Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Tannic Acid Inhibits Cholangiocyte Proliferation after Bile Duct Ligation via a Cyclic Adenosine 5',3'-Monophosphate-Dependent Pathway

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Chronic cholestatic diseases are characterized by morphological changes involving cholangiocyte proliferation and functional alterations of secretory capacity. The plant polyphenol tannic acid inhibits the growth of malignant human cholangiocytes. However, the mechanisms by which tannic acid limits excessive cholangiocyte proliferation are unknown. In this study we assessed the effect of tannic acid on cholangiocyte proliferation after bile duct ligation in rats. Tannic acid feeding decreased cholangiocyte proliferation and ductal mass *in vivo* after bile duct ligation. These changes were associated with functional changes in bile secretion and with decreases of intracellular cyclic adenosine 5',3'-monophosphate. The anti-proliferative effect of tannic acid was associated with a reduction of ERK1,2 phosphorylation. Additionally, tannic acid feeding decreased protein kinase A phosphorylation and activity. Similar changes were observed in isolated cholangiocytes during *in vitro* incubation with tannic acid. Furthermore, forskolin abolished the anti-proliferative effect of tannic acid on cholangiocyte proliferation after bile duct ligation. In conclusion, the anti-proliferative effects of tannic acid in cholangiocytes involve modulation of ERK1,2 by a cyclic adenosine 5',3'-monophosphate-protein kinase A-dependent pathway. These data suggest that tannic acid may be useful in limiting excessive cholangiocyte proliferation and modulating secretion during cholestasis. (*Am J Pathol* 2005, 166:1671–1679)

A diverse and heterogeneous group of diseases affecting the liver are characterized by progressive hepatic damage resulting from chronic cholestasis, often leading to the need of liver transplantation.^{1–4} These conditions are characterized by morphological and functional alterations involving cholangiocytes, the biliary epithelial cells.^{4,5} Cholangiocytes play an important role in the modification of pH, water, and electrolyte composition of the bile.⁶ Thus, alterations in the number of functional cholangiocytes or cholangiocyte dysfunction can result in alterations in bile secretion and contribute to progressive liver damage. Current therapies for these conditions do not modulate cholangiocyte proliferation.

Plant polyphenols are known to inhibit malignant cell proliferation in many cancer cell lines.^{7–13} Tannic acid is a hydrolysable plant polyphenol present in a variety of foods such as nuts, red wine, tea, and coffee. This natural product is generally regarded as safe and widely used as a food additive.^{14,15} We have recently showed that tannic acid inhibits the growth of malignant human cholangiocytes *in vitro* and *in vivo*.⁷ However, the precise mechanisms by which tannic acid decreases excessive cholangiocyte proliferation are unknown.

Ductal bile secretion can be regulated by gastrointestinal hormones/peptides such as secretin. The receptor for this gastrointestinal hormone is expressed only by cholangiocytes in rat livers. After binding to its receptor, secretin increases intracellular cyclic adenosine 3',5'-monophosphate (cAMP) levels, with subsequent opening of CFTR Cl[−] channels, activation of the apically located Cl[−]/HCO₃[−] exchanger and secretion of HCO₃[−] ions into bile.^{16–18} In physiological conditions, cholangiocytes are

Supported by the Public Health Service (grant awards DK60637 to T.P. and DK58411 to G.A.), and a Veteran's Administration merit award to G.A.), Japan Society for the Promotion of Science [grant-in-aid for scientific research (C) 13670488 to Y.U.], and the Scott and White Memorial Hospital Foundation.

Accepted for publication March 1, 2005.

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mitotically quiescent. However, after bile duct ligation (BDL), biliary epithelial cells undergo rapid and dramatic proliferation.⁶ Increased proliferation is associated with enhanced secretin-stimulated cAMP levels and increased ductal secretion.¹⁹ Thus, BDL is a useful model for the study of cholangiocyte proliferation and bile ductular secretion.

