P-glycoprotein Expression, Localization, and Function in Sandwich-Cultured Primary Rat and Human Hepatocytes: Relevance to the Hepatobiliary Disposition of a Model Opioid Peptide

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Purpose. The isolation of hepatocytes from intact liver involves collagenase digestion of the tissue, resulting in loss of cell polarization and functional vectorial excretion. These studies examined repolarization, localization of P-glycoprotein (P-gp) to the canalicular domain of the hepatocyte, and re-establishment of vectorial transport in sandwich-cultured (SC) rat and human primary hepatocytes.

Methods. Protein localization and expression were determined in SC hepatocytes by confocal microscopy and Western blotting, respectively. Transporter function was evaluated by measuring [D-penicillamine^{2,5}]enkephalin (^{3}H-DPDPE) and 5 (and 6)-carboxy-2′,7′-dichlorofluorescein (CDF) biliary excretion in SC hepatocytes.

Results. P-gp and the canalicular marker protein dipeptidyl peptidase IV (DPPIV) co-localized by Day 3 and Day 6 in SC rat hepatocytes and SC human hepatocytes, respectively, consistent with canalicular network formation visualized by light microscopy. Co-localization of multidrug resistance associated protein 2 (MRP2) and P-gp in SC human hepatocytes was observed on Day 6 in culture. Expression levels of P-gp increased slightly in both species over days in culture; similar expression was observed for MRP2 in SC human hepatocytes. Oatp1a1 expression in SC rat hepatocytes was maintained over days in culture, whereas Oatp1a4 expression decreased. OATP1B1 expression decreased slightly on Day 3 in SC human hepatocytes. OATP1B3 expression was constant in SC human hepatocytes. *In vitro* biliary excretion of the opioid peptide ^{3}H-DPDPE correlated with the proper localization of canalicular proteins in both species. Excretion of CDF in SC human hepatocytes confirmed network formation and MRP2 function.

Conclusions. These studies indicate that SC hepatocytes repolarize and traffic functional canalicular transport proteins to the appropriate cellular domain.

KEY WORDS: cell culture; biliary excretion; drug transport; drug transport models; hepatocytes; P-glycoprotein.

INTRODUCTION

P-glycoprotein (P-gp) is a 170-kDa ATP-dependent transport protein encoded by the *MDR1* gene in humans and by the *Mdr1a* and *Mdr1b* genes in rodents. P-gp is expressed in several tissues including the brain, kidneys, intestine, and liver (1). In the liver, P-gp is localized to the canalicular domain of the hepatocyte, where it facilitates excretion of exogenous organic cations and toxicants into bile (2–4). The polarized architecture of the hepatocyte, critical to hepatic function, makes direct investigation of P-gp activity and resultant vectorial transport into the bile somewhat difficult. Primary hepatocytes have been used extensively for investigating the uptake of bile acids, therapeutic agents, and toxicants. However, upon isolation by collagenase digestion of the liver, primary hepatocytes lose both polarization and the ability to excrete compounds across the cell in a vectorial manner (5). This problem may be circumvented by culturing primary hepatocytes in a gelled collagen sandwich configuration. Sandwich-cultured (SC) hepatocytes repolarize over time in culture, form bile canalicular networks, synthesize liver specific proteins, and excrete a variety of endogenous and exogenous compounds into the formed bile canalicular space (6–8). Previous investigations have focused on SC rat hepatocytes. Establishing the expression, localization, and function of transport proteins in SC hepatocytes would provide a basis for studying the effect of disease states or xenobiotic insult on transport proteins in the liver and defining the consequences of such alterations. The use of SC human hepatocytes is of particular interest for studying clinically relevant vectorial transport of bile salts and drugs, and potentially predicting *in vivo* hepatic drug-drug interactions mediated by transport proteins based on *in vitro* data.

