Heterogeneity of the proliferative capacity of rat cholangiocytes after bile duct ligation

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We previously introduced the concept that intrahepatic bile duct epithelial cells, or cholangiocytes, are functionally heterogeneous. This concept is based on the observation that secretin receptor (SR) gene expression and secretin-induced cAMP synthesis are present in cholangiocytes derived from large (>15 µm in diameter) but not small (<15 µm in diameter) bile ducts. In work reported here, we tested the hypothesis that cholangiocytes are heterogeneous with regard to proliferative capacity. We assessed cholangiocyte proliferation in vivo by measurement of [3H]thymidine incorporation and in vitro by both [3H]thymidine incorporation and H3 histone gene expression in small (fraction 1) and large (fraction 2) cholangiocytes isolated from rats after bile duct ligation (BDL). In the two cholangiocyte subpopulations, we also studied basal somatostatin receptor (SSTR2) gene expression as well as the effects of somatostatin on 1) SR gene expression and secretin-induced cAMP synthesis and 2) [3H]thymidine incorporation and H3 histone gene expression. In normal rat liver, cholangiocytes, unlike hepatocytes, were mitotically dormant; after BDL, incorporation of [3H]thymidine markedly increased in cholangiocytes but not hepatocytes. When subpopulations of cholangiocytes were isolated after BDL, DNA synthesis assessed by both techniques was limited to large cholangiocytes, as was SSTR2 steady-state gene expression. In vitro, somatostatin inhibited SR gene expression and secretin-induced cAMP synthesis only in large cholangiocytes. Moreover, compared with no hormone, somatostatin inhibited DNA synthesis solely in large cholangiocytes. These results support the concept of the heterogeneity of cholangiocytes along the biliary tree, extend this concept to cholangiocyte proliferative activity, and imply that the proliferative compartment of cholangiocytes after BDL is located principally in the cholangiocytes lining large (>15 µm) bile ducts.

secretin; somatostatin; adenosine 3’5’-cyclic monophosphate; [3H]thymidine incorporation; H3 histone

Intrahepatic bile duct epithelial cells, or cholangiocytes, are simple epithelia that line the intrahepatic biliary tree, a complex three-dimensional network of tubular conduits of different diameters within the liver (8). A number of studies (2–5, 7–12, 14, 18, 19, 28, 35) have demonstrated that cholangiocytes modify bile of canalicular origin by a coordinated series of hormone-regulated secretory and absorptive processes. For example, secretin stimulates ductal bile secretion by interacting with specific receptors [present in the liver only on cholangiocytes (11)] through increases in the cAMP second messenger system (2, 4, 9, 10, 14, 18, 19, 35). In addition to secretin (2, 4, 9, 10, 14, 18, 19, 35), other gastrointestinal hormones, including gastrin and somatostatin (see below), have the capacity to regulate ductal absorptive and secretory activity (14, 27, 35).

Under basal conditions, cholangiocytes are thought to be mitotically dormant (2, 5, 19). However, cholangiocytes proliferate markedly in response to a number of perturbations including bile duct ligation (BDL), α-naphthylsulfohydroxamate feeding, and 70% hepatectomy (5–8, 19, 35). Not surprisingly, this proliferative response is accompanied by functional changes (5–8, 10, 19, 35). For example, cholangiocyte proliferation after BDL or 70% hepatectomy is coupled with an increase in secretin receptor (SR) gene expression (10, 11, 19) and secretin-inhibited cAMP synthesis (11, 19) and is associated with a secretin-stimulated, bicarbonate-rich choleresis (5, 7, 14, 19, 35).

In several series of experiments, we have previously demonstrated that cholangiocytes lining the intrahepatic biliary tree of normal rats are heterogeneous. These studies have employed both in vivo quantitative morphological techniques (3, 9) and in vitro functional assays using distinct subpopulations of cholangiocytes (9, 10) and intrahepatic bile duct fragments (3, 4) of different sizes. Indeed, we recently reported a technique for isolating subpopulations of cholangiocytes of different sizes from normal rat liver and showed (9) that both SR gene expression and secretin-induced increases in intracellular cAMP levels are differentially distributed among cholangiocyte subpopulations derived from different portions of the rat biliary tree (4, 9). Moreover, we demonstrated that the increased ductal secretory activity observed after BDL reflects transport processes restricted to selected cholangiocyte subpopulations (3, 10). Thus the morphological and functional heterogeneity of the epithelia lining the intrahepatic biliary tree has become an accepted concept (2–4, 9, 10).