To evaluate the effect and mechanisms by which tannic acid modulates cholangiocyte proliferation, we studied the effects of tannic acid feeding on cholestasis induced by BDL in rats. We tested the hypothesis that tannic acid decreases hyperplastic cholangiocyte proliferation during cholestasis by modulation of cAMP-dependent pathways. We examined this hypothesis in experimental cholestasis resulting from BDL, and posed the following questions: 1) Does tannic acid feeding administration affect proliferation of cholangiocytes during cholestasis resulting from BDL in rats? 2) Does tannic acid feeding modulate ductal secretion after BDL? 3) If so, are these effects mediated by a cAMP-dependent mechanism? 4) Do these changes result from direct effects of tannic acid on cholangiocytes?

Materials and Methods

Animal Model

Male Fisher rats (150 to 175 g) were purchased from Charles River (Wilmington, MA). Animals were maintained in a temperature-controlled environment (20 to 22°C) with a 12:12-hour light-dark cycle, fed *ad libitum* standard rat chow, and had free access to drinking water. For selected studies, animals underwent BDL, bile duct incannulation (BDI), or no surgery, and subsequently received either sterile water or sterile water containing 0.05% tannic acid. For *in vitro* studies, cholangiocytes were isolated from rats 7 days after BDL. BDL or BDI were performed as described.^{20,21} Before each experimental procedure, the animals were anesthetized with sodium pentobarbital (50 mg/kg weight, i.p.) according to the regulations of the panel on euthanasia of the American Veterinarian Medical Association. Animal protocols were approved by the Institutional Animal Care and Use Committee.

Purification of Cholangiocytes

The isolation of pure cholangiocytes from the selected groups of animals was performed by immunoaffinity separation using a mouse monoclonal antibody (IgM; kindly provided by Dr. R. Faris, Brown University, Providence, RI) against an unidentified membrane antigen expressed by all rat intrahepatic cholangiocytes.¹⁸ Cell viability was determined by trypan blue exclusion, and ranged from 95 to 98% in all experiments.

Evaluation of Cholangiocyte Proliferation

Cholangiocyte proliferation was evaluated by quantitative measurement of the number of proliferating cellular nu-

clear antigen (PCNA)- and CK-19-positive cholangiocytes and γ -glutamyltranspeptidase (γ -GT)-positive bile ducts in liver sections. After staining for PCNA or CK-19, the sections were counterstained with hematoxylin and examined under a light microscope (BX 40; Olympus Optical Co., Tokyo, Japan). Approximately 200 cells per slide were counted in seven nonoverlapping fields. Intrahepatic ductal mass was evaluated by light microscopy in sections stained for γ -GT by assessing the percentage of γ -GT-positive bile ducts in seven nonoverlapping fields.¹⁸ Proliferation in isolated cholangiocytes stimulated *in vitro* was assessed by quantitating PCNA protein expression (an index of cell replication) by immunoblot analysis.

Immunoblot Analysis

Immunoblot analysis was performed as previously described.²² Briefly, proteins (5 μ g/lane) were resolved by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated overnight at 4°C with primary antibodies followed by incubation with an IgG-horseradish peroxidase anti-mouse antibody diluted 1:2000 with 5% nonfat dry milk in TBST (50 mmol/L Tris, 150 mmol/L NaCl, and 1% Tween 20). The primary antibodies used were a rabbit anti-PCNA antibody (1:10,000), a mouse anti- β -actin antibody (1:10,000), a rabbit anti-protein kinase A (PKA) and anti-phospho PKA, a goat anti-ERK1,2, a mouse monoclonal anti-phospho ERK1,2, a mouse anti-Src, a goat anti-phospho Src Tyr⁵³⁰, and a rabbit anti-phospho Src Tyr¹³⁹ and were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). After several washes, bands were visualized using chemiluminescence (ECL Plus kit; Amersham Life Science, Little Chalfont, Buckinghamshire, UK), and quantitated using the Chemilmager 4000 low-light imaging system (Alpha Innotech Corp., San Leandro, CA).

