Portal Fibroblasts Regulate the Proliferation of Bile Duct Epithelia via Expression of NTPDase2*

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Bile duct epithelia are the target of a number of “cholangiopathies” characterized by disordered bile ductular proliferation. Although mechanisms for bile ductular proliferation are unknown, recent evidence suggests that extracellular nucleotides regulate cell proliferation via activation of P2Y receptors. Portal fibroblasts may regulate bile duct epithelial P2Y receptors via expression of the ecto-nucleotidase NTPDase2. Thus, we tested the hypothesis that portal fibroblasts regulate bile duct epithelial proliferation via expression of NTPDase2. We generated a novel co-culture model of Mz-ChA-1 human cholangiocarcinoma cells and primary portal fibroblasts. Cell proliferation was measured by bromodeoxyuridine uptake. NTPDase2 expression was assessed by immunofluorescence and quantitative real-time reverse transcription PCR. NTPDase2 expression in portal fibroblasts was blocked using short interfering RNA. NTPDase2 overexpression in portal myofibroblasts isolated from bile duct-ligated rats was achieved by cDNA transfection. Co-culture of Mz-ChA-1 cells with portal fibroblasts decreased their proliferation to 26% of control. Similar decreases in Mz-ChA-1 proliferation were induced by the soluble ecto-nucleotidase apyrase and the P2 receptor inhibitor suramin. The proliferation of Mz-ChA-1 cells returned to baseline when NTPDase2 expression in portal fibroblasts was inhibited using NTPDase2-specific short interfering RNA. Untransfected portal myofibroblasts lacking NTPDase2 had no effect on Mz-ChA-1 proliferation, yet portal myofibroblasts transfected with NTPDase2 cDNA inhibited Mz-ChA-1 proliferation. We conclude that portal fibroblasts inhibit bile ductular proliferation via expression of NTPDase2 and blockade of P2Y activation. Loss of NTPDase2 may mediate the bile ductular proliferation typical of obstructive cholestasis. This novel cross-talk signaling pathway may mediate pathologic alterations in bile ductular proliferation in other cholangiopathic conditions.

Bile duct epithelia (BDE), also known as biliary epithelia or cholangiocytes, are the targets of a number of chronic liver diseases. These cholangiopathies include such diverse conditions as primary biliary cirrhosis, primary sclerosing cholangitis, cystic fibrosis hepatopathy, AIDS cholangiopathy, biliary atresia, and many others (1, 2). Together these conditions comprise a significant health burden, with a disproportionate effect on the health of children. Although the causes and presentations of cholangiopathies are diverse, they are all characterized by disordered proliferation of BDE (3).

Factors regulating proliferation of BDE are not well understood. Although it has long been known that extrahepatic biliary obstruction (either via neoplasms in patients or bile duct ligation (BDL) in experimental animals) induces massive bile ductular proliferation, the mechanism by which this occurs is unknown (4). Recent experimental work has suggested that the hydrophilic bile acid tauoursodeoxycholic acid and the peptide hormone gastrin attenuate BDE proliferation (5, 6). However, neither of these mechanisms has been shown to mediate bile ductular proliferation after neoplastic biliary obstruction or BDL. Moreover, the mechanisms by which these occur are just beginning to be elucidated.

Recently, we reported that signaling in BDE may be regulated by a novel liver cell type, the portal fibroblast (PF), via expression of the ecto-nucleotidase NTPDase2 (also known as ecto-ATPase or CD39L1) (7). Several lines of evidence have directed us to examine the role of NTPDase2 in the regulation of bile ductular proliferation. First, BDE express basolateral P2Y receptors for extracellular nucleotides that are tonically regulated by nucleotide hydrolysis that is likely mediated by NTPDase2 (8). Second, the role of these basolateral BDE P2Y receptors is unknown. Third, strong evidence has been provided over the past several years demonstrating that activation of P2Y receptors has marked effects on cell proliferation (9). Finally, BDL induces marked down-regulation of NTPDase2 expression by PF (10). Thus, we tested the hypothesis that the physiologic role of basolateral P2Y receptors is to regulate BDE proliferation, and PF regulate this process via NTPDase2 expression. We further tested whether this hypothesis explains bile ductular proliferation induced by BDL.

MATERIALS AND METHODS

Chemical Reagents—Apyrase, suramin, and alkaline phosphatase-conjugated anti-rabbit antibody were purchased from Sigma. Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies and TOPRO-3 nuclear stain were purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest quality available.

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1 The abbreviations used are: BDE, bile duct epithelia; BDL, bile duct ligation; PF, portal fibroblast; PMF, portal myofibroblast; siRNA, short interfering RNA; RT, reverse transcription.