Somatostatin, a cyclic tetradecapeptide, was first isolated from rat hypothalamus where it was shown to inhibit the secretion of growth hormone (37). Somatostatin is also inhibitory in a variety of other organs (25,
30); for example, it decreases secretory processes in the intestine (30) and pancreas (25) and inhibits growth of a variety of epithelial cells (32, 34, 36). In the liver, studies by us (35) and Ricci and Favery (27) using in vivo models have shown that somatostatin is cholestatic and inhibits both basal and secretin-stimulated ductal bile secretion. Furthermore, we have shown that somatostatin inhibits both basal and secretin-induced exocytosis and secretin-stimulated increases in cAMP levels in isolated, highly purified cholangiocytes (35). Somatostatin is also antiproliferative in at least two circumstances: 1) it inhibits cholangiocyte proliferation in rats in response to BDL (36), and 2) we have recently shown that somatostatin prevents the growth of human cholangiocarcinoma cells in vitro and in vivo (32). Taken together, these observations strongly suggest that the cholangiocyte is the major target for somatostatin in the liver. All of these inhibitory actions of somatostatin occur through interaction with one of its receptor subtypes (i.e., SSTR2). The specific portion of the intrahepatic biliary tree where SSTR2 resides and where somatostatin exerts its effects on ductal secretory activity and on the proliferation of cholangiocytes is unknown. Indeed, no information currently exists as to whether or not cholangiocytes are heterogeneous with regard to their proliferative capacity (i.e., is there a specific proliferative compartment within the intrahepatic biliary tree).

To begin to explore these issues, we isolated from BDL rat liver two subpopulations of cholangiocytes of different sizes, i.e., fraction 1 or small cholangiocytes (~8 µm in size and originating from ducts <15 µm in diameter (3, 9, 10)) and fraction 2 or large cholangiocytes (~14 µm in size and originating from ducts >15 µm in diameter (3, 9, 10)), and assessed proliferative capacity of the two subpopulations by measurement of both [3H]thymidine incorporation and H3 histone gene expression. In the two subpopulations, we also studied in vitro basal SSTR2 gene expression and the effects of somatostatin (10^{-7} M) on SR gene expression, secretin-induced cAMP synthesis, and cholangiocyte proliferation.

**MATERIALS AND METHODS**

**Animal Model**

We used male Fischer 344 rats (200–225 g) from Charles River (Wilmington, MA). The animals were kept in a temperature-controlled room (20–22°C) with a 12:12 h light-dark cycle and fed ad libitum with standard rat food. The present experiments were performed in normal rats and in rats with cholangiocyte hyperplasia induced by BDL for 28 days. BDL was performed as previously described (5–7). Before each experiment, animals were anesthetized with pentobarbital sodium (50 mg/kg ip). Study protocols were performed in compliance with institutional guidelines.

**Materials**

Reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Both secretin and somatosta
tin were purchased from Peninsula Laboratories (Belmont, CA). The mouse anticytokeratin 19 (CK-19) antibody was purchased from Amersham (Arlington Heights, IL). Radiommoassay kits for the determination of intracellular cAMP levels in purified cholangiocytes were purchased from Amersham. Dulbecco’s PBS was obtained from Celox (Hopkins, MN). The monoclonal antibody to vimentin, an IgG1 against the 57-kDa intermediate filament vimentin (38), was purchased from BioGenex (San Ramon, CA). BSA was purchased from Calbiochem-Novabiochem (La Jolla, CA).

**In Situ Assessment of Cholangiocyte Proliferation in both Normal and BDL Rat Liver**

In frozen liver sections (~6 µm thick) obtained randomly from both normal and BDL rat livers, immunohistochemistry for CK-19 was performed as previously described by us (6, 19). Ninety minutes after an intraperitoneal injection of 1 µCi/g body wt [methyl-3H]thymidine (6.70 Ci/mmol, Du Pont-New England Nuclear Products, Boston, MA), in situ nuclear labeling was determined in paraffin-embedded sections (~6 µm thick) from both normal and BDL rat livers. Briefly, after coating in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY), the paraffin-embedded sections were exposed for 2 days, processed for standard autoradiography, and stained with hematoxylin and eosin by standard procedures.

