modulating the proliferation of cholangiocytes; this further supports the concept of neuroendocrine features acquired by the intrahepatic epithelium during proliferation. Together with the observation that NGF was also able to modulate portal inflammation, this should stimulate further studies aimed at managing the progression of chronic cholestatic liver disease by using NGF or modulators of neurotrophin receptors. Chronic cholestatic liver diseases are, in fact, characterized by both compensatory proliferation of cholangiocytes and portal inflammation, which heavily conditions the evolution of the disease toward the terminal ductopenic stage. The pharmacologic modulation of the neurotrophin network could represent a future therapeutic strategy for the management of cholangiopathies.

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cAMP stimulates the secretory and proliferative capacity of the rat intrahepatic biliary epithelium through changes in the PKA/Src/MEK/ERK1/2 pathway

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***Background/Aims*: To evaluate if increased cholangiocyte cAMP levels alone are sufficient to enhance cholangiocyte proliferation and secretion.**

***Methods*: Normal rats were treated in vivo with forskolin for two weeks. Cholangiocyte apoptosis, proliferation and secretion were evaluated. Purified cholangiocytes from normal rats were treated in vitro with forskolin in the absence or presence of Rp-cAMPs (a PKA inhibitor), PP2 (an Src inhibitor) or PD98059 (a MEK inhibitor). Subsequently, we evaluated cholangiocyte proliferation by determination of proliferating cellular nuclear antigen (PCNA) protein expression by immunoblots. We evaluated if the effects of forskolin on cholangiocyte functions are associated with changes in the cAMP/PKA/Src/MEK/ERK1/2 pathway.**

***Results*: Chronic administration of forskolin to normal rats increased the number of ducts, cAMP levels, and secretin-induced choleresis compared to controls. Forskolin-induced increases in cholangiocyte proliferation and secretion were devoid of cholangiocyte necrosis, inflammation and apoptosis. In vitro, in pure isolated cholangiocytes, forskolin increased cholangiocyte proliferation, which was ablated by Rp-cAMPs, PP2 and PD98059. The effects of forskolin on cholangiocyte proliferation were associated with increased activity of PKA, Src Tyrosine 139 (Tyr 139) and ERK1/2.**

***Conclusions*: Modulation of the PKA/Src/MEK/ERK1/2 pathway may be important in the regulation of cholangiocyte growth and secretion observed in cholestatic liver diseases.**

***Keywords*: Bicarbonate secretion; Biliary epithelium; Proliferation; Secretin receptor**

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Abbreviations: cAMP, cyclic adenosine 3′,5′-monophosphate; BSA, bovine serum albumin; CK-19, cytokeratin-19; ERK, extracellular signal-regulated kinase; γ-GT, γ-glutamyltranspeptidase; PCNA, proliferating cellular nuclear antigen; PKA, protein kinase A.

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1. Introduction

Cholangiocytes are the target cells in several cholestatic liver diseases characterized by cholangiocyte proliferation or loss [1]. In animal models, cholangiocyte proliferation is achieved by pathological maneuvers including bile duct ligation (BDL) [1–4], partial hepatectomy [5], or feeding α-naphthylisothiocyanate (ANIT) [6], whereas cholangiocyte loss occurs after acute carbon tetrachloride (CCl_4) administration [7]. While cholangiocyte proliferation is associated with increased ductal secretion [1–6,8,9], cholangiocyte loss is characterized by impaired ductal secretion [7].

The cAMP system regulates cellular proliferation in several epithelia [10,11]. Cell proliferation is regulated by a series of phosphorylation events through the cAMP/PKA/Src/MEK/ERK1/2 signaling cascade [12]. Cholangiocyte proliferation is associated with increased cAMP levels whereas cholangiocyte loss is characterized by reduced cAMP levels [1,3–6,8,9]. Although these studies suggest a link between cholangiocyte proliferation and cAMP levels [1,3–6,8,9], direct evidence for the role of cAMP in the regulation of cholangiocyte proliferation is lacking. The aims of the studies were to demonstrate that: (i) increased cAMP levels (by forskolin) alone are sufficient to activate cholangiocyte proliferation and secretion; and (ii) forskolin activation of cholangiocyte proliferation and secretion is associated with changes in the PKA/Src/MEK/ERK1/2 pathway.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). RIA kits for the determination of intracellular cAMP levels were purchased from Amersham (Arlington Heights, IL). The rabbit anti-phospho-Src-Tyr 139, goat antiphospho-Src-Tyr 530, mouse anti total Src, mouse anti-phospho-ERK1/2, goat anti-ERK1 or anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2. Animal model

Male Fischer 344 rats (150–175 g) were purchased from Charles River (Wilmington, MA). The animals were kept in a temperature-controlled environment (22 °C) with a 12-hour light-dark cycle. The in vivo studies were conducted in normal rats treated for 2 weeks with: (i) DMSO-soluble forskolin (0.04 mg/100 g BW) twice a day IP dissolved in 0.9% NaCl:DMSO 1:1 [4] or 0.9% NaCl:DMSO 1:1 control solution [4]; or (ii) water-soluble (i.e. forskolin, 7-deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-, dihydrochloride) forskolin [13] (Calbiochem, San Diego, CA) or 0.9% NaCl. The in vitro studies were performed in purified (98–99% pure by γ-GT histochemistry) [14] cholangiocytes from normal rats. Before each procedure, animals were anesthetized with sodium pentobarbital (50 mg/kg IP). Study protocols were performed in compliance with the institution guidelines.

2.3. Purification of cholangiocytes

Purified cholangiocytes (see Fig. 1) were isolated by immunoaffinity separation [3–5,15] using a monoclonal antibody against an unidentified antigen expressed by all intrahepatic cholangiocytes [15]. Purity was assessed by γ-GT histochemistry [14]. In previous studies [16], we have shown that other cells including hepatocytes and mesenchymal cells do not contaminate our cholangiocyte preparations.

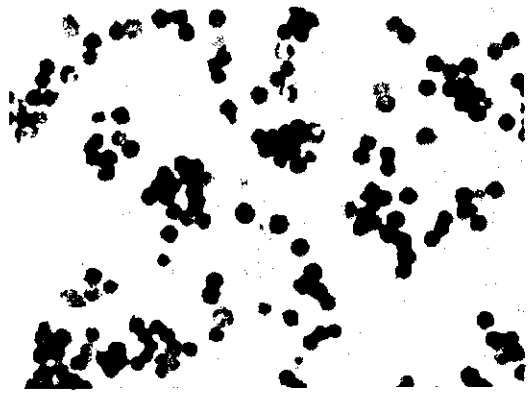

Fig. 1. Histochemistry for γ-GT (a biliary-specific marker) in virtually pure cholangiocytes from normal rats. In this field, all cholangiocytes are positive for γ-GT (arrow). Similar to our previous studies [16], other cells including hepatocytes, mesenchymal cells and vascular cells do not contaminate our cholangiocyte preparations. Orig. magn., ×125.

2.4. In vivo studies

We determined if: (i) chronic administration of forskolin to normal rats alters cholangiocyte functions; and (ii) forskolin-induced changes in cholangiocyte functions are associated with changes in the cAMP/PKA/Src/MEK/ERK1/2 pathway.

2.5. Cholangiocyte proliferation

Cholangiocyte proliferation was evaluated by: (i) quantitative immunohistochemistry for PCNA [6] or cytokeratin-19 (CK-19) or histochemistry for γ-GT [14] in liver sections; and (ii) measurement of PCNA protein expression by immunoblots [3] in purified cholangiocytes from the selected group of animals. Paraffin-embedded sections (5 μm thick, $n = 3$ for each group) were used for PCNA immunohistochemistry [6]. Immunohistochemistry for CK-19 [5] and histochemistry for γ-GT [14] was performed in frozen sections (5 μm thick, $n = 3$ for each group). In each liver section stained for γ-GT, the entire area was examined by multiple photographs taken in a mosaic fashion [5]. From the total number of points over hepatic tissue and the number of points over γ-GT positive ducts, we calculated the volume percent of liver occupied by ducts [17]. Following the selected staining, sections were counterstained with hematoxylin and examined in a random, blinded fashion with an Olympus BX 40 microscope (Olympus Optical Co., Ltd, Tokyo, Japan). Data were expressed as number of: (i) PCNA- or CK-19 positive cholangiocytes per each 100 cholangiocytes; and (ii) γ-GT-positive ducts counted in seven different fields. DNA replication was also evaluated by measurement of PCNA protein expression [3,6] by immunoblots in cholangiocytes from the selected groups of animals. The intensity of the bands was determined by scanning video densitometry using the Chemilmager™ 4000 low light imaging system (Alpha Innotech Corp., San Leandro, CA).