Evaluation of Ductal Secretion

Ductal secretion *in vivo* was assessed by measurement of secretin-stimulated bicarbonate-rich choleresis. After anesthesia, rats were weighed and surgically prepared for bile collection as described.²³ One jugular vein was incannulated with a PE 50-cannula to infuse Krebs-Ringer-Henseleit (KRH) and secretin. Body temperature (37°C) was monitored with a rectal thermometer. When steady-state bile flow was reached (60 to 70 minutes from the infusion of KRH), BDI rats were subsequently infused with 100 nmol/L secretin for 30 minutes followed by a final infusion of KRH for 30 minutes. Bile was collected every 10 minutes and bile flow was determined by weight, assuming a density of 1.0 g/ml. Bicarbonate concentration (measured as total CO₂) in bile from BDI rats was determined by an ABL 520 blood gas system (Radiometer Medical A/S, Copenhagen, Denmark).

Measurement of Intracellular cAMP Levels

Basal and secretin-stimulated intracellular cAMP levels were measured in pure, freshly isolated cholangiocytes from BDL rats treated *in vivo* with 0.05% tannic acid or sterile water and from BDL animals for the *in vitro* stimulation with or without tannic acid (50 μ g/ml). Cholangiocytes (1×10^5 cells per stimulation) were incubated for 1 hour at 37°C to restore surface proteins damaged by the purification technique²⁰ and subsequently stimulated for 5 minutes with 0.2% bovine serum albumin (basal), or secretin (100 nmol/L) in the presence of 0.2% bovine serum albumin at room temperature. After ethanol extraction, cAMP levels were measured by a commercial radioimmunoassay kit (Amersham) following instructions provided by the vendor.

Src Kinase Activity Assay

Cholangiocytes were lysed in lysis buffer and protein concentration of lysates was quantified. Protein (30 μ g) was incubated with 5 μ g of Src monoclonal antibody (Upstate, Charlottesville, VA) for 2 hours, followed by the addition of protein A agarose (20 μ l) and incubation for an additional 2 hours. Immunoprecipitated proteins were washed three times in lysis buffer, then once in kinase buffer (0.1 mol/L HEPES, 0.02 mol/L $MgCl_2$, 0.01 mol/L $MnCl_2$, and 0.02 mol/L dithiothreitol) and tyrosine kinase activity was assayed using a commercial assay kit (Chemicon Int., Temecula, CA).

Materials

Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). Radioimmunoassay kits for the determination of intracellular cAMP levels were purchased from Amersham (Arlington Heights, IL). The substrate for γ -GT, *N*-(γ -L-glutamyl)-4-methoxy-2-naphthylamide was purchased from Polysciences (Warrington, PA). The mouse anti-cytokeratin 19 (CK-19) antibody was purchased from Amersham. The monoclonal mouse antibody against PCNA was purchased from DAKO (Kyoto, Japan). Antibodies (IgG) against PKA and phosphorylated PKA, total and phosphorylated ERK1,2, were purchased from Santa Cruz Biotechnology Inc. Kits for the detection of Raf-1 and B-Raf activity were purchased from Upstate (Charlottesville, VA).

Statistical Analysis

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

Results

Tannic Acid Decreases Cholangiocyte Proliferation in Vivo

BDL results in a dramatic increase in cholangiocyte proliferation and an increase in bile ductal mass. To assess the effect of tannic acid on cholangiocyte proliferation *in vivo*, PCNA- and CK-19-positive cholangiocytes and the number of γ -GT-positive ducts were identified by immunohistochemistry and quantitated in normal rats, as well as after BDL. There was no significant difference in PCNA, CK-19, or γ -GT staining in histological sections from normal rats receiving water containing 0.05% tannic acid compared to those from control rats receiving sterile water alone (Figure 1A). In contrast, there was a marked reduction in the number of PCNA- and CK-19-positive cholangiocytes in liver sections from BDL rats treated with 0.05% tannic acid as compared with rats administered sterile water alone (Figure 1B). Likewise, histochemistry for γ -GT on liver sections showed a significant decrease in ductal mass in BDL rats receiving drinking water with tannic acid compared to control rats. Thus, cholangiocyte proliferation after BDL was almost completely abrogated. These findings were further supported by studies demonstrating that PCNA protein expression (a marker of cell replication) was significantly decreased in cholangiocytes from BDL rats fed with tannic acid compared to controls fed with sterile water only (Figure 2A).