**Isolation and Phenotypic Characterization of Hepatocytes and Cholangiocytes**

Hepatocytes were obtained from both normal and BDL rats as previously described (5, 6). After isolation, the purity of hepatocytes was assessed by histochemistry for glucose-6-phosphatase (33), a marker for hepatocytes (2, 5, 6), and γ-glutamyltranspeptidase (γ-GT) (29), a cholangiocyte-specific marker (5, 16). Pooled or cholangiocyte subpopulations (i.e., fraction 1 or small cholangiocytes, ~8 µm in size, and fraction 2 or large cholangiocytes, ~14 µm in size) were purified by immunoaffinity separation as previously described by us (2, 9–11, 16, 18, 19, 35). The two subpopulations (i.e., fractions 1 and 2) were obtained by counterflow elutria
tion (at the flow rate of 25 and 55 ml/min, respectively) using a Beckman J-2–21M centrifuge equipped with a J E-6B rotor (Beckman Instruments, Fullerton, CA) before immunobead purification. Cholangiocyte purity was determined by γ-GT histochemistry (29). To assess the degree of cellular contamination by hepatocytes or mesenchymal cells, cholangiocytes were stained for glucose-6-phosphatase (33) and vimentin (2, 6), respectively. Diameters of the two subpopulations were assessed by a technique recently developed by us (2, 9, 10). Cell number and viability were assessed by trypan blue exclusion.

**Measurement of Proliferative Capacity of Hepatocytes and Pooled Cholangiocytes**

DNA synthesis in hepatocytes and pooled cholangiocytes from both normal and BDL rat livers was assessed by measurement of both [3H]thymidine incorporation (19) and H3 histone gene expression [an index of DNA synthesis (2, 3)] using RNase protection assays (9–11, 35). Both approaches have been previously used by us (2, 3, 19) to measure DNA synthesis in normal and proliferating cholangiocytes. Briefly, 90 min after an intraperitoneal injection of 1 µCi/g body wt [methyl-3H]thymidine, pure preparations of hepatocytes and pooled cholangiocytes from normal and BDL rat livers (~1.0 × 10^6 cells) were first treated with 3 M KOH at 37°C for 30 min, then with a solution containing 15% trichloroacetic acid and 6 N HCl at 4°C for 12 h. After the solution was centrifuged at 10,000 g for 20 min at 4°C, DNA was extracted from the cholangiocyte cell pellet with HClO4 at 80°C for 15 min. After
the solution was spun, the supernatant was transferred, and the radioactivity incorporated into DNA was measured. Before they were counted, samples were kept at 4°C in the dark overnight to avoid chemiluminescence. The results were expressed as $1 \times 10^6$ cpm/108 cholangiocytes. Data are means ± SE of 3-5 experiments.

The quantitative expression of H3 histone was assessed by RNase protection assays with the RPA II kit (Ambion, Austin, TX) using total cellular RNA (20 µg) obtained from hepatocytes or pooled cholangiocytes (50 × 106) according to the method of Chomczynski and Sacchi (13). The amount of the total cellular RNA assayed was determined by both ultraviolet detection of ethidium bromide-stained blots and absorbance at 260 nm. The quality of the total RNA samples was assessed by the ratio of absorbance at 260 nm to absorbance at 280 nm. The equality of the total RNA used was assessed by hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. After exposure for 2 days, autoradiograms were quantified by densitometry. Antisense riboprobes were transcribed from linearized cDNA templates with either T7 or SP6 RNA polymerase using [c-32P]UTP (800 Ci/mmol, Amersham). We used the following 32P-labeled single-stranded antisense riboprobes: a 204-bp riboprobe encoding for the H3 histone gene was obtained from Dr. S. Gupta (Albert Einstein Hospital, Bronx, NY) and a 316-bp riboprobe encoding a complementary sequence for rat GAPDH mRNA was purchased from RNA (20 µg) obtained from fraction 1 and fraction 2 (stimulated with BSA or somatostatin) according to the method of Chomczynski and Sacchi (13). The comparability of the total cellular RNA used in our molecular studies was determined by hybridization with GAPDH, a housekeeping gene (9–11, 35). The 318-bp riboprobe (encoding for SR mRNA) was generated from our SR cDNA clone (11); a 316-bp riboprobe encoding a complementary sequence for rat GAPDH mRNA was transcribed from cDNA purchased from Ambion. We used the following controls: rat spleen (positive) and yeast transfer RNA (negative) for the H3 histone gene as well as rat kidney (positive) and yeast transfer RNA (negative) for the GAPDH gene.