2.6. Effect of forskolin on liver morphology, serum chemistry and hormone serum levels

Similar to our previous studies in BDL rats [2], although not specific only to cholangiocyte growth, we utilized the liver to body weight ratio as an index [2] of increased hepatic growth after forskolin administration. We determined if administration of forskolin alters cholangiocyte apoptosis and portal inflammation compared to control rats. Sections (5 μm thick) were stained with H&E or TUNEL to evaluate cholangiocyte apoptosis [4,7]. We

evaluated the effects of forskolin on portal inflammation [18] and liver morphology. Following the selected staining, liver sections were examined by light microscopy with an Olympus BX-40 microscope equipped with a camera. We evaluated the effect of forskolin on liver damage by assessing transaminase serum levels [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)]. To determine that the effects of forskolin on cholangiocyte proliferation are not due to the release of gastrointestinal hormones in the blood potentially affecting cell growth [4], we evaluated the serum levels of gastrin, somatostatin and insulin by radioimmunoassay using commercially available kits obtained, respectively, from Diagnostic Products Corporation (DPC, Los Angeles, CA), Phoenix Pharmaceuticals (Belmont, CA), and Linco Research Inc. (St. Charles, MO).

2.7. Ductal secretion

Following anesthesia with sodium pentobarbital, rats were surgically prepared for bile collection [2]. When steady-state bile flow was achieved (60–70 min from the beginning of bile collection), 100 nM secretin was infused for 30 min followed by a final infusion of Krebs Ringer Henseleit for 30 min. Bile was collected every 10 min and immediately stored at −70 °C before determining bicarbonate concentration. Biliary bicarbonate concentration (measured as total CO_2) was determined by an ABL™ 520 Blood Gas System (Radiometer Medical A/S, Copenhagen, Denmark).

2.8. In vivo effects of forskolin on cholangiocyte functions are associated with changes in the cAMP/PKA/Src/MEK/ERK1/2 pathway

Basal and secretin-stimulated intracellular cAMP levels of cholangiocytes were determined by RIA [5,19]. Following purification, cholangiocytes were incubated for 1 h at 37 °C [5,19], and stimulated (1×10^5 cells) with 0.2% BSA (basal) or 100 nM secretin for 5 min at 22 °C [5,19]. Following ethanol extraction, cAMP samples were dried under nitrogen and stored at −70 °C before using.

We evaluated in purified cholangiocytes if forskolin administration increases PKA activity. PKA assay was performed according to the manufacturer's instructions using PepTag Assay Protein Kinase Kits (Promega, Madison, WI) for PKA [20,21]. Phosphorylated and non-phosphorylated peptide bands were visualized on a 0.8% agarose gel. Phosphorylated peptide bands were quantitated by scanning densitometry using the ChemiImager™ 4000 low light imaging system (Alpha Innotech Corp.).

The expression of total and phosphorylated Src and ERK1/2 in purified cholangiocytes was evaluated by immunoblotting. Membranes were stripped to evaluate the protein expression of both the phosphorylated and total amounts of protein. Stripping buffer from Pierce was utilized according to the manufacturer's instructions. Expression of Src in cholangiocytes was evaluated by measuring phosphorylation of Src at both the Tyr-139 and Tyr-530 residues by immunoblots [3]. The phosphorylation at Tyr-139 is associated with a significant increase in Src kinase activity [22], whereas the phosphorylation of Tyr-530 negatively regulates the kinase activity [23]. The intensity of the bands was determined by scanning video densitometry using the ChemiImager™ 4000 low light imaging system (Alpha Innotech Corp.).

2.9. In vitro studies

In in vitro studies in normal cholangiocytes, we determined if: (i) cAMP analogues directly interact with cholangiocytes altering proliferative capacity of these cells; and (ii) the cAMP/PKA/Src/MEK/ERK1/2 pathway is involved in forskolin stimulation of cholangiocyte proliferation.

2.10. Evaluation of cholangiocyte proliferation

Purified normal cholangiocytes (3×10^6) were incubated at 37 °C with: (i) 0.2% BSA (basal value) for 60 min; (ii) forskolin (10^{-4} M) for 60 min; or (iii) Rp-cAMPs (a PKA inhibitor, 100 μM) [24], PP2 (a Src inhibitor, 1 μM) [25] or PD98059 (a MEK inhibitor, 10 nM) [26] for 30 min in the absence or presence of forskolin. We evaluated cholangiocyte proliferation by determination of PCNA protein expression by immunoblots [3,6]. The intensity of the bands was determined by scanning video densitometry (ChemiImager™ 4000 low light imaging system, Alpha Innotech Corp.).

2.11. In vitro effect of forskolin on the cAMP/PKA/Src/MEK/ERK1/2 pathway

Normal cholangiocytes (1×10^5) were incubated in vitro at 22 °C [5, 19] with: (i) 0.2% BSA (basal value) for 5 min; or (ii) forskolin (10^{-4} M) for 5 min. cAMP levels were evaluated by RIA [5,19]. To evaluate the in vitro effects of forskolin on the cAMP/PKA/Src/MEK/ERK1/2 pathway, we measured PKA activity and protein expression for total and phosphorylated Src-Tyr 139 and ERK1/2 in normal cholangiocytes stimulated with: (i) 0.2% BSA (basal value) for 60 min; or (ii) forskolin (10^{-4} M) for 60 min. The intensity of the bands was determined by scanning video densitometry using the ChemiImager™ 4000 low light imaging system (Alpha Innotech Corp.).

2.12. Statistical analysis

All data are expressed as mean ± SEM. The differences between groups were analyzed by Student's *t*-test when two groups were analyzed or analysis of variance (ANOVA) if more than two groups were analyzed.

3. Results

3.1. In vivo administration of forskolin increases cholangiocyte proliferation

Administration of DMSO-soluble forskolin to normal rats increased the number of PCNA-positive cholangiocytes compared to rats receiving control solution (Fig. 2a). Similarly, both DMSO-soluble and water-soluble significantly increased the number of CK-19-positive cholangiocytes (Fig. 2b) and γ-GT-positive ducts (Fig. 2c) compared with control-treated rats. PCNA protein expression was also increased in purified cholangiocytes (Fig. 1) from rats treated with DMSO-soluble forskolin compared to cholangiocytes from control-treated rats (Fig. 3).

3.2. Effect of forskolin on liver morphology, serum chemistry and gastrointestinal serum levels

Morphological evaluation of liver parenchyma showed absence of inflammatory infiltrate and necrosis in portal tracts and in the lobule of normal as well as forskolin treated rats. Evidence of apoptosis in the biliary epithelium was low in control as well as in forskolin treated animals (0.012 ± 0.009% vs. 0.015 ± 0.006%), $n = 3$, (not significant), respectively. The increase in body and liver weight (observed following forskolin administration) was not significant compared to the body and liver weight of normal rats treated with control solution (Table 1). We found that the liver to body weight ratio (an index of liver growth) [2] was significantly higher in forskolin-treated rats compared to control-treated rats (Table 1). Consistent with the concept that forskolin stimulation of cholangiocyte proliferation is devoid of liver damage, there was no difference in transaminase serum levels between forskolin- or control-treated rats (Table 2). Serum levels of insulin, gastrin

