BRIEF METHOD

Modified Culture Conditions Enhance Expression of Differentiated Phenotypic Properties of Normal Rat Cholangiocytes

Kelli D. Salter, Richard M. Roman, Nicholas R. LaRusso, J. Gregory Fitz, and R. Brian Doctor

Department of Medicine (KDS, RMR, JGF, RBD), University of Colorado Health Sciences Center, Denver, Colorado; and Department of Medicine (NRL), Mayo Clinic, Rochester, Minnesota (Lab Invest 2000, 80:1775–1778).

Cholangiocyte secretion contributes importantly to the formation of bile by the liver. Under normal conditions, increases in cellular cAMP levels increase cholangiocyte Cl^- and HCO_3^- secretion across the apical membrane, resulting in alkalinization and increased bile volume. Cholangiocytes also represent an important target of injury in a number of disease states, and impaired cell and epithelial function contributes to the cholestasis associated with primary biliary cirrhosis, sclerosing cholangitis, and posttransplant liver failure (Fitz, 1996; Mennone et al, 1995; Nathanson and Boyer, 1991; Tavaloni, 1987). Despite their physiologic and pathophysiologic importance, the intrahepatic location and limited number (4% of the liver nuclear mass) of cholangiocytes has limited direct definition of the cellular mechanisms involved in secretion. A model of normal rat cholangiocytes (NRC) in culture was recently introduced in *Laboratory Investigation* (Vroman and LaRusso, 1996). When cultured on collagen in the presence of empirically defined supplements, these cells retain many of the phenotypic features of cholangiocytes in situ, including a polarized morphology and the ability to form monolayers amenable to transport and electrophysiologic studies (Doctor et al, 1999; Vroman and LaRusso, 1996). Consequently, NRC cells have been used by a number of laboratories to broaden molecular and cellular insights into cholangiocyte function, including the characterization of phlorizin-sensitive glucose transport, Na^+ -dependent bile acid transport, and cAMP-stimulated Cl^- secretion (Lazaridis, 1997a, 1997b; Roberts et al, 1994; Spirli et al, 1998; Tietz et al, 1997). Despite these advances, the use of NRC

cells has been limited in part by (a) the formation in monolayer culture of relatively low transepithelial resistances, (b) the requirement for a number of expensive supplements, and (c) the need for prolonged culture before development of the differentiated epithelial phenotype. Consequently, the present studies were performed to critically assess the culture requirements of NRC cells, using transepithelial resistance (Rt) (Epithelial Volt-Ohm Meter; World Precision Instruments, Sarasota, Florida; confirmed by Ussing analysis) and transepithelial H^+ gradient ($\Delta[\text{H}^+]$) (Accu pHast; Fisher Scientific Pittsburgh, Pennsylvania) as primary assays of differentiation. To evaluate net H^+ flux, a pH/ H^+ titration curve was established for the HEPES/ HCO_3^- buffered media and measured pH values were converted to $[\text{H}^+]$. Among the different supplements tested, basolateral addition of dexamethasone (DEX) resulted in a dramatic increase in transepithelial resistance and greatly reduced cell culture expenses by decreasing the time required for cellular differentiation. Moreover, other measures of cholangiocyte function were enhanced in parallel. Appropriate modifications of NRC culture conditions are likely to facilitate the use of NRC cells as a model for investigation of the cellular and epithelial mechanisms responsible for cholangiocyte secretion and bile formation.

NRC cells (passages 9–25) were propagated in flasks as previously described (Vroman and LaRusso, 1996). Experimental studies were performed on NRC cells plated on collagen-coated semipermeable supports (Costar Corporation, Cambridge, Massachusetts; media exchanged every 48 hours). In the original studies (Vroman and LaRusso, 1996), the NRC media contained a significant number of supplements (Table 1, Control). These were individually evaluated for their effect on cell growth and epithelial differentiation. Cell growth and confluence were scored microscopically;

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Address reprint requests to: Dr. R. Brian Doctor, Campus Box B158, 4200 E. 9th Avenue, Denver, CO 80262. Fax: 303 315 3507; E-mail: brian.doctor@uchsc.edu