Measurement of Genetic Expression of SSTR2 in Cholangiocyte Subpopulations from BDL Rat Liver

In the two cholangiocyte subpopulations, we measured the transcript for SSTR2 mRNA by RNase protection assays with the RPA II kit (Ambion) using 20 µg total cellular RNA (9–11) (see above). Total RNA was extracted by the acid guanidium thiocyanate-phenol chloroform single-step extraction method (13). The equality of total cellular RNA assayed was determined by hybridization with a cDNA encoding for the GAPDH gene (9–11, 35). A 252-bp riboprobe encoding for a complementary sequence for rat GAPDH mRNA was transcribed from cDNA purchased from Dr. S. Gupta (Albert Einstein Hospital, Bronx, NY) and a 316-bp riboprobe encoding a complementary sequence for rat GAPDH mRNA was obtained from cDNA purchased from Ambion. We used the following controls: rat spleen (positive) and yeast transfer RNA (negative) for the GAPDH gene. Steady-state levels of selected messages were quantified by densitometry.

Determination of Proliferative Capacity of Cholangiocyte Subpopulations

To determine if somatostatin has differential inhibitory effects on proliferative processes of cholangiocyte subpopulations originating from different portions of the intrahepatic biliary tree, we studied in vitro the effect of somatostatin on DNA synthesis of small and large cholangiocytes isolated from BDL rat livers. Ninety minutes after an intraperitoneal injection of 1 µCi/g body wt [methyl-3H]thymidine, fraction 1 and fraction 2 were isolated by immunofluorescence and subsequently treated with 0.2% BSA (control) or somatostatin (10−7 M) in 0.2% BSA for 15 min at 37°C. The nuclear incorporation of [3H]thymidine in fraction 1 and fraction 2 was measured as previously described by us (19) (see above).

After incubation with 0.2% BSA (control) or somatostatin (10−7 M) in 0.2% BSA for 15 min at 37°C, proliferative capacity of fraction 1 and fraction 2 was assessed by measuring H3 histone gene expression by RNase protection assay (9–11, 35) (see above). The comparability of the total RNA used was assessed by hybridization with GAPDH, the housekeeping gene (9–11, 35). We used rat spleen (positive) and yeast transfer RNA (negative) for the H3 histone gene as well as rat kidney (positive) and yeast transfer RNA (negative) for the GAPDH gene. Steady-state levels of selected messages were quantified by densitometry.

In Vitro Effect of Somatostatin on Ductal Secretory Activity of Cholangiocyte Subpopulations from BDL Rat Liver

Measurement of SR gene expression. After purification, both fraction 1 and fraction 2 (50 × 106 cells) from BDL rat liver were stimulated in vitro with 0.2% BSA (control) or somatostatin (10−7 M) in 0.2% BSA for 15 min at 37°C. After stimulation, the quantitative genetic expression of the SR gene was assessed by RNase protection assays with the RPA II kit (Ambion) (see above) using total cellular RNA (20 µg) obtained from fraction 1 and fraction 2 (stimulated with BSA or somatostatin) according to the method of Chomczynski and Sacchi (13). The comparability of the total cellular RNA used in our molecular studies was determined by hybridization with GAPDH, a housekeeping gene (9–11, 35). The 318-bp riboprobe (encoding for SR mRNA) was generated from our rat SR cDNA clone (11); a 316-bp riboprobe encoding a complementary sequence for rat GAPDH mRNA was transcribed from cDNA purchased from Ambion. In our molecular analysis, we used the following controls: rat heart (positive) and kidney (negative) for the SR gene and rat kidney (positive) and yeast transfer RNA (negative) for the GAPDH gene.