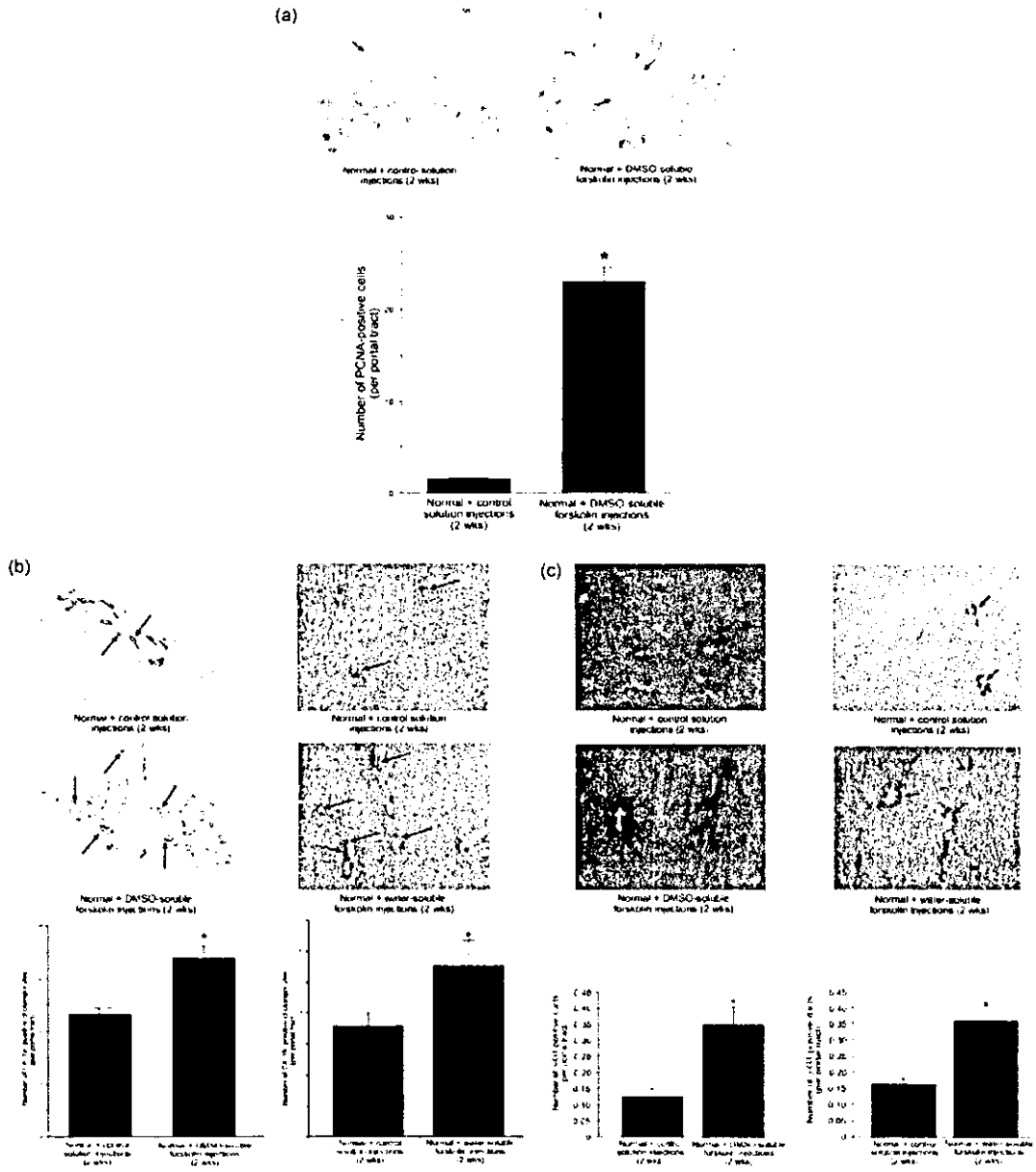

Fig. 2. Measurement of the number of PCNA- (a) and CK-19- (b) positive cholangiocytes and γ-GT positive ducts (c) in normal rats treated in vivo with DMSO- or water-soluble forskolin (0.04 mg/100 g BW, twice daily IP) or control solution for 2 weeks. Forskolin treatment induces a marked increase in the number of PCNA-, and CK-19-positive cholangiocytes and γ-GT-positive ducts per portal tracts. Data are mean ± SEM of six experiments for PCNA and CK-19 staining. Data are mean ± SEM of eight experiments for γ-GT staining. *$P < 0.05$ vs. the corresponding basal value.

and somatostatin were similar in forskolin- or control-treated rats (Table 3). These findings demonstrate that the stimulatory effects of forskolin on cholangiocyte proliferation are due to forskolin-induced increases in cAMP and not from systemic effects or the release of gastrointestinal hormones by forskolin.

3.3. Administration of forskolin increases cholangiocyte secretion

In control rats, basal bile flow, bicarbonate concentration and secretion were similar to that of previous studies (Table 4) [2,5]. Secretin, administered via the jugular vein,

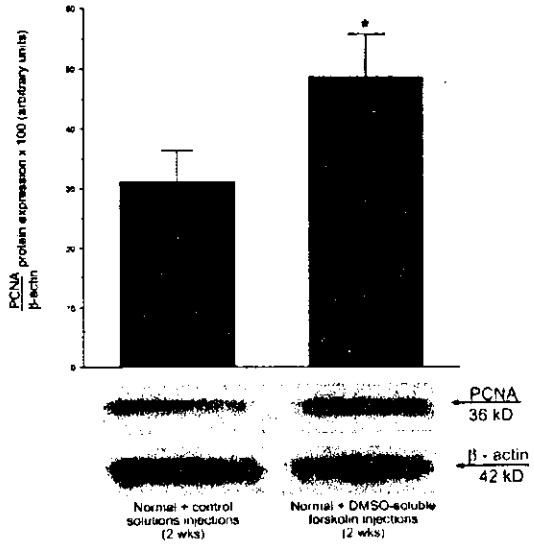

Fig. 3. Immunoblots for PCNA in cholangiocytes from normal rats treated in vivo for 2 weeks with DMSO-soluble forskolin or control solution. Administration of forskolin to rats increased PCNA protein expression in purified cholangiocytes compared with cholangiocytes from rats treated with control solution. Autoradiograms were quantitated by densitometry. Data are mean ± SEM of five experiments. *$P < 0.05$ vs. its corresponding basal value.

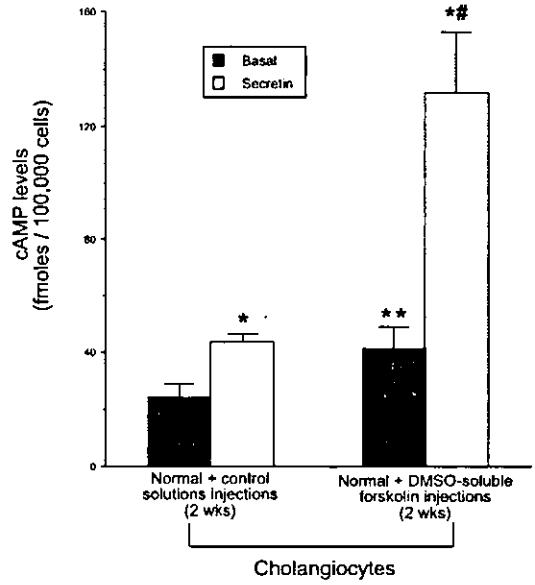

Fig. 4. Measurement of basal and secretin-stimulated cAMP levels in cholangiocytes from normal rats treated in vivo for 2 weeks with DMSO-soluble forskolin or control solution. Data are mean ± SEM of 14 experiments. *$P < 0.05$ vs. its corresponding basal value. **$P < 0.05$ vs. basal cAMP levels of cholangiocytes from control-treated rats. #$P < 0.05$ vs. secretin-stimulated cAMP levels of cholangiocytes from control rats.

did not alter bile flow, bicarbonate concentration or secretion in control rats (Table 4). Administration of forskolin induced a significant increase in secretin-stimulated bile flow, bicarbonate concentration and secretion (Table 4).

3.4. Forskolin-induced increase of cholangiocyte proliferation is associated with changes in cAMP levels and the PKA/Src/MEK/ERK1/2 pathway

Basal and secretin-stimulated cAMP levels were increased in cholangiocytes from forskolin-treated rats compared with cholangiocytes from control-treated rats (Fig. 4). In vivo administration of forskolin increased PKA activity compared with cholangiocytes from control-treated rats (Fig. 5a). There was a decrease in Src-Tyr 530 and an increase in Src-Tyr 139 phosphorylation (expressed as ratio to total Src protein expression) in cholangiocytes from forskolin-treated rats compared to control-treated rats (Fig. 5b). Both forms of phosphorylated ERK1/2 (expressed as ratio to total ERK1/2 protein expression) were increased in cells from rats treated with forskolin for 2 weeks compared to cells from normal rats treated with control solution (Fig. 5c).

3.5. Forskolin in vitro increases proliferation of normal cholangiocytes by changes in the PKA/Src/MEK/ERK1/2 Pathway

We performed in vitro studies in purified cholangiocytes to determine that the stimulatory effects of forskolin on cholangiocyte proliferation are due to a direct interaction with cholangiocytes. In vitro, forskolin increased cAMP levels of normal cholangiocytes compared to cholangiocytes treated with BSA (Fig. 6a). Consistent with the concept that forskolin increases cholangiocyte proliferation by changes in the PKA/Src/MEK/ERK1/2 pathway, forskolin stimulation of PCNA protein expression was blocked by Rp-cAMPs, PP2 and PD98059 (Fig. 6b). Similar to that observed in vivo, forskolin increased PKA activity compared to cholangiocytes treated with BSA (Fig. 7a). There

Table 1
Liver weight, body weight and liver to body weight ratio in normal rats treated for 2 weeks with DMSO-soluble forskolin or forskolin control solution

Treatment	Liver weight (g)	Body weight (g)	Liver to body weight ratio (%)
Normal + control injections (2 weeks)	10.25 ± 0.11 ($n = 27$)	216.00 ± 3.50 ($n = 27$)	4.78 ± 0.16 ($n = 27$)
Normal + forskolin injections (2 weeks)	11.28 ± 0.57 ($n = 24$)	221.49 ± 3.10 ($n = 24$)	5.07 ± 0.23* ($n = 24$)

Values are mean ± SEM. *$P < 0.05$ vs. corresponding values of control rats.