Tannic Acid Decreases ERK1,2 Phosphorylation

The extracellular signal-regulated kinases (ERK1,2) are critical intracellular mediators of proliferation in cholangiocytes, as well as in several other cell types.^{24–27} These kinases can be activated by a protein kinase cascade initiated by activation of the serine/threonine protein kinase Raf-1.^{28–30} Once activated, Raf-1 phosphorylates and activates the mitogen and extracellular signal-regulated kinase kinases 1 and 2 (MEK 1 and 2), which subsequently phosphorylate and activate ERK 1 and 2.^{25,26} Activated ERKs can phosphorylate several nuclear and cytoplasmic proteins involved in growth and cell division.^{25,26} Cholangiocyte proliferation during BDL is associated with increased phosphorylation of ERK1,2. To assess if the effects of tannic acid on cholangiocyte proliferation involved activation of ERK1,2, we quantitated ERK1,2 phosphorylation in BDL cholangiocytes from rats fed with 0% or 0.05% tannic acid. Tannic acid administration inhibited ERK1,2 active-site phosphorylation compared to controls (Figure 2B). Moreover, administration of tannic acid resulted in decreased activities of the upstream cellular kinases B-raf and Raf-1 (Figure 2, C and D). Thus, the inhibitory effect of tannic acid on cholangiocyte proliferation after BDL involves intracellular signaling pathways that culminate in ERK1,2 activation.

Figure 1. Tannic acid feeding decreases bile duct proliferation after BDL. Cholangiocyte proliferation was assessed by quantitating the number of PCNA- and CK-19-positive cholangiocytes and γ -GT-positive ducts in normal (**A**) or BDL (**B**) rats fed with either sterile water (control) or sterile water containing 0.05% tannic acid for 7 days. In normal rats, tannic acid feeding did not alter the number of PCNA- or CK-19-positive cholangiocytes or the number of γ -GT-positive ducts compared to control rats fed with water. However, after BDL, there was a dramatic reduction in the number of PCNA- and CK-19-positive cholangiocytes in liver sections from rats fed with tannic acid compared to those fed with water. Similarly, there was a significant decrease in ductal mass in BDL rats receiving drinking water with tannic acid compared to control rats. Representative histological sections are shown along with quantitative data representing the mean $\pm$ SEM of at least three experiments for each group. * $P < 0.05$ compared to controls.

Figure 2. Cholangiocyte proliferation is decreased by tannic acid feeding. **A:** PCNA protein expression, a marker of cell proliferation, was evaluated in pure cholangiocytes from BDL rats fed with sterile water with or without 0.05% tannic acid. A significant decrease in PCNA protein expression was observed in purified cholangiocytes from BDL rats fed with tannic acid compared with cholangiocytes from control rats. Data are mean $\pm$ SEM of at least three experiments. * $P < 0.05$ compared to BDL control rats fed with sterile water alone. **B:** Total and phosphorylated ERK1,2 protein expression was quantitated by immunoblot analysis in cholangiocytes from BDL rats fed with water containing 0 or 0.05% tannic acid. Tannic acid feeding decreases the ratio of phosphorylated to total ERK1,2 consistent with a decrease in kinase activation. Data are mean $\pm$ SEM of at least three experiments. * $P < 0.05$ versus the corresponding basal values. **C and D:** Measurement of B-Raf and Raf-1 kinase activity in cholangiocytes from BDL, receiving water with or without 0.05% tannic acid. **C:** Tannic acid treatment caused a strong reduction of B-Raf activity in cholangiocytes from treated rats as compared with cholangiocytes from the untreated group of rats. **D:** Tannic acid administration also triggered a significant reduction of Raf-1 activity in cholangiocytes from the treated group of animals. Data are mean $\pm$ SEM of at least three experiments. * $P < 0.02$ versus the corresponding basal values.

Tannic Acid Decreases Ductal Secretion

Next, we assessed the extent to which these morphological effects correlated with functional effects in cholangiocytes. Secretin stimulation results in increased bicarbonate secretion and bile flow in BDL rats (Table 1). However, these effects on bile flow and on bicarbonate secretion were not observed in rats receiving tannic acid water (Table 1). The functional effects of tannic acid on bile secretion suggested that tannic acid may act on intracellular signaling mediators of secretion such as the second messenger cyclic AMP (cAMP). Secretin stimulation results in an increase in cAMP levels in cholangiocytes from BDL rats. In cholangiocytes from BDL rats fed with tannic acid, cAMP levels were not

increased in contrast to those from rats fed with sterile water only (Figure 3A). Taken together, these observations suggest that tannic acid modulation of bile flow and bicarbonate secretion after BDL involve the modulation of intracellular cAMP levels.