Assessment of intracellular cAMP levels. In BDL rat liver, intracellular cAMP levels of fraction 1 and fraction 2 were measured by commercially available radioimmunoassay kits (Amersham) as previously reported by us (2, 9, 10, 18, 19, 28, 35). Purified cholangiocytes were incubated at 37°C for 1 h to restore membrane proteins that may have been damaged by proteolytic enzyme digestion and subsequently incubated for 5 min at 22°C (2, 9, 10, 18, 19, 28, 35) in the presence of 1% BSA with secretin (10−7 M) or secretin plus somatostatin (both at 10−7 M) before assessing intracellular cAMP levels. Control cholangiocytes were incubated with 0.2% BSA under the same experimental conditions. In both control and hormone-treated cholangiocytes, phosphodiesterase activity was inhibited by addition of 3-isobutyl-1-methylxanthine (0.5 mM).

Statistical Analysis

All data are expressed as means ± SE. The differences between groups were analyzed by Student’s unpaired t-test when two groups were analyzed or ANOVA if more than two groups were analyzed.

RESULTS

In Situ Immunohistochemistry and Autoradiography

To validate our model of cholangiocyte hyperplasia after BDL, we estimated cholangiocyte proliferative capacity by both in situ immunohistochemistry for CK-19 [a cholangiocyte-specific marker (6, 9, 10, 19)] and standard in vivo autoradiography. Immunohistochemical studies show that only two to three intrahepatic bile ducts (stained for CK-19) were present in a
normal rat liver section (Fig. 1A) in agreement with other studies (6, 19). After BDL, a marked increase in the number of bile ducts (stained for CK-19) was seen within portal areas (Fig. 1B), findings consistent with our previous studies (6, 19). In both normal and BDL rat livers, the lobular hepatic architecture was normal, indicating that BDL does not induce proliferative or morphological changes in hepatic lobules. In normal rats (Fig. 1C), no cholangiocyte nuclei were labeled with [3H]thymidine, whereas silver grains were observed in hepatocytes, the only liver epithelia that replicate in the normal state. After BDL (Fig. 1, D and E), cholangiocytes lining bile ducts had labeled nuclei, whereas no change in labeling was seen in other liver cells. The data are consistent with the concept that cholangiocytes selectively and markedly proliferate after BDL (5–7, 14, 35).

**Purification and Characterization of Hepatocytes and Cholangiocytes**

Hepatocytes from both normal and BDL rats were $\geq 99\%$ pure by glucose-6-phosphate histochemistry (data not shown). By $\gamma$-GT histochemistry, we did not detect any cholangiocyte contamination in our hepatocyte preparations (data not shown). In agreement with our previous studies (9–11, 14, 16, 18, 19, 35), we obtained, by immunoaffinity bead purification, virtually pure preparations of both pooled and fractionated small (i.e., fraction 1) and large (i.e., fraction 2) cholangiocytes (data not shown). No hepatocytes or mesenchymal cells were detected in our preparations of cholangiocytes (data not shown), a finding that agrees with our previous studies (2, 6, 14, 19). In agreement with our previous studies (2, 9, 10), diameter distribution analysis showed that we were able to isolate two distinct subpopulations (i.e., fractions 1 and 2) of cholangiocytes from BDL rat liver with virtually no overlap (data not shown). Cell viability of both hepatocytes and cholangiocytes was $\geq 97\%$.