Table 2
Serum enzyme levels in normal rats treated with DMSO-soluble forskolin or forskolin control solution for 2 weeks

Treatment	AST (International units/ml)	ALT (International units/ml)
Normal + control injections (2 weeks)	26.11 ± 2.45	27.85 ± 3.21
Normal + forskolin injections (2 weeks)	26.55 ± 2.31[a]	21.70 ± 1.93[a]

Values are mean ± SEM of six values from animals treated with DMSO-SOLUBLE forskolin or a control solution for 2 weeks. Serum levels were measured by commercially available kits. AST, aspartate aminotransferase; ALT, alanine aminotranferase.
[a] ns vs. the corresponding value of normal rats treated with a control solution for 2 weeks.

Table 3
Hormone serum levels (gastrin, insulin and somatostatin) in rats treated with DMSO-soluble forskolin or forskolin control solution for 2 weeks measured by RIA

Treatment	Gastrin levels (pg/ml)	Insulin levels (ng/ml)	Somatostatin levels (pg/ml)
Normal + control injections (2 weeks)	127.2 ± 2.27	8.79 ± 1.02	15.9 ± 1.35
Normal + forskolin injections (2 weeks)	120.9 ± 5.06[a]	7.81 ± 0.68[a]	15.9 ± 1.19[a]

Values are mean ± SEM of six samples.
[a] ns vs. the corresponding value of normal rats treated with a control solution for 2 weeks.

Table 4
Basal and secretin-stimulated bile flow, bicarbonate concentration and secretion in normal rats treated for 2 weeks with DMSO-soluble forskolin or forskolin control solution

	Bile flow		Bicarbonate concentration		Bicarbonate secretion	
Treatment	Basal (μl/min/kg)	Secretin (μl/min/kg)	Basal (mequiv./l)	Secretin (mequiv./l)	Basal (μequiv./min/kg)	Secretin (μequiv./min/kg)
Control solution ($n = 8$)	61.67 (±5.31)	64.29 (±5.85)	24.87 (±1.01)	25.25 (±0.96)	1.48 (±0.12)	1.63 (±0.18)
Forskolin ($n = 8$)	61.12 (±4.70)	74.92 (±2.51)[a]	23.52 (±0.74)	26.40 (±0.92)[b]	1.39 (±0.10)	2.00 (±0.12)[c]

Data are mean ± SE of eight rats.
[a] $P < 0.05$ vs. corresponding basal value of bile flow.
[b] $P < 0.05$ vs. corresponding basal value of bicarbonate concentration.
[c] $P < 0.05$ vs. corresponding basal value of bicarbonate secretion. Statistical analysis was performed by both unpaired *t*-student test.

was an increase in Src Tyr 139 phosphorylation in normal cholangiocytes treated in vitro with forskolin compared with cholangiocytes treated with 0.2% BSA (Fig. 7b). Phosphorylation of ERK1/2 increased in normal cholangiocytes treated in vitro with forskolin compared with cholangiocytes treated with BSA (Fig. 7c).

4. Discussion

Elevated cAMP levels alone are sufficient to increase cholangiocyte proliferation and secretion through changes in the PKA/Src/MEK/ERK1/2 pathway. Following in vivo administration of forskolin to normal rats, there was increased number of PCNA- and CK-19-positive cholangiocytes and γ-GT-positive ducts in liver sections and augmented PCNA protein expression in cholangiocytes. Forskolin-induced increase in cholangiocyte proliferation was devoid of apoptosis, necrosis or inflammation. The serum levels of gastrin, somatostatin and insulin were not altered by administration of forskolin to normal rats. This excludes that our findings were caused by the release of these hormones into the bloodstream but rather by a direct effect of forskolin on cholangiocyte functions by changes in cholangiocyte cAMP levels. Administration of forskolin to normal rats increased secretin-stimulated bicarbonate rich-choleresis in vivo. The in vivo effects of forskolin on cholangiocyte proliferation and secretion were associated with increased cholangiocyte cAMP levels and increased PKA activity, and enhanced Src-Tyr 139 and ERK1/2 phosphorylation, but a decrease in Src-Tyr 530 phosphorylation. Similar to in vivo studies, in vitro experiments in normal cholangiocytes demonstrated that forskolin increases cholangiocyte proliferation. Consistent with the concept that forskolin increases cholangiocyte proliferation by changes in the PKA/Src/MEK/ERK1/2 pathway; forskolin stimulation of PCNA protein expression in vitro was blocked by Rp-cAMPs, PP2 and PD98059. In vitro stimulatory effects of forskolin on PCNA protein expression were associated with increased activity of PKA, and enhanced phosphorylation of Src Tyr 139 and ERK1/2.

Forskolin is a ubiquitous activator of eukaryotic adenylyl cyclase (AC) [27]. Forskolin binds to AC since studies of [^{3}H] forskolin derivatives identified AC binding sites [28]. The functional unit of AC is formed by two domains (termed C1 and C2) arranged in a semi-symmetrical mirror

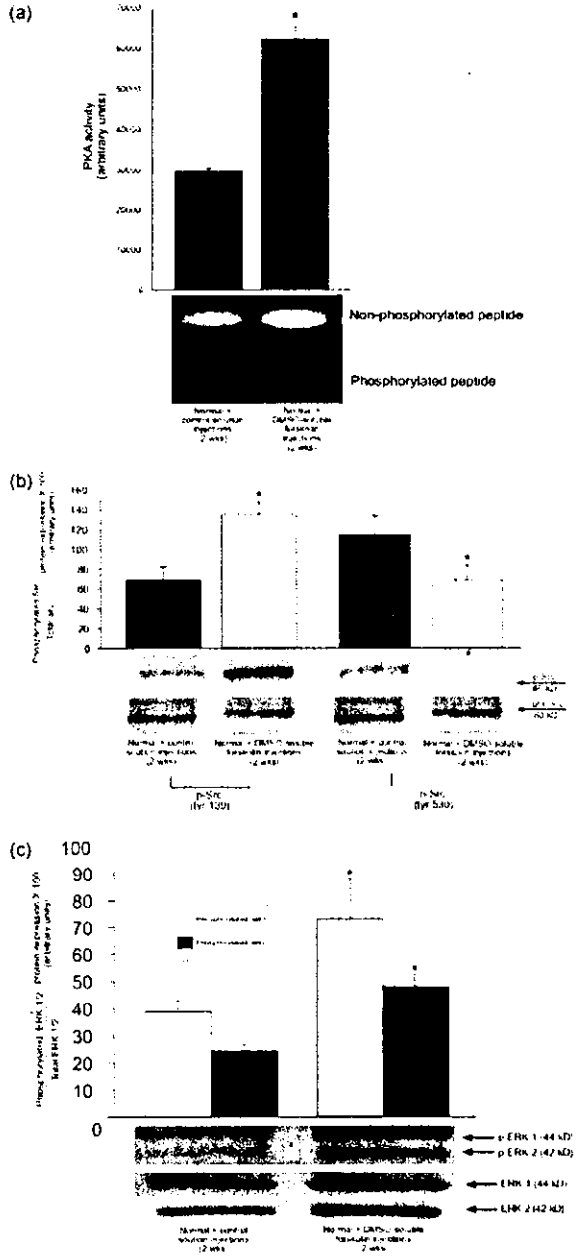

Fig. 5. Effect of in vivo administration of forskolin on PKA activity and the protein expression of total and phosphorylated form of Src and ERK1/2. (a) Forskolin increased the PKA activity compared with cholangiocytes isolated from control-treated rats. Data are mean ± SEM of three experiments. $^*P < 0.05$ vs. its corresponding basal value. (b) There was a significant decrease in Src-Tyr 530 and an increase in Src-Tyr 139 phosphorylation (which decrease and increase Src activity, respectively) in cholangiocytes from forskolin-treated rats compared to control-treated rats. Phosphorylation of Src-Tyr 530 and Src-Tyr 139 was expressed as ratio to total Src protein expression. Total Src protein expression was unchanged in cells from normal rats treated with forskolin compared to the control. (c) Both forms of the phosphorylated ERK1/2 were increased in cells from rats treated with forskolin for 2 weeks compared to cells from normal rats treated with a control solution. Total ERK protein expression was unchanged in cells from normal rats treated with forskolin compared to the control. Autoradiograms were quantitated by densitometry. Data are mean ± SEM of five experiments. $^*P < 0.05$ vs. its corresponding basal value.