Effect of Tannic Acid on cAMP-Dependent Signaling Pathways

cAMP-dependent pathways have been implicated in the regulation of cell proliferation in a number of epithelial cells including cholangiocytes. Indeed, cholangiocyte hyperplasia is associated with increased cAMP levels whereas loss

Table 1. Bile Flow and Bicarbonate Secretion in BDL Rats Receiving Water with or without 0.05% Tannic Acid

Treatment	Bile flow ($\mu\text{l}/\text{min}/\text{kg}$)		Bicarbonate concentration ($\mu\text{Eq}/\text{liter}$)		Bicarbonate secretion ($\mu\text{Eq}/\text{min}/\text{kg}$)	
	Basal	Secretin	Basal	Secretin	Basal	Secretin
Controls	73.08 (± 6.74)	135.39 (± 22.43)*	27.78 (± 1.67)	33.45 (± 1.19)*	2.01 (± 0.25)	4.61 (± 0.87)*
Tannic acid	81.32 (± 1.78)	82.50 (± 1.34)	29.35 (± 0.55)	31.55 (± 1.04)	2.35 (± 0.05)	2.52 (± 0.06)

* $P < 0.05$.

of cholangiocytes is characterized by decreased intracellular cAMP levels.¹⁹ Thus, we evaluated the role of cAMP-dependent signaling mechanisms in mediating the anti-proliferative effects of tannic acid. First we assessed the effect of tannic acid feeding on basal cAMP levels. Basal cAMP levels were decreased in cholangiocytes isolated from rats fed with tannic acid compared to controls after BDL (Figure 3A). Because the effects of cAMP on cell proliferation can involve signaling via activation of PKA, we next assessed the role of tannic acid on PKA activation (Figure 3, A and B). Tannic acid feeding decreased the phosphorylation as well as the activation of PKA. Finally, we evaluated the effect of forskolin, a stimulator of adenylyl cyclase that increases cAMP on growth inhibition by tannic acid. Forskolin completely abolished the effects of tannic acid on the proliferation of BDL cholangiocytes *in vitro* sup-

porting a crucial role of cAMP in mediating growth inhibition by tannic acid. In combination, these observations indicate that tannic acid can modulate phosphorylation of ERK1/2,

Figure 3. Tannic acid reduces secretin-stimulated cAMP stimulation and PKA phosphorylation and activation in bile duct-ligated rats. **A:** Basal and secretin-stimulated cAMP levels was measured *in vivo* in rats, after BDL, fed with sterile water with or without 0.05% tannic acid. Secretin stimulation increased cAMP levels after BDL, but this effect was decreased in rats fed with tannic acid. **B:** Quantitative measurement of total and phosphorylated PKA protein expression in cholangiocytes isolated from BDL rats fed water with or without 0.05% tannic acid. Tannic acid feeding decreases the ratio of phosphorylated to total PKA. **B:** PKA kinase activity was measured in cholangiocytes isolated from BDL rats fed water with or without 0.05% tannic acid, and was significantly decreased in the former. Data are mean $\pm$ SEM of at least three experiments. * $P < 0.05$ versus the corresponding basal values.

Figure 4. Tannic acid feeding decreases Src activation in cholangiocytes. After BDL, rats were fed with sterile water containing 0 or 0.05% tannic acid for 7 days. Cholangiocytes were then isolated, and the expression of Src evaluated using phosphorylation state-independent antibodies (total Src) or Src Tyr530 or Src Tyr139 phosphorylation site-specific antibodies. In cholangiocytes from tannic acid-fed rats, there is an increase in phosphorylation of Src at the inhibitory Tyr530 site (**A**) and a decrease in phosphorylation of Src at the stimulatory Tyr139 site (**B**). Thus, tannic acid administration *in vivo* increases activation of Src in cholangiocytes. Data are mean $\pm$ SEM of at least three experiments. * $P < 0.05$ compared to basal controls.

by a cAMP-dependent mechanism involving activation of PKA.