**Measurement of [3H]thymidine Incorporation in Isolated Hepatocytes and Isolated Pooled Cholangiocytes**

In hepatocytes isolated from normal rat liver, [3H]thymidine incorporation was 238.31 ± 21.96 cpm/10⁶ cells (Fig. 2), consistent with the concept that in normal rat liver hepatocytes have the capacity to replicate (see also Fig. 1C). In pooled cholangiocytes isolated from normal rat liver, [3H]thymidine incorporation was virtually absent (0.70 ± 0.48 cpm/10⁶ cells) (Fig. 2), consistent with the notion that cholangiocytes are mitotically dormant under basal conditions (2, 5, 6, 19). After BDL, [3H]thymidine incorporation into purified hepatocytes did not change significantly (156.65 ± 44.07 cpm/10⁶ cells, P < 0.05 vs. normal hepatocytes) (Fig. 2) concordant with the notion that hepatocytes do not proliferate after BDL (5–7). In contrast, radioactivity incorporated into the DNA of pooled cholangiocytes isolated after BDL markedly increased (97.89 ± 6.50 cpm/10⁶ cells, P < 0.05 vs. normal pooled cholangiocytes), supporting the concept that cholangiocytes are the major proliferative target of BDL in rat liver (see also Fig. 1, D and E) (5–7, 10, 35). Similarly, H3 histone gene expression was
present in normal hepatocytes and was not affected by BDL (Fig. 2). The expression of H3 histone gene was very low in normal pooled cholangiocytes but markedly increased (90-fold) in proliferating cholangiocytes from BDL rat liver (Fig. 2). The expression of the housekeeping gene GAPDH was similar between hepatocytes and pooled cholangiocytes obtained from BDL rat liver (Fig. 2).

SSTR2 mRNA in Cholangiocyte Subpopulations From BDL Rat Liver

As shown in Fig. 3, the message for GAPDH, the housekeeping gene, was similarly expressed in fraction 1 and fraction 2 of cholangiocytes obtained from BDL rat liver. In contrast, we found no expression of the message for SSTR2 in fraction 1, but unequivocal expression of this message in fraction 2 (Fig. 3).

Effect of Somatostatin on Proliferative Capacity of Cholangiocyte Subpopulations From BDL Rat Liver

Measurement of [3H]thymidine incorporation. After purification, DNA synthesis was active principally in fraction 2 (186.05 ± 13.63 cpm/10⁶ cells, P < 0.05 vs. [3H]thymidine uptake of fraction 1) obtained from BDL rat livers (Fig. 4). Indeed, DNA synthesis was virtually absent in fraction 1 (1.56 ± 0.95 cpm/10⁶ cells) (Fig. 4). In the presence of somatostatin (10⁻⁷ M), DNA synthesis significantly decreased only in fraction 2 (84.06 ± 33.94 cpm/10⁶ cells, P < 0.05 vs. [3H]thymidine uptake of untreated cholangiocytes, 186.05 ± 13.63 cpm/10⁶ cells) (Fig. 4). The inhibitory effect of somatostatin on DNA synthesis of fraction 2 (Fig. 4) closely parallels the molecular data on SSTR2 mRNA distribution obtained by the RNase protection assay showing that the transcript for SSTR2 is expressed by fraction 2 but not fraction 1 (see Fig. 3).
State-steady levels of H₃ histone mRNA. As shown in Fig. 5, H₃ histone mRNA was principally expressed in fraction 2 after BDL, being present at only very low levels in fraction 1. The data closely parallel the studies on [³H]thymidine incorporation (Fig. 4). In a fashion similar to that shown for [³H]thymidine incorporation in purified cholangiocytes (Fig. 4), somatostatin caused an approximately sixfold decrease in H₃ histone gene expression in fraction 2 as compared with BSA-treated (control) cholangiocytes (Fig. 5). Consistent with the concept that SSTR₂ mRNA is present principally in fraction 2 in rat liver (see Fig. 3), somatostatin did not decrease the expression of H₃ histone mRNA in fraction 1 (Fig. 5).

Effect of Somatostatin on Secretin-Induced Ductal Secretory Activity of Cholangiocyte Subpopulations From BDL Rat Liver

SR gene expression. Because of the close coupling between cholangiocyte proliferation and increased ductal bile secretion (5, 7, 14, 19), we next determined if somatostatin also differentially inhibits ductal secretory activity in small (fraction 1) and large (fraction 2) cholangiocytes from BDL rat livers. In agreement with our previous studies in BDL rat liver (11), SR mRNA was principally expressed in fraction 2 (Fig. 6). Parallel with decreases in the proliferative capacity of cholangiocytes, somatostatin induced an ~50% decrease in SR gene expression in fraction 2. Consistent with the cellular distribution of SSTR₂ mRNA in cholangiocytes (Fig. 3), somatostatin did not alter the genetic expression of SR in fraction 1.