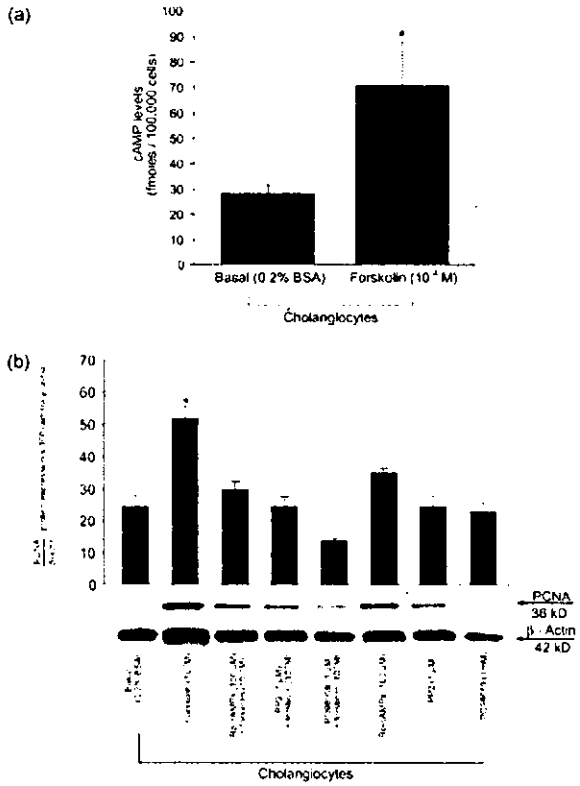

Fig. 6. In vitro effect of forskolin on (a) cAMP levels and (b) PCNA protein expression of normal purified cholangiocytes. (a) Forskolin increased intracellular cAMP levels of normal cholangiocytes compared to normal cholangiocytes treated with 0.2% BSA. Data are mean ± SEM of seven experiments. $^*P < 0.05$ vs. its corresponding basal value. (b) Forskolin induced a significant increase in PCNA protein expression of normal cholangiocytes compared with normal cholangiocytes treated with 0.2% BSA. The intensity of the bands was determined by scanning video densitometry. Data are mean ± SEM of three experiments. $^*P < 0.05$ vs. its corresponding basal value.

image of each other [29]. The C1 domain contains the catalytic and Gi binding sites, whereas the C2 domain provides the Gs binding site as well as a 'pseudo-catalytic' site at which forskolin binds [29]. Binding of forskolin at the pseudocatalytic site increases Gs interaction with AC and increases substrate catalysis at the active site [29]. Therefore, activation of Gs contributes to forskolin-stimulated cAMP generation in cells because of Gs-forskolin potentiation of AC activity. Response of cells to forskolin can involve activation of G-coupled protein receptors (GPCRs), such as nucleotide and prostaglandin receptors that link to Gs activation [30]. Thus, cellular generation of cAMP in response to forskolin can include a contribution by GPCR

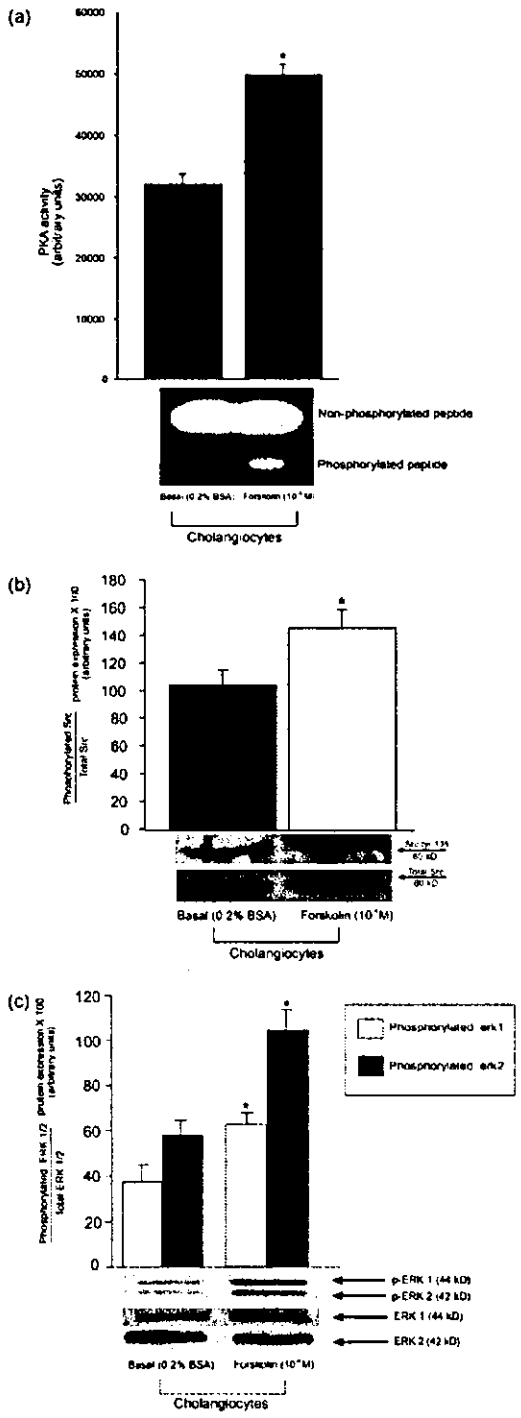

Fig. 7. In vitro effect of forskolin on the PKA/Src//MEK/ERK1/2 pathway. (a) Forskolin increased PKA activity in normal cholangiocytes compared to normal cholangiocytes treated with BSA. (b) There was a significant increase in Src-Tyr 139 phosphorylation in normal cholangiocytes treated in vitro with forskolin compared with cholangiocytes treated with 0.2% BSA. Total Src protein expression was unchanged. (c) Phosphorylation of ERK1/2 (expressed as ratio to total ERK1/2 protein expression) increased in normal cholangiocytes treated in vitro with forskolin compared with cholangiocytes treated with BSA. Total ERK protein expression was unchanged. The intensity of the bands was determined by scanning video densitometry. Data are mean ± SEM of three experiments. *$P < 0.05$ vs. its corresponding basal value.

activation even though GPCR agonist is not present. Forskolin activates AC in many cells including rat cerebral cortex neurons and adipocytes [27,31]. There are no extensive studies on the pharmacokinetics of forskolin in animals or humans. Water-soluble forskolin derivatives such as colforsin daropate or 7-deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-, dihydrochloride are positive inotropic agents and vasodilators used to treat acute ventricular dysfunction after cardiopulmonary bypass. These water-soluble derivatives are mainly metabolized in the liver in humans. The major metabolite produced by *N*-demethylation of colforsin daropate is cleared by the kidneys [32].

Cholangiocyte proliferation and secretion is regulated by: (i) parasympathetic [4,33], sympathetic [34], and dopaminergic [20] nerves; (ii) gastrointestinal hormones (e.g. somatostatin) [35]; and (iii) cAMP, whose levels increase with enhanced cholangiocyte proliferation but decrease with cholangiocyte loss [3–7,9,35]. In this study, we demonstrated that the stimulatory action of cAMP (by forskolin) on cholangiocyte proliferation might be partly mediated by increased PKA activity. The increase in cholangiocyte proliferation was produced by both DMSO- and water-soluble forskolin, demonstrating that the effect of proliferation was not due to DMSO (in which forskolin is dissolved), but by the activation of cAMP by forskolin itself. Parallel to previous findings [36–38] that demonstrate that PKA regulates epithelial functions, our studies produce evidence that: (i) the PKA pathway plays a role in the regulation of cholangiocyte functions; (ii) increased cAMP levels are sufficient to stimulate cholangiocyte proliferation and secretion; (iii) similar to other cells [36–38], PKA (shown to regulate cholangiocyte functions) [24] is the major substrate of cAMP, since forskolin-induced increases in cAMP levels are coupled with enhanced PKA activity; (iv) increased proliferation was blocked in vitro by Rp-cAMPs, demonstrating the direct effect of PKA on cholangiocyte proliferation; and (v) cAMP stimulation of cholangiocyte proliferation occurs by changes in the Src/MEK/ERK1/2 pathway. In the cAMP/PKA/Src/MEK/ERK1/2 pathway a major role is played by the MAPK isoforms, ERK1 and ERK2 [39]. The cAMP system stimulates proliferation of an IL-3 dependent leukemic cell line through an ERK-dependent mechanism [39]. Adrenomedullin promotes proliferation and migration of endothelial cells via a cAMP/PKA dependent pathway [38]. Similarly, our study supports the concept that forskolin-induced increases in cAMP levels stimulates cholangiocyte proliferation by: (i) increased activity of PKA, Src-Tyr-139

and ERK1/2; and (ii) decreased Src-Tyr-530 phosphorylation. Manipulation of intracellular cAMP may be important in managing cholangiocyte proliferation/loss in liver diseases. As direct outgrowth of the studies, we will evaluate the effect of forskolin on the proliferation of small and large cholangiocytes, which differentially regulate cholangiocyte functions [1,7,16,35,40,41]. Preliminary data demonstrates that in vivo forskolin administration increases the proliferation of large cholangiocytes and induces the de novo proliferation of small cholangiocytes, which are normally mitotically dormant but able to proliferate (by a cAMP dependent mechanism) following bile acid feeding or administration of CCl4 or ANIT [6,7,35,41].