Table 1. Polarized Supplement Additions to CONTROL and EPITH Media

	Concentration	CONTROL		EPITH	
		Ap	BL	Ap	BL
Stock components					
DMEM/F12 (Gibco, Grand Island, NY)		+	+	+	+
Nonessential amino acids (Gibco, Grand Island, NY)	0.01 ml/ml	+	+	+	+
Lipid concentrate (Gibco, Grand Island, NY)	0.01 ml/ml	+	+	+	+
Vitamin solution (Gibco, Grand Island, NY)	0.01 ml/ml	+	+	+	+
L-glutamine (Gibco, Grand Island, NY)	2 mM	+	+	+	+
Soybean trypsin inhibitor (Gibco, Grand Island, NY)	0.05 mg/ml	+	+	+	+
Fetal bovine serum (Hyclone Lab, Logan, UT)	5%	+	+		
Tested supplements					
Dexamethasone (Sigma, St. Louis, MO)	393 ng/ml	+	+		+
Epidermal growth factor (UBI, Lake Placid, NY)	25 ng/ml	+	+		
Bovine pituitary extract (UBI, Lake Placid, NY)	30 μg/ml	+	+		+
Triiodothyrodine (Sigma, St. Louis, MO)	3.4 μg/ml	+	+		+
Insulin-transferrin-Se (Gibco, Grand Island, NY)	0.01 ml/ml	+	+	+	
Forskolin (Sigma, St. Louis, MO)	4.11 mg/ml	+	+		

Ap, apical chamber; BL, basolateral chamber; +, substance added.

Rt and $\Delta[H^+]$ were measured at 48-hour intervals as markers of differentiation. Of the supplements tested, only DEX and epidermal growth factor (EGF) were capable of inducing cell growth to confluence. Basolateral addition of DEX increased Rt from control values of $217 \pm 2 \Omega\text{cm}^2$ to $2,623 \pm 10 \Omega\text{cm}^2$ after 14 days and increased $\Delta[H^+]$ from control values of $0.6 \pm 0.6 \text{ mm}$ to $101.0 \pm 0 \text{ mm}$ over the same period. Apical addition of DEX also had substantial effects, increasing Rt to $1,420 \pm 11 \Omega\text{cm}^2$ and $\Delta[H^+]$ to $82 \pm 1 \text{ mm}$, but these values were significantly less than those observed with basolateral DEX addition. Addition of EGF caused a smaller, but still significant, increase in $\Delta[H^+]$ over control values ($21.0 \pm 0.9 \text{ mm}$). Notably, culture in the presence of all other supplements, including bovine pituitary extract (BPE), triiodothyrodine (T3), forskolin (FSK), and insulin-transferrin-selenium (ITS), had no measurable effect. Taken together, these findings indicate that DEX is a potent inducer of NRC differentiation and, despite its putative intracellular receptor, the findings suggest that the polarity of addition (basolateral vs apical) has differential effects.

To assess whether the other additives might function synergistically with DEX, additional studies were performed in DEX-containing media supplemented

with EGF, BPE, T3, FSK, and ITS, alone and in combination. Further, the effects from polarized addition (apical vs basolateral) were evaluated. The highest degree of differentiation was obtained using media selectively supplemented with ITS in the apical chamber and DEX, BPE, and T3 in the basolateral chamber (Table 1). Under these conditions, cells grew to confluence more rapidly, and Rt and $\Delta[H^+]$ values increased further to $3,226 \pm 9 \Omega\text{cm}^2$ and $126 \pm 0 \text{ mm}$, respectively. This modified media is referred to as EPITH because of its ability to promote the epithelial properties of NRC cells. Additional studies were performed to assess whether these changes induced by EPITH media were reversible. NRC cells were grown for an initial 12-day period in either the control ($n = 24$) or EPITH ($n = 24$) media. As above, EPITH media led to rapid and substantial increases in Rt ($2,493 \pm 27 \Omega\text{cm}^2$ vs $240 \pm 6 \Omega\text{cm}^2$) and $\Delta[H^+]$ ($88 \pm 1 \text{ mm}$ vs $12 \pm 1 \text{ mm}$) as compared with control media (Fig. 1). After 12 days, continued culture in EPITH led to maintenance of these properties, whereas switching to control media led to a rapid decline in both Rt and $\Delta[H^+]$. After 12 days in control media, transfer to EPITH media permitted the increase in Rt and $\Delta[H^+]$ to levels measured in cells continuously cultured in EPITH media (Fig. 1).