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Experimental Animals—Adult male Sprague-Dawley rats (180–250 g) were used for all experiments. For production of portal myofibroblasts (PMF), rats underwent BDL for 1 week (10). Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The abdomen was washed with isopropyl alcohol, and midline laparotomy was performed. The common bile duct was exposed using blunt dissection and ligated with dual sutures. Treatment of animals was within the prescribed guidelines of the Yale University Institutional Care and Use Committee.

Isolation of Portal Fibroblasts and Portal Myofibroblasts—PF were isolated as described previously (11). Briefly, rat nonparenchymal cell fractions were obtained by collagenase and pronase digestion of rat livers. Cell suspensions were separated using serial mesh filtration. The resulting suspension of nonparenchymal cells was plated in medium containing Dulbecco’s modified Eagle’s medium/F12 containing 2% penicillin-streptomycin, 10% fetal calf serum, 0.3% gentamycin, and 0.1% fungizone. Cells were used 96 h after isolation, at which time cell purity approaches 100%.

PMF (12, 13) were isolated from rats that had undergone BDL as described above using identical conditions (10). Cells from each batch were demonstrated to be myofibroblasts by staining for a-smooth muscle actin (not shown).

Culture of Mz-ChA-1 Cells—Mz-ChA-1 human cholangiocarcinoma cells were maintained in Dulbecco’s modified Eagle’s medium/F12 medium with identical additives to the culture medium for PF, so that observed changes would not be because of differences in cell culture conditions. Cells were used at ~75% confluence at 2–4 days after splitting.

Isolation of Rat Bile Duct Epithelia Cells—Rat BDE were prepared and characterized in the Cell Isolation Core Facilities of the Yale Liver Center as described previously (8). This preparation results in a bile duct epithelial preparation that is ~98% pure as assessed by staining positively for the biliary epithelial markers cytokeratin-19, and cytokeratin-7 (14).

Measurement of BrdUrd Incorporation—Cell proliferation was measured by BrdUrd enzyme-linked immunosorbent assay according to the manufacturer’s instructions (Cell Proliferation ELISA, BrdUrd Colorimetric, Roche Diagnostics GmbH). For experiments in Mz-ChA-1 and Portal Fibroblasts (PF), not shown). After co-culture, cells were maintained in the same medium described in the section titled “Isolation of Portal Fibroblasts and Portal Myofibroblasts.”

Co-culture of Mz-ChA-1 or BDE and PF—PF were split and plated onto individual wells of 6-well cell culture plates. After 2 days, PF were quantitated, and cells were added at an approximate 2:1 ratio to PF (basal stratified column preparations demonstrated stratification in the co-culture with PF, not shown). After co-culture, cells were maintained in the same medium described in the section titled “Isolation of Portal Fibroblasts and Portal Myofibroblasts.”

For experiments in Mz-ChA-1 cells in co-culture, cell proliferation was measured in Mz-ChA-1 cells only by loading the cells with BrdUrd-labeled Mz-ChA-1 cells. Medium alone was added to control cells. After 24 h, determination of BrdUrd incorporation was determined by enzyme-linked immunosorbent assay as described in the preceding paragraph.

Treatment of PF with siRNA—NTPDase2 protein expression in PF was down-regulated using siRNA. NTPDase2-specific siRNA was designed on the published sequence of rat NTPDase2 (GenBank™ accession number NM_172030) (18). The siRNA, 5′-GUUGUGUGUGUGACUGUGCCGTT-3′ and a control siRNA known not to correspond to any published cDNA sequences (“nonsense siRNA”) were labeled with fluorescein on the 3′-end (Qiagen, Mississauga, Canada) as a marker of successful transfection. The siRNAs were provided as annealed double-stranded siRNA. PF were transfected with siRNAs using TransMesse using QIAprep Spin Miniprep kit (Qiagen), and orientation of the insert was verified by restriction mapping. One clone obtained with each of primers was amplified and fully sequenced in one direction. Both sequences were identical. The corresponding and combined sequence is provided in GenBank™ accession number AY57674. The clone obtained with the first set of primers was verified using ecdot-A TPase activity assays. PMF were grown to 75–90% confluence and transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Real-time RT-PCR—Alterations in expression of NTPDase2 mRNA in PF were determined using real-time RT-PCR (18). PF were isolated from rats as described above and treated with either vehicle alone (control), nonsense siRNA, or NTPDase2-specific siRNA for 96 h. Total RNA was isolated using chustrophic methods and subjected to real-time PCR using the ABI-PRISM 7700 (Applied Biosystems, Foster City, CA). Detection of NTPDase2 was accomplished by labeling with 6-FAM. PCR was performed using the following cycling parameters: reverse transcription at 48 °C × 30 min; activation of AmpliTaq polymerase (Applied Biosystems) at 95 °C × 10 min; PCR cycling 40 cycles of 95 °C × 15 s (denaturation), and 60 °C × 1 min (annealing/extension). Experiments were performed in triplicate.
Statistical Analysis—Changes in proliferation were determined by two-tailed Student’s t test. Data are represented as mean ± S.D.