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Peptide Antibiotic Human Beta-Defensin-1 and -2 Contribute to Antimicrobial Defense of the Intrahepatic Biliary Tree

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Human beta-defensins (hBDs) are important antimicrobial peptides that contribute to innate immunity at mucosal surfaces. This study was undertaken to investigate the expression of hBD-1 and hBD-2 in intrahepatic biliary epithelial cells in specimens of human liver, and 4 cultured cell lines (2 consisting of biliary epithelial cells and 2 cholangiocarcinoma cells). In addition, hBD-1 and hBD-2 were assayed in specimens of bile. hBD-1 was nonspecifically expressed immunohistochemically in intrahepatic biliary epithelium and hepatocytes in all patients studied, but expression of hBD-2 was restricted to large intrahepatic bile ducts in 8 of 10 patients with extrahepatic biliary obstruction (EBO), 7 of 11 with hepatolithiasis, 1 of 6 with primary biliary cirrhosis (PBC), 1 of 5 with primary sclerosing cholangitis (PSC), 0 of 6 with chronic hepatitis C (CH-C), and 0 of 11 with normal hepatic histology. hBD-2 expression was evident in bile ducts exhibiting active inflammation. Serum C reactive protein levels correlated with biliary epithelial expression of hBD-2. Real-time PCR revealed that in all of 28 specimens of fresh liver, including specimens from patients with hepatolithiasis, PBC, PSC, CH-C and normal hepatic histology, hBD-1 messenger RNA was consistently expressed, whereas hBD-2 messenger RNA was selectively expressed in biliary epithelium of patients with hepatolithiasis. Immunobloting analysis revealed hBD-2 protein in bile in 1 of 3 patients with PSC, 1 of 3 with PBC, and each of 6 with hepatolithiasis; in contrast, hBD-1 was detectable in all bile samples examined. Four cultured biliary epithelial cell lines consistently expressed hBD-1; in contrast these cell lines did not express hBD-2 spontaneously but were induced to express hBD-2 by treatment with *Escherichia coli*, lipopolysaccharide, interleukin-1β or tumor necrosis factor-α. In conclusion, these findings suggest that in the intrahepatic biliary tree, hBD-2 is expressed in response to local infection and/or active inflammation, whereas hBD-1 may constitute a preexisting component of the biliary antimicrobial defense system. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2004; 40:925–932.)

Human bile is sterile under normal conditions. However, there have been several reports that bacterial components, such as lipopolysaccharide (LPS), may be detected in normal bile.[1–3] Furthermore, in inflammatory biliary diseases, bacteria have been cultured from bile.[1,4–6] The biliary tract drains directly into the duodenum, where the biliary epithelium may potentially be exposed to bacteria and bacterial components. Duodenal microorganisms are believed to be a major source of bacterial infection in several biliary diseases. In particular, enteric bacteria, demonstrable in bile, may be responsible for chronic proliferative cholangitis associated with hepatolithiasis.[7,8] Several defense mechanisms tend to protect the biliary tract from bacterial invasion; these include physical, chemical, and immunological factors.[6,9,10]

The first line of defense against invading pathogens is the innate immune system.[11] Key elements of this system are defensins, which are antimicrobial peptides.[12] Structurally, defensins are a family of cationic antimicrobial peptides; they are divided into α- and β-subfamilies.[11,12] The β-defensin family is found in the epithelia of several

Abbreviations: LPS, lipopolysaccharide; hBD, human beta-defensin; TNF, tumor necrosis factor; hBECs, human biliary epithelial cells; ICC, intrahepatic cholangiocarcinoma; EBO, extrahepatic biliary obstruction; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; CH-C, chronic hepatitis; ISH, in situ hybridization; IgG, immunoglobulin G; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; E. coli, Escherichia coli; IL, interleukin; IFN, interferon; CRP, C-reactive protein; PBS, phosphate-buffered saline.

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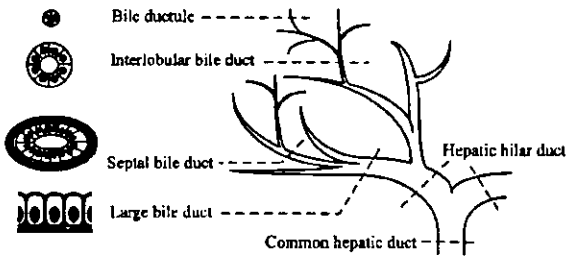

Fig. 1. Schema of the intrahepatic biliary tree. The intrahepatic biliary tree is classified into large intrahepatic bile ducts, septal bile ducts, interlobular bile ducts, and bile ductules. Large bile ducts are recognizable by the naked eye, and they are consistently associated with peribiliary glands. Septal bile ducts are microscopic; they consist of tall columnar cells, which have oval nuclei at their basal pole, and thick fibrous walls. Interlobular bile ducts are composed of cuboidal cells, in the absence of fibrotic periductal walls; they are accompanied by a small branch of the portal vein and a small branch of the hepatic artery. Bile ductules are the smallest ductal element; they occur in the periphery of portal tracts and are directly associated with hepatocytes.

organs; it constitutes an important barrier at mucosal surfaces.[11] So far, at least 6 human β-defensins (hBD-1 to -6) have been identified.[13] Among them, hBD-1 and hBD-2 are well recognized to mediate salt-sensitive microbicidal activity.[14] hBD-1 is extensively expressed in broad epithelia, including that of the gastrointestinal tracts.[11,15–18] hBD-2 is most abundant in inflammed skin and lung; its expression in other epithelial organs is low.[19,20] Interestingly, hBD-2 expression is induced by exposure to bacteria or cytokines, such as tumor necrosis factor (TNF)-α.[14]

Several studies have demonstrated the importance of the antimicrobial actions of hBD-1 and hBD-2 in several organs, such as the bronchi and gastrointestinal tract.[12,15,18,21,22] So far, the expression of hBD-1 and hBD-2 in the hepatobiliary system and their roles in hepatobiliary pathophysiology have been poorly evaluated.

In this study, we examined immunohistochemically the distribution of hBD-1 and hBD-2 along intrahepatic bile ducts in certain hepatobiliary diseases. Also, using cultured human biliary epithelial cells (hBECs) and intrahepatic cholangiocarcinoma (ICC) cells, we assessed whether hBD-1 and hBD-2 expression in hBECs is influenced by bacterial products and inflammatory cytokines.

Patients and Methods

Anatomy of the Intrahepatic Biliary Tree

The intrahepatic biliary tree was classified into large and small bile ducts (Fig. 1).[23] The former are macroscopically visible (diameter >300 μm); they have a fibrous wall and are associated with peribiliary glands. They represent the first to the fourth branches of the right and left hepatic ducts. The small bile ducts are recognized under a microscope; they are classified into septal and interlobular bile ducts and bile ductules, based on their size and location within portal tracts.

Patients and Tissue Preparation

All tissue specimens were obtained from our Department of Human Pathology. Consent for research on human subjects was obtained from each patient that had provided a specimen before undertaking experimental procedures. The protocol of this study was approved by the Ethics Committee of Kanazawa University.

Formalin-fixed Specimens. A total of 49 surgical, autopsy, or explant liver specimens were obtained from 10 patients with extrahepatic biliary obstruction (EBO), 11 with hepatolithiasis, 6 with primary biliary cirrhosis (PBC), 5 with primary sclerosing cholangitis (PSC), 6 with chronic hepatitis C (CH-C), and 11 with normal hepatic histology. Characteristics of these patients are given in Table 1. Explanted livers were obtained at liver transplantation. In patients with hepatolithiasis, stones contained brown pigment, and stone-containing ducts exhibited fibrosis, an inflammatory cell infiltrate, and peribiliary proliferation (chronic proliferative cholangitis).[7] Three patients with hepatolithiasis had stones at the bifurcation of common hepatic duct and cholestasis. Liver

Table 1. Main Clinicolaboratory Features of Hepatobiliary Diseases Studied

Disease	Case Number	Average age (yrs)	Sex M:F	Sample S:A:E	CRP (mg/dL)	ALP (IU/L)	γGTP (IU/L)	T.bil (mg/dL)
Hepatolithiasis	11	65	5:6	11:0:0	3.5 (0.3-9.6)	315 (176-479)	125 (39-174)	2.0 (0.8-4.5)
EBO	10	75	6:4	2:8:0	8.8 (2.3-18.3)	612 (209-1557)	102 (24-260)	6.3 (0.7-13.6)
PBC*	6	52	0:6	0:1:5	3.4 (1.2-5.4)	486 (265-898)	54 (37-107)	18.4 (1.2-32.6)
PSC*	5	36	2:3	0:1:4	3.0 (0.5-4.8)	498 (311-732)	66 (58-80)	13.7 (1.5-21.6)
CVH	6	64	4:2	5:0:1	<0.4¶	249 (182-366)	54 (26-83)	0.8 (0.3-1.8)
Normal liver	11	62	7:4	3:8:0	<0.4	233 (154-276)	25 (20-33)	0.7 (0.2-1.5)

NOTE. Laboratory data shown as the mean of each category and parenthesis is range.