Figure 1.

The modified media (EPITH) induces reversible changes in normal rat cholangiocytes (NRC) epithelial properties. The culture of NRC cells in selectively supplemented EPITH media led to rapid increases in transepithelial resistance as compared with control media. After 12 days, these effects were sustained in cells maintained in EPITH media (filled circles) but rapidly reversed when cells were transferred to control media (open circles). Similarly, transfer of cells from control to EPITH media led to a rapid increase in transepithelial resistance (filled triangles).

Three additional indicators indicate EPITH media permits expression of differentiated epithelial properties in NRC cells when compared with control media.

Figure 2.

Culture in EPITH media enhances Cl^- secretory responses. Ussing chamber analysis of the short-circuit current across NRC cells shows monolayers cultured in control media had lower resistances and minimal secretory responses to the P2 receptor agonist ATP (300 nM) or the membrane-permeable cAMP analog 8-(4-chlorophenylthio)-cAMP (500 μM) (top tracings). In contrast, monolayers cultured in EPITH media had higher resistances and exhibited secretory responses to both stimuli. The upward deflections indicate the current response to a 10 mV test voltage used to assess transepithelial resistance.

First, cholangiocytes in vivo alkalinize bile through bicarbonate secretion (Kato et al, 1992; McGill et al, 1994). Consistent with enhanced apical bicarbonate secretion, NRC cells cultured in EPITH media had increased bicarbonate in the apical chamber ($10.0 \pm 0.2 \text{ mM}$ vs $2.0 \pm 0.0 \text{ mM}$) (ABL5; Radiometer America Inc., Copenhagen, Denmark) and an increase in the apical:basolateral $[\text{HCO}_3^-]$ ratio when compared with cells in control media (3.4 ± 0.1 vs 2.0 ± 0.0). Second, cholangiocytes in vivo release ATP as a versatile autocrine/paracrine purinergic signal to regulate cholangiocyte secretion (Roman et al, 1999; Salter et al, 2000). Culture of NRC cells in EPITH media resulted in an increase in regulated ATP release (measured as arbitrary light units, ALU) (Turner Designs, Sunnyvale, California) across both the apical ($78.2 \pm 0.1 \text{ ALU}$ vs $0.5 \pm 0.1 \text{ ALU}$) and basolateral ($15.2 \pm 0.3 \text{ ALU}$ vs $0.2 \pm 0.1 \text{ ALU}$) membranes when compared with cells in control media. Third, channel-mediated Cl^- secretion represents the electrophysiologic basis of cholangiocyte secretion (Roman et al, 1999). Measuring epithelial ion fluxes across NRC monolayers by Ussing short-circuit current analysis (ΔIsc), the culture of NRC cells in EPITH media resulted in the detection of robust secretory responses (Figure 2) to the P2 receptor agonist ATP (300 nM, $\Delta \text{Isc} = 2.0 \pm 0.3 \mu\text{A}/\text{cm}^2$) and the membrane-permeable cAMP analog 8-cpt-cAMP (500 μM , $\Delta \text{Isc} = 4.0 \pm 0.3 \mu\text{A}/\text{cm}^2$).

Collectively, these findings indicate that culture of NRC cells in EPITH media induces the expression of functional properties anticipated for differentiated

cholangiocytes in vivo and suggest that DEX is the primary agent responsible for the differentiated phenotype. This approach to NRC cell culture has two important advantages. First, there are substantial cost and effort savings because of the use of selected supplements and the shorter time to epithelial confluence. Second, the functional effects of the EPITH protocol are substantial, greatly enhancing the ability to directly assess the molecular and cellular basis of cholangiocyte transport, secretion, and bile formation.

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