RESULTS

Co-culture of Mz-ChA-1 Cells with PF Markedly Attenuates Their Proliferation—To determine the effect of PF on bile ductular proliferation, we developed a novel model of co-culture of Mz-ChA-1 cholangiocarcinoma cells and primary PF. Successful co-culture of Mz-ChA-1 cells and PF was determined using confocal immunofluorescence. As seen in Fig. 1, MCL-1-expressing Mz-ChA-1 cells and vimentin-expressing PF closely abutted one another.

Mz-ChA-1 proliferation was assessed by BrdUrd uptake. Mz-ChA-1 cells were loaded with BrdUrd prior to co-culture, so that all changes in BrdUrd uptake reflected changes in Mz-ChA-1 proliferation. Relative to control Mz-ChA-1 cells, Mz-ChA-1 in co-culture with PF had a marked decrease in proliferation to 26 ± 12% of control (p < 0.002) as seen in Fig. 2A. This finding demonstrates that co-culture of Mz-ChA-1 cells...
we have previously described (7).

**NTPDase2 levels can be markedly reduced in PF using siRNA transfection.** The feasibility of reducing NTPDase2 protein levels in primary PF using siRNA transfection was determined using confocal immunofluorescence and real-time quantitative RT-PCR. A–H, confocal immunofluorescence. A–D, cells were transfected with fluorescein isothiocyanate-labeled nonsense siRNA for 48 h and fixed. Cells were stained for NTPDase2 using immunofluorescence and nuclei using TO-PRO staining. A, NTPDase2 staining (red) is shown in the pattern we have previously described (7). B, nonsense siRNA uptake in PF (green). C, TO-PRO staining of PF nuclei (blue). As seen in the merged image (D), PF transfected with nonsense siRNA express high levels of NTPDase2 and have taken up nonsense siRNA. E–H, cells were transfected with fluorescein isothiocyanate-labeled NTPDase2 siRNA as described above. E, weak NTPDase2 staining (red) is shown. Nuclear staining is likely because of bleed-through of TO-PRO (G). F, NTPDase2 siRNA uptake in PF (green) is demonstrated. G, TO-PRO staining of PF nuclei. As seen in the merged image (H), PF transfected with NTPDase2 siRNA express negligible levels of NTPDase2 and have taken up with PF markedly attenuates proliferation of these cells.

To confirm that Mz-ChA-1 cells are appropriate models for these experiments, we repeated these experiments on primary immuno-isolated rat BDE (14). Co-culture of BDE with PF decreased proliferation to 34 ± 4% of control, suggesting that Mz-ChA-1 cells are appropriate cell models for these studies (Fig. 2B).

P2Y Receptor Blockade Attenuates Mz-ChA-1 Proliferation—To test the hypothesis that the effect of PF on Mz-ChA-1 proliferation might be mediated via blockade of P2Y receptors, we examined the effect of the soluble ecto-nucleotidase apyrase and the P2 receptor inhibitor suramin on BrdUrd uptake in Mz-ChA-1 cells. Compared with control, both apyrase (65 ± 20%) and suramin (49 ± 16%) attenuated Mz-ChA-1 proliferation (p < 0.01), though not to the extent of co-culture with PF (Fig. 3). These data suggest that P2 receptor activation regulates Mz-ChA-1 proliferation and that this is mediated by endogenous nucleotide release.

**NTPDase2 Mediates PF-regulated Attenuation of Mz-ChA-1 Proliferation**—To test directly whether the attenuation of Mz-ChA-1 cell proliferation induced by PF was mediated by NTPDase2, we “knocked out” expression of NTPDase2 in PF using NTPDase2-specific siRNA. As seen in Fig. 4, transfection of PF with NTPDase2 siRNA (but not nonsense siRNA) markedly decreased the expression of NTPDase2 in these cells, demonstrating the effectiveness of siRNA in specifically down-regulating NTPDase2 mRNA and protein expression using this system.