Abbrevations: EBO, extrahepatic biliary obstruction; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; CVH, chronic viral hepatitis; CRP: C-reactive protein; ALP: alkaline phosphatase; γGTP: γ-glutamyltranspeptitase; T.bil: total bilirubin; M:F, male:female; S:A:E, surgical:autopseid:explanted.

*Histological stage IV.

¶Except one case (CRP 1.3mg/dL).

tissue of 5 of 6 patients with CH-C was obtained from tumor-free parts of liver that had been surgically resected for hepatocellular carcinoma. Specimens of liver tissue that showed no histological abnormality were obtained from tumor-free parts of specimens of liver from 3 patients who had undergone surgical resection of hepatic metastases; these specimens were designated "normal hepatic histology." Large intrahepatic bile ducts were present in all specimens.

Among the patients with hepatolithiasis, 3 surgically resected gallbladders that did not contain stones were available. In addition, liver specimens were obtained at autopsy of 3 patients (65-86 years old; 2 males and 1 female) who had had fatal extrahepatic sepsis (pulmonary infection in 2, and urinary tract infection in 1).

All of the liver and gallbladder specimens were fixed in neutral formalin and embedded in paraffin. Four-micrometer-thick sections were prepared for routine histology and immunohistochemistry.

Fresh Specimens. Fresh surgical or explanted liver specimens containing large intrahepatic bile ducts were available from 6 patients with hepatolithiasis, 6 with PBC, 4 with PSC, 6 with CH-C, and 6 with normal hepatic histology. Two of the patients with hepatolithiasis had cholestasis. Fresh liver specimens were cut into several pieces for RNA extraction. The remaining tissue was fixed in 4% paraformaldehyde and embedded in paraffin; 4-μm-thick sections were prepared for *in situ* hybridization (ISH).

Immunohistochemistry of hBD-1 and hBD-2

Expression of hBD-1 and hBD-2 was assessed immunohistochemically in formalin-fixed sections. Briefly, for assessment of hBD-1, deparaffinized and rehydrated sections were microwaved in 10 mmol/L citrate buffer for 20 minutes; for assessment of hBD-2, similar sections were treated with 0.1 % trypsin for 20 minutes.[24] After blocking endogenous peroxidase, sections were incubated with polyclonal antibodies against hBD-1 (goat immunoglobulin G (IgG), 1 μg/mL, a gift from coauthors T.H. and A.W.) or hBD-2 (rabbit IgG, Nagasaki University, Japan[24]) at 4°C overnight and then incubated with rabbit anti-goat immunoglobulin conjugated to peroxidase-labeled polymer (Simple Staining Kit, Nichirei, Tokyo, Japan) or goat anti-rabbit immunoglobulin conjugated to peroxidase-labeled dextran polymer (Envision, Dako, Tokyo, Japan) at room temperature for 1 hour, respectively. After reacting with benzidine, sections were lightly counterstained with hematoxylin. To generate a negative control, normal rabbit IgG or goat IgG was used instead of the primary antibody.

Detection of Messenger RNA (mRNA) of hBD-1 and hBD-2 in Liver Tissue

To confirm the presence of hBD-1 and hBD-2 mRNA in liver tissue, reverse transcription-polymerase chain reaction (RT-PCR), real-time PCR, and ISH were undertaken.

RT-PCR and Real-Time PCR. Total RNA was isolated from fresh liver using the RNeasy Total RNA System (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For RT, 1 μg of total RNA was reverse transcribed with oligo-dT primers and RTase in a total volume of 20 μL; 0.5 μL was used as a template for PCR. Specific primers were designed as follows: hBD-1 forward, 5′-CATGAGAACTTCCTACCTTCTG-3′, and reverse, 5′-GCTCACTTGCAGCACTTGGCC-3′; hBD-2, forward, 5′-CCAGCCATCAGCCATGAGGGT-3′, and reverse, 5′-GGAGCCCTTTCTGAATCCGCA-3′. The predicted product sizes were 210 base pairs for hBD-1 and 255 base pairs for hBD-2. To generate a positive control, the primers for β-actin mRNA (forward, 5′-CAAGAGATGGCCACGGCTGCT-3′; reverse, 5′-TCCTTCTGCATCCTGTCGGCA-3′) were used to amplify a 275 base-pair portion. The PCR profile consisted of an initial denaturation at 94°C for 3 minutes followed by 28 cycles (hBD-1 and hBD-2) or 20 cycles (β-actin): 30-second denaturation at 94°C, 30-second annealing of primers at 62°C, and 60-second extension at 72°C. After PCR, 5-μL aliquots of the products were subjected to 1.5% agarose gel electrophoresis. One of the amplicons was reamplified using the primers containing T7- and Sp6-RNA polymerase promoter to transcribe RNA *in vitro*. Real-time PCR was undertaken for quantitative analysis using the SYBER Green PCR Master Mix and ABI PRISM 7700 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan) according to the standard protocol.

ISH. Using the PCR-amplified products containing T7- and Sp6-RNA polymerase promoter, antisense and sense digoxigenin-labeled RNA probes for hBD-1 and hBD-2 mRNA were generated by *in vitro* transcription using the Digoxigenin RNA Transcription Kit (Roche Diagnostics, Indianapolis, IN) according to the standard protocol. Deparaffinized sections of paraformaldehyde-fixed specimens were incubated with 10 μg/mL proteinase K for 10 minutes at 37°C and then dehydrated in ethanol and air. The sections were incubated with a hybridization solution containing digoxigenin-labeled probes (1 μg/mL) at 50°C for 16 hours. After washing in a sodium chloride–sodium citrate solution (SSC, final concentration 0.2 × SSC, Sigma, St. Louis, MO), the sections were incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (Roche) for 1 hour. Color development was achieved by adding a prepared substrate

solution that contained nitroblue tetrazolium salt (450 μg/mL) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (175 μg/mL) to the slides for 3 hours.

Preparation of Bile and Immunoblot Analysis

Bile samples were obtained from the affected bile ducts of 6 of the patients with hepatolithiasis, and from the gallbladders of 3 of the patients with PBC and 3 of those with PSC.

Protein Extraction. Bile samples were homogenized in protein lysis solution (Tissue Protein Extraction Reagent, Pierce, Rockford, IL). After centrifugation, the supernatant was transferred to a fresh tube; the concentration of total protein in the supernatant was measured using a Bradford-based assay and the concentration was then adjusted to 1 μg/μL.

Dot Blotting. Two-microgram protein solution extracted from bile or 3-μL medium of cell culture were directly applied to a nitrocellulose membrane. After blocking in 5% bovine serum albumin, the membrane was first incubated with mouse monoclonal anti-human CD64 (diluted 1:10, Dako) for 30 minutes, to eliminate nonspecific binding to Fc receptors of human immunoglobulin in bile,[25] and then probed for 1 hour with polyclonal antibodies against hBD-1 or hBD-2 (goat IgG, 1 μg/mL, Santa Cruz Technologies). After washing, the membranes were incubated with rabbit anti-goat immunoglobulin conjugated to peroxidase-labeled dextran polymer (Nichirei) for 1 hour and then reacted with benzidine.