Intracellular cAMP levels. Basal levels of cAMP were similar among fractions 1 and 2 (Fig. 7), a finding that agrees with our previous studies (10). Also in agreement with our previous reports (2, 10, 11), secretin had no significant effect on intracellular cAMP levels in fraction 1 (Fig. 7) but increased the intracellular cAMP levels approximately fivefold over basal in fraction 2 (Fig. 7). When cells were exposed simultaneously to both somatostatin and secretin (both at 10⁻⁷ M), there was approximately a fourfold decrease in intracellular cAMP levels of fraction 2 compared with secretin only. The inhibitory effect of somatostatin on cAMP synthesis was essentially absent in fraction 1 (Fig. 7).

DISCUSSION

The major findings of these in vivo and in vitro studies relate to 1) the capacity of cholangiocytes
isolated from different portions of the intrahepatic biliary tree to differentially proliferate in response to BDL and 2) the differential inhibitory effect of somatostatin on secretory and proliferative processes of cholangiocyte subpopulations of different sizes originating from different portions of the intrahepatic biliary tree.

We have isolated two distinct subpopulations of cholangiocytes (fraction 1, or small cholangiocytes, and fraction 2, or large cholangiocytes) from BDL rat liver and have shown that the proliferative capacity of cholangiocytes assessed by two independent methods is found principally in large cholangiocytes (i.e., fraction 2); our previous work has indicated that these cholangiocytes line bile ducts of diameters >15 µm (3). After BDL, no proliferative activity was detected in small cholangiocytes (i.e., fraction 1) that line bile ducts <15 µm. SSTR2 mRNA was expressed in fraction 2 but not in fraction 1. Consistent with the presence of the SSTR2 only in fraction 2, we found that the inhibitory effect of somatostatin on SR gene expression and on secretin-induced cAMP synthesis (two indexes of ductal bile secretion; Refs. 2–5, 7–11, 14, 18, 19, 35) occurred solely in fraction 2. In parallel with these data, somatostatin inhibited DNA synthesis in vitro principally in fraction 2. These data, taken together, provide strong additional evidence for the concept of cholangiocyte heterogeneity and suggest that the proliferative compartment for cholangiocytes after BDL is present principally in large cholangiocytes that line bile ducts >15 µm in diameter (3). Together with our previous studies (10, 11), we propose that a gradient for somatostatin responsiveness in BDL rats exists along the length of the intrahepatic biliary tree and that regulation of ductal secretion and cholangiocyte proliferation by somatostatin occurs principally in large cholangiocytes lining large bile ducts (3). Moreover, we propose that, after BDL, the resulting proliferative response of cholangiocytes is tightly coupled with several hormone-regulated ductal secretory processes within the intrahepatic biliary segments undergoing proliferation.

Normally, cholangiocytes are mitotically inactive (2, 5, 19). However, after a proliferative stimulus (e.g., BDL, partial hepatectomy), cholangiocyte proliferation is vigorous (5, 7, 8, 10, 14, 19, 35) and is tightly coupled to an increase in intrahepatic bile ductal mass (5, 7, 19), SR gene expression (10, 11, 19), and both basal and secretin-induced cAMP synthesis (10, 14, 19, 35). Indeed, it appears that the proliferating cholangiocyte is also more responsive to hormones (i.e., secretin and somatostatin) that are major regulators of ductal bile secretion (2–11, 14, 19, 27, 35).