To examine downstream-signaling intermediates involved in the anti-proliferative effects of tannic acid, we assessed activation of the tyrosine kinase Src. Src has been shown to be involved downstream of cAMP/PKA and to have a role in modulating several different cell functions including cell growth.^{31,32} Tannic acid feeding induced an increase of Src Tyr⁵³⁰ phosphorylation with a concomitant decrease in Src Tyr¹³⁹ phosphorylation in BDL cholangiocytes. Phosphorylation at the Tyr⁵³⁰ and Src Tyr¹³⁹ sites are associated with decreased and increased Src activity, respectively (Figure 4, A and B). Thus, tannic acid decreased Src activation *in vivo*. These observations suggest that Src activation may mediate the inhibitory effects of tannic acid on cholangiocyte proliferation after BDL.

Tannic Acid Has a Direct Effect on Intracellular Signaling Mechanisms Mediating Cholangiocyte Proliferation

Our previous studies were performed in cholangiocytes isolated from rats after tannic acid feeding. However, tannic acid can be metabolized after ingestion to gallic acid, and other related compounds, raising the possibility that the observed effects observed *in vivo* could arise because of metabolites. To examine whether the observed effects resulted from a direct effect of tannic acid, we assessed the effect of incubation with tannic acid on cholangiocytes *in vitro*. Incubation of cholangiocytes obtained after BDL with tannic acid (50 μ g/ml for 72 hours) *in vitro* decreased both basal and secretin-stimulated intracellular cAMP levels compared to controls (Figure 5A). Furthermore, there was a

Figure 5. Tannic acid has a direct effect on intracellular signaling mechanisms mediating cholangiocyte proliferation. Cholangiocytes were isolated from BDL rats and incubated *in vitro* with either 0.2% bovine serum albumin or tannic acid (50 μ g/ml) in 0.02% bovine serum albumin for 72 hours. **A:** cAMP levels were assessed under basal conditions or after secretin stimulation. Data are mean $\pm$ SEM of at least three experiments. * P < 0.05 versus the corresponding basal values. **B:** Cells were incubated in the presence or absence of forskolin (10^{-4} mol/L), and cholangiocyte proliferation was assessed by measuring PCNA protein expression. The reduction in PCNA expression by tannic acid was inhibited by forskolin. **C:** ERK1,2 activation was assessed by quantitating total and phosphorylated ERK1,2 expression. Data are mean $\pm$ SEM of at least three experiments. * P < 0.05 versus the corresponding basal values. **D:** Activation of Src was assessed by an *in vitro* kinase assay after immunoprecipitation using monoclonal Src antibodies. Data represent mean $\pm$ SEM of seven separate assays. * P < 0.05 versus the corresponding basal values.

decrease in PCNA expression during the *in vitro* incubation of BDL cholangiocytes with tannic acid (50 $\mu\text{g/ml}$ for 72 hours) (Figure 5B). The decrease in PCNA expression was reduced by preincubation with forskolin (10^{-4} mol/L), a stimulator of cAMP. In addition, ERK1,2 phosphorylation was decreased during incubation of BDL cholangiocytes with tannic acid *in vitro* (Figure 5C). Src kinase activity was decreased in BDL cholangiocytes incubated with tannic acid *in vitro* (Figure 5D). Taken together, these findings demonstrate that the effects of tannic acid administration *in vivo* are related to a direct effect of tannic acid on intracellular signaling in cholangiocytes.

Discussion

The major findings of the present work relate to the demonstration of tannic acid as a potent inhibitor of cholangiocyte proliferation and bile ductal mass after experimental cholestasis induced by BDL in rats. The anti-proliferative effect of tannic acid was associated with a functional reduction in bile secretory capacity and loss of responsiveness to secretin stimulation. Furthermore, we demonstrate that the anti-proliferative effects of tannic acid were mediated by a cAMP-dependent mechanism, and that these effects involved modulation of the downstream proliferative signaling through ERK1,2. Additionally, tannic acid feeding was associated with decreased PKA phosphorylation and activity. Similar changes were observed in isolated cholangiocytes *in vitro*. The anti-proliferative effect of tannic acid on cholangiocyte proliferation after BDL was completely abolished by forskolin, which stimulates adenylyl cyclase and increases cAMP levels. These findings identify a potent effect of tannic acid on cholangiocyte proliferation involving a cAMP-dependent mechanism (Figure 6).