The effect of PF expressing or lacking NTPDase2 expression on Mz-ChA-1 proliferation was assessed by BrdUrd uptake (Fig. 5). Consistent with the findings in Fig. 2, untransfected PF (54 ± 10%) and PF transfected with nonsense siRNA (48 ± 20%) decreased Mz-ChA-1 proliferation (p < 0.03). Importantly, when NTPDase2 expression was decreased in PF using NTPDase2-specific siRNA, the ability of Mz-ChA1 cells to proliferate (NTPDase2 siRNA, I, real-time quantitative RT-PCR. PF were treated with either vehicle alone (control), nonsense siRNA, or NTPDase2 siRNA as described above for 96 h. Cells were lysed for mRNA isolation. Changes in NTPDase2 mRNA were determined using real-time quantitative RT-PCR. Nonsense siRNA did not change levels of NTPDase2 mRNA, but NTPDase2-specific siRNA decreased NTPDase2 mRNA to ~60% of control (n = 6; *, p = 0.001 by two-tailed t test), which is equivalent to siRNA-sensitive transcriptional changes in mRNA levels noted by earlier investigators (49, 50).
Epithelial/Mesenchymal Interaction in the Liver

Fig. 6. NTPDase2 may be transfected into portal myofibroblasts with low NTPDase2 expression. NTPDase2 expression in PMF was detected using confocal immunofluorescence. NTPDase2 is seen in green, and nuclear staining by TOPRO is seen in blue. As reported previously, expression of NTPDase2 in (untransfected) PMF is markedly reduced or absent (10). However, after transfection with NTPDase2, almost all cells in the low power field are NTPDase2-positive and express NTPDase2 in the distribution previously described (7). This demonstrates the feasibility of up-regulating NTPDase2 protein expression in PMF.

Mz-ChA-1 Proliferation Is Not Attenuated by PMF Lacking NTPDase2 Expression because of BDL—To test the hypothesis that the bile ductular proliferation characteristic of BDL is mediated by loss of NTPDase2 expression, we determined Mz-ChA-1 proliferation in the presence of PMF lacking and expressing NTPDase2. We transfected BDL PMF lacking NTPDase2 expression with an NTPDase2 plasmid. As seen in Fig. 6, transfection of BDL PMF with NTPDase2 induced a marked increase in NTPDase2 expression at the plasma membrane.

Proliferation of Mz-ChA-1 cells was determined using BrdUrd as described above. As seen in Fig. 7, untransfected BDL PMF had no significant effect on Mz-ChA-1 proliferation (82 ± 40%; p was not significant), whereas BDL PMF transfected with NTPDase2 decreased Mz-ChA-1 proliferation (41 ± 20%; p < 0.001). These findings show that BDL PMF lacking NTPDase2 do not attenuate Mz-ChA-1 proliferation, whereas BDL PMF expressing NTPDase2 do, and support the hypothesis that bile ductular proliferation after BDL may be regulated by loss of NTPDase2 expression.

DISCUSSION

Cholangiopathies are conditions in which BDE are the target of disease. These conditions contribute greatly to the burden of liver disease in the United States and throughout the world, yet there are few effective therapies for them (19). In recent years, understanding of BDE physiology has increased because of improved techniques for their study (2). One of the areas in which BDE physiology has been well characterized is the role of extracellular nucleotides acting via P2Y nucleotide receptors expressed on BDE (20). Extracellular nucleotides are known to regulate electrolyte transport in BDE (21) and are important mediators of cell volume autoregulation (22). Because BDE express diverse P2Y receptors (8), it is likely that extracellular nucleotides regulate other BDE cell functions as well. Because P2Y receptors are thought to be of greatest importance for autocrine/paracrine communication, it is likely that these receptors mediate communication between BDE and their adjacent stroma.

Epithelial/mesenchymal interactions are of great importance in the regulation of a variety of cell functions. Specifically, recent reports have highlighted the importance of underlying stroma in the regulation of epithelial proliferation and/or differentiation (23–27). However, few studies have demonstrated a cross-talk loop created by a functional interaction between an ecto-enzyme expressed by one cell type and a receptor on the other. Here we have demonstrated directly that PF regulate the proliferation of BDE via expression of the ecto-nucleotidase NTPDase2. Furthermore, we showed that this regulatory mechanism is lost in the best-known model of bile ductular hyperproliferation (Fig. 8). This change in the NTPDase2-dependent regulation of BDE proliferation appears to parallel transdifferentiation of portal fibroblasts to myofibroblast-like, α-smooth muscle actin-positive cells known as portal myofibroblasts. This transdifferentiation is similar to the one observed in hepatic stellate cells (28) and other fibrogenic cells in which pro-fibrogenic stimuli induce myofibroblastic transdifferentiation. Thus, the interaction between PF and BDE may be a model of these critical interactions throughout the body.

In fact, an interaction between epithelium and mesenchyme is necessary for development of the liver. If the primordial liver epithelium contained in the liver diverticulum does not interact with septum transversum mesenchyme, the liver fails to form
representative human diseases to generate novel pharmaco-logic treatments for critical diseases within and outside of the liver.

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