BDL is an experimental maneuver that induces selective and marked proliferation of normal-appearing cholangiocytes (5–7, 14, 35). In a fashion similar to that shown by us (2, 19) in other models of cholangiocyte proliferation, we found in the present studies that an excellent correlation exists between [3H]thymidine incorporation and H3 histone gene expression as measures of the proliferative capacity of cholangiocytes. The data support the concept that the increased number of intrahepatic bile ducts and the enhanced agonist-induced ductal bile secretion after BDL both reflect activities of preexisting, large cholangiocytes. Importantly, when cholangiocyte hyperplasia is induced by perturbations other than BDL (e.g., partial hepatectomy and acute exposure to CCl4), the proliferative response involves small cholangiocytes (20–22). These data emphasize the plasticity of cholangiocytes to respond to different stimuli (2, 5–8, 10, 11, 14, 19, 20, 22, 35). Indeed, for reasons that are currently obscure, it appears that although all cholangiocytes (i.e., small and large) have the capacity to proliferate in response to mitotic stimuli, those cholangiocytes that actually do proliferate depend on the nature of the stimulus. That different stimuli can induce a differential proliferative response in epithelia lining a particular organ has been previously observed (23, 24, 26, 31). For example, prolonged hormone stimulation of pancreatic cell replication is restricted to intralobular but not interlobular ducts (23). In the kidney, potassium depletion induces hyperplasia of epithelium only in the collecting duct and not in the rest of the nephron (31). In the intestine, gastrin stimulates proliferation of crypt cells only in the duodenum and the colon (24). In the liver, periporal hepatocytes have greater proliferative capacity compared with perivenous hepatocytes after partial hepatectomy (26). Obviously, additional experiments are...
necessary to understand the molecular mechanisms that underlie this interesting phenomenon.

We (35) and Ricci and Fevery (27) have shown that somatostatin, by interacting specifically with SSTR2 on cholangiocytes inhibits both spontaneous and secretin-stimulated choleretic in vivo and basal and secretin-induced exocytosis and increases in cAMP levels in vitro. Furthermore, Tracy et al. (36) have shown that somatostatin inhibits cholangiocyte proliferation in rats in response to BDL. However, no information exists regarding the anatomic sites of somatostatin’s action and its receptor (i.e., SSTR2) along the length of the intrahepatic biliary tree or how somatostatin affects proliferative and secretory processes of distinct subpopulations of cholangiocytes. Parallel to our findings on the distribution of cholangiocyte proliferative activity in different cholangiocyte subpopulations, we found SSTR2 gene expression to be present only in fraction 2. Our data represent the first evidence of the cellular localization of SSTR2 receptor in distinct portions of the intrahepatic biliary tree. Consistent with somatostatin functioning as a specific antagonist for secretin (35), we found SSTR2 distribution among cholangiocyte subpopulations to be similar to the distribution of SR gene expression and to the secretin-induced increases in cAMP synthesis (i.e., present in fraction 2 but not fraction 1) (2–4, 9, 10). Moreover, the data demonstrate that somatostatin inhibits secretory processes of cholangiocytes by specifically interacting with large cholangiocytes through a coordinated inhibition of SR gene expression and secretin-induced cAMP synthesis, two major determinants of ductal bile secretion (2–5, 7–11, 14, 19, 35). The decrease in SR gene expression observed in vitro after somatostatin treatment closely parallels previous in vivo studies (17) showing that somatostatin decreased levels of gastrin mRNA and partially inhibited gastrin gene transcription in dog antral mucosa.

Further studies are necessary to understand the physiological function of small cholangiocytes. In the present studies, we have shown that small cholangiocytes apparently do not proliferate after BDL and do not respond physiologically in this hyperplastic model. In contrast, preliminary data from our laboratory (12) indicate that small cholangiocytes participate in the modulation of ductal bile secretion via Ca2+- and inositol 1,4,5-trisphosphate-dependent (but not cAMP-dependent) mechanisms through a pathway different from that shown for secretin (2–5, 7–11, 14, 18, 19, 35). Also, we have evidence that in other models of ductal hyperplasia (e.g., partial hepatectomy and acute exposure to CCl4), small cholangiocytes proliferate robustly (20–22).

We believe our studies have important physiological and pathophysiological implications. In contrast to other organs (1, 15), little information exists with regard to the mechanisms by which specific segments of the intrahepatic biliary tree proliferate. Information on the proliferative responses of different segments of the biliary tree in response to different pathological perturbations may ultimately offer insight into certain human diseases (e.g., primary biliary cirrhosis or primary sclerosing cholangitis) where only certain size ducts are damaged (8).

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-24031 (to N. LaRusso), a grant from the Mayo Foundation (to N. F. LaRusso), a grant from Scott & White Hospital and Texas A&M University Health Science Center (to G. Alpini and G. LeSage), and a grant from the Department of Veterans Affairs (to G. Alpini).}

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Received 2 September 1997; accepted in final form 9 January 1998.

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