Cell Cultures

Two human ICC cell lines (CCKS1[26,27] and HuCCT1[28]), and 2 hBEC lines (1 isolated from explanted liver of a patient with CH-C, and the other isolated from explanted liver of a patient with PBC) (hBEC1 and hBEC2, respectively) were used. HuCCT1 was purchased from Health Science Research Resources Bank (Osaka, Japan). CCKS1 was established in our laboratory.[26,27] hBEC1 was a gift from Dr. Takashi Kamihira (Kyushu University, Japan).[29] hBEC2 was established in our laboratory. CCKS1 and HuCCT1 lines were grown as monolayers in suspension cultures using a standard medium—RPMI 1640 or D-MEM/F-12 (Gibco, Rockville, MD), respectively; the media were supplemented with 10% fetal calf serum and an antibiotic/antimycotic solution (Invitrogen Japan, Tokyo, Japan). hBECs were incubated in a culture medium that contained D-MEM/F-12, Nu-Serum (Becton Dickinson, Bedford, MA), ITS+ (Becton Dickinson), 5 μmol; forskolin (Wako, Osaka, Japan), 12.5 mg/mL bovine pituitary extract (Gibco), 1 μmol dexamethasone (Sigma), 5 μmol triiodo-thyronine (Sigma), 5 mg/mL glucose (Sigma), 25 mmol/L sodium bicarbonate (Sigma), 1 % antibiotic-antimycotic solution, 20 ng/mL of human epidermal growth factor (Gibco), and 10 ng/mL human hepatocyte growth factor (Gibco). A HepG2 cell line (derived from a human hepatocellular carcinoma, RCB1648, RIKEN Cell Bank, Tsukuba, Japan) was used as a control. The cell lines were cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Treatment with* Escherichia coli (E. coli), *LPS, and Cytokines. The effects of *E. coli*, LPS, and inflammatory cytokines on the expression of hBDs in cultured cells were determined. A strain of *E. coli*, ATCC25922 (American Type Culture Collection, Rockville, MD), grown in Luria-Bertani medium (Wako), was collected at the exponential growth phase, and then killed by autoclaving. LPS (derived from *E. coli* O55:B5, Sigma), and recombinant human cytokines, interleukin (IL)-1β (PeproTech, London, UK), IL-4 (PeproTech), IL-5 (PeproTech), IL-6 (PeproTech), interferon (IFN)-γ (Sigma), and TNF-α(Sigma), were used to stimulate cell lines.

HuCCT1, CCKS1, hBEC1, hBEC2, and HepG2 grown to semiconfluence in culture plates were treated with no stimulants (medium only), LPS (1 μg/mL), or autoclaved *E. coli* (1 μg/mL) in fresh minimal standard medium for 3 and 12 hours. The cells were then washed 3 times before isolation of their RNA. Expression of mRNA of hBDs in RNA extracts of cultured cells was assessed by RT-PCR and real-time PCR. In addition, these 5 cell lines, grown to semiconfluence in plates, were treated with each of the 6 cytokines at a concentration of 1,000 U/mL in fresh minimal standard medium for 3 hours; the same approach was adopted to assess expression of mRNA of hBDs in cytokine-treated cells.

HuCCT1 and hBEC1 grown to semiconfluence in culture plates were treated with LPS (1 μg/mL) in fresh minimal standard medium for 12 hours; proteins of hBDs were then assessed in culture supernatants as described in Preparation of Bile and Immunoblot Analysis.

RT-PCR and Real-Time PCR for mRNA of hBD-1 and hBD-2. Total RNA was isolated from cultured cells using the RNeasy Total RNA System (Qiagen) according to the manufacturer's instructions. The methods used for applying RT-PCR and real-time PCR were the same as those applied to fresh liver tissue.

Statistical Analysis

Welch's *t* test or Mann-Whitney *U* test was applied when appropriate. A *P* value of less than .05 was considered to be significant.

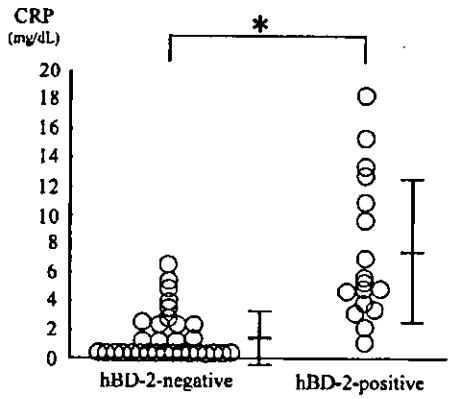

Fig. 2. Mean values and SDs (**bars**) of serum C-reactive protein (CRP) levels in human beta defensin (hBD)-2-positive and -negative groups. **Open circles** indicate individual values for all the patients studied using immunohistochemistry. The patients who expressed hBD-2 had higher levels of CRP than those who did not express hBD-2 (*$P < .05$). **Bars** are SDs.

Results

Tissue Expression of hBD-1 and hBD-2

Diffuse and granular immunohistochemical patterns of expression of hBD-1 occurred in the cytoplasm of intrahepatic bile duct epithelial cells. In all patients there was no gradient in the intesity of staining at different anatomical sites or in hepatocytes. The staining intensity in hBECs was similar to or weaker than that in hepatocytes (Supplementary Fig. 1). In contrast, expression of hBD-2 was restricted to large intrahepatic bile ducts and peribiliary glands (Supplementary Fig. 1); no expression of hBD-2 was detected in hepatocytes in any liver specimen. Expression of hBD-2 in large bile ducts was found in 8 of 10 patients with EBO, 7 of 11 with hepatolithiasis, 1 of 6 with PBC, 1 of 5 with PSC, 0 of 6 with CH-C, and 0 of 11 with normal hepatic histology; its prevalence in EBO and hepatolithiasis was higher than that in other liver diseases ($P < .05$). hBD-2 expression was heterogeneous in the intrahepatic biliary tree; it occurred particularly in large bile ducts in which there was an appreciable polymorphonuclear inflammatory infiltrate but not in minimally inflamed portions of bile ducts (Supplementary Fig. 1). hBD-2 was often detectable in the luminal secretion of large intrahepatic bile ducts. Moreover, hBD-1- and hBD-2-positive mononuclear cells were found in inflammed portal tracts; this finding seemed to correlate with mRNA-positive cells demonstrated by ISH (see ISH Study).[30]

The serum level of C-reactive protein (CRP) was higher in patients with biliary epithelial expression of hBD-2 (7.4 ± 5.1 mg/dL SD) than in those without such expression (1.5 ± 1.7 mg/dL) (Table 1 and Fig. 2). In 3 patients with hepatolithiasis who expressed hBD-1 and hBD-2 in their stone-containing intrahepatic bile ducts, hBD-1 but not hBD-2 was expressed in the lining epithelium of their gallbladders. Furthermore, in autopsy livers from 3 patients with extrahepatic sepsis, biliary epithelial expression of hBD-2 was not detected.

Detection of hBD-1 and hBD-2 mRNAs in Tissue

RT-PCR and Real-Time PCR. Amplification of hBD-1 mRNA occurred in all liver specimens. However, amplification of hBD-2 mRNA occurred in 1 of 4 patients with PSC, 3 of 6 with hepatolithiasis, and in none of the other patients. Real-time PCR revealed that the level of hBD-1 mRNA was not different among the patient groups (Supplementary Fig. 2). However, the level of hBD-2 mRNA was significantly greater in hepatolithiasis (particularly in 3 patients) than in PBC, CH-C, or normal liver (Supplementary Fig. 2). The serum level of CRP was particularly high in the 3 patients with hepatolithiasis who had the highest levels of hBD-2 mRNA; 2 of these patients had cholestasis. There were no other correlations between levels of hBD-2 mRNA and other routine laboratory findings.

ISH Study. Low levels of hBD-1 mRNA were found in the cytoplasm of large bile ducts and hepatocytes in all specimens; such levels were appreciable in large bile ducts of patients with hepatolithiasis associated with active cholangitis (Fig. 3). hBD-2 mRNA was detectable in large bile ducts in 2 of 6 patients with hepatolithiasis, but it was not detectable in bile ducts of other patients; these ISH-positive patients also exhibited increased levels of hBD-2

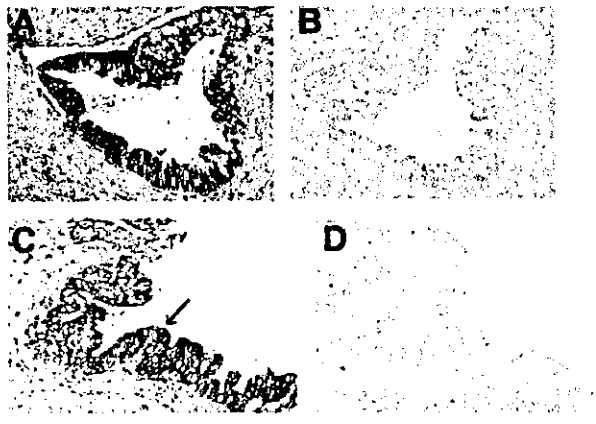

Fig. 3. *In situ* hybridization staining for human beta defensin (hBD)-1 messenger RNA (mRNA) with antisense (A) and sense (B) probes and for hBD-2 with antisense (C) and sense (D) probes. (A and C) In hepatolithiasis, large intrahepatic bile ducts that exhibit cholangitis and biliary epithelial hyperplasia are positive for both hBD-1 and -2 mRNA. Moreover, several hBD-1 and hBD-2 mRNA-positive mononuclear cells are present in the periductal zone. (B and D) Semiserial sections of (A) and (C), respectively. The specific signals are eliminated when the slides are hybridized with sense probes (B and D).