Figure 6. Tannic acid inhibits cAMP-mediated proliferation and bile secretion during experimental cholestasis. BDL increases both secretin-stimulated bile secretion as well as cholangiocyte proliferation. These effects may be mediated by alterations in expression of the second messenger cAMP. Tannic acid modulation of cAMP expression results in inhibition of downstream PKA activation, Src phosphorylation, and ERK1,2 phosphorylation with a decrease in proliferation.

The role of cAMP in cell proliferation has been extensively studied.^{33,34} Most of the effects of cAMP are mediated via phosphorylation and activation of PKA. Activation of PKA by the intracellular cAMP messenger system has been implicated in the regulation of cell proliferation in several different epithelial cell types, including cholangiocytes.^{19,23,35} Cholangiocyte hyperplasia has been associated with increased cAMP levels, whereas loss of cholangiocytes is characterized by decreased intracellular cAMP levels. We have recently reported that interruption of the parasympathetic innervation by vagotomy impairs cholangiocyte proliferation and enhances apoptosis leading to a decreased ductal mass in response to BDL.^{36,37} Maintenance of cAMP levels prevents the effects of vagotomy on cholangiocyte proliferation and apoptosis. These data, along with the results of the current study are consistent with the concept that activation of cAMP contributes to the regulation of cholangiocyte proliferation after BDL. Regulation of cell growth by the cAMP-dependent PKA pathway has been shown to involve ERK1,2 signaling.³⁸ However, the effects of cAMP on ERK1,2 are cell-type-specific and can involve either positive or negative regulation. Involvement of cAMP/PKA in the anti-proliferative effects of tannic acid in cholangiocytes is supported by our findings that tannic acid decreases active site phosphorylation for ERK1,2. Furthermore, stimulation with forskolin abolishes the inhibitory effect of tannic acid on PCNA protein expression as well as in ERK1,2 phosphorylation. Activation of ERK1,2 by cAMP/PKA can involve the Src kinase.³⁸ Thus the reduction in Src kinase activity by tannic acid is consistent with a potential role for Src kinase in the regulation of ERK1,2 in cholangiocytes.

cAMP is a central mediator and integrator of signaling in response to activation of diverse cell surface receptors. Identification of the mechanisms by which cAMP is regulated by tannic acid thus warrants further study. A better understanding of these mechanisms may eventually allow the design of novel pharmaceutical approaches to treat conditions characterized by aberrant cell proliferation. We speculate that tannic acid modulates stimulatory pathways resulting in cAMP activation after a proliferative stimulus. Tannic acid has been shown to reduce autophosphorylation of insulin receptor as well as activation of intracellular kinase signaling.^{39–41} The possibility that tannic acid directly modulates intracellular signaling is supported by our studies in which *in vitro* administration of tannic acid decreases intracellular kinase phosphorylation. Direct effects of tannic acid on adenylyl cyclase activity may also account for our observations. Because cAMP can be modulated by controlling its degradation, another potential site for the effect of tannic acid is at the level of modulation of phosphodiesterase activity.

These findings have important clinical implications for cholestatic diseases such as extrahepatic biliary atresia, idiopathic adulthood ductopenia, primary biliary cirrhosis, and primary sclerosing cholangitis. These and other diseases are characterized by alterations in secretin-induced bicarbonate secretion and bile flow. Although the precise relationship between abnormalities in bile flow and progressive liver disease is unknown, the use of

tannic acid in early stages of chronic cholestasis may help restore physiological bile ductal mass and secretion. Additional potential benefits of tannic acid may include antioxidant effects that may serve to limit fibrosis similar to those reported for other plant polyphenols.⁴² For these reasons, tannic acid supplementation may be useful in persons with chronic cholestatic diseases.

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