[D-penicillamine^{2,5}]enkephalin (^{3}H-DPDPE) is a metabolically stable opioid peptide that is a substrate for P-gp (9, 10). Very recent work also suggests that Mrp2 may play a role in the biliary excretion of ^{3}H-DPDPE (11). In cRNA microinjected *Xenopus laevis* oocytes, rat Oatp1a1, Oatp1a4, and Oatp1b2 actively transport ^{3}H-DPDPE and likely are responsible for the rapid hepatic uptake of ^{3}H-DPDPE in the rat liver (12). Similar studies in oocytes expressing human OATP isoforms suggest OATP1B1 and OATP1B3 transport ^{3}H-DPDPE in the human liver (12, 13). Functional excretion of ^{3}H-DPDPE into the bile canaliculi of Day 4 SC rat hepatocytes suggests that transport proteins involved in ^{3}H-DPDPE vectorial transport at both the sinusoidal and canalicular domains of the hepatocyte are present and functional (14). In the current study, P-gp expression, localization, and function were investigated in SC rat hepatocytes and SC human hepatocytes. Repolarization of SC rat hepatocytes and SC human hepatocytes was determined by light microscopy and immunofluorescent localization of P-gp and dipeptidyl peptidase IV (DPPIV) over days in culture. The reestablishment of P-gp–mediated vectorial transport in SC rat hepatocytes and SC human hepatocytes was evaluated with the probe sub-

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ABBREVIATIONS: CDF, 5 (and 6)-carboxy-2',7'-dichlorofluorescein; DMEM, Dulbecco's Modified Eagle's Medium; DPPIV, dipeptidyl peptidase IV; ^{3}H-DPDPE, [D-penicillamine^{2,5}]enkephalin; MRP2, multidrug resistance associated protein 2; P-gp, P-glycoprotein; PXR, pregnane X receptor; Oatp1a1, rat organic anion transporting polypeptide 1 (Oatp1); Oatp1a4, rat organic anion transporting polypeptide 2 (Oatp2); OATP1B1, human organic anion transporting polypeptide C (OATP-C); OATP1B3, human organic anion transporting polypeptide 8 (OATP8); SC, sandwich-cultured.

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strate ^{3}H-DPDPE. MRP2 function in SC human hepatocytes was confirmed using 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF).

MATERIALS AND METHODS

Chemicals and Reagents

Unlabeled DPDPE was obtained from American Peptide Company (Sunnyvale, CA, USA); ^{3}H-DPDPE (44 Ci/mmol, >95% purity) was from Perkin Elmer Life Sciences (Boston, MA, USA). Collagenase (type I, class I) was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). Liberase was obtained from Roche Biosciences (Indianapolis, IN, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, insulin, penicillin-streptomycin, nonessential amino acids, and L-glutamine were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Rat tail collagen (type I) and ITS^{+} were purchased from BD Biosciences (Bedford, MA, USA). Anti-human CD26 (human DPPIV) and anti-rat CD26 (rat DPPIV) antibodies were obtained from Pharmingen (San Diego, CA, USA). Anti-human MDR1 (Ab-1) that cross-reacts with rodent Mdr1a/b was purchased from Oncogene/EMD Biosciences (Darmstadt, Germany). Anti-human MRP2 (M_2III-6) was obtained from Alexis Biochemical (San Diego, CA, USA). Anti-rat Oatp1a4 and anti-human/rat actin were purchased from Chemicon (Temecula, CA, USA); polyclonal antibody against Oatp1a1 was raised in rabbits using a C-terminal fusion protein (aa 631-670) as antigen (15). Rabbit polyclonal antibodies for OATP1B1 and OATP1B3 were raised against the C-terminal peptide of OATP1B1 (ESLNKNKHFVPSAGADSETHC) and OATP1B3 (SKTCNLDMQDNAAAN). These antibodies were custom-made by Research Genetics (Huntsville, AL, USA). Dexamethasone, bovine serum albumin, rabbit serum, goat serum, rabbit IgG, and mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alexa fluor 488 and Alexa fluor 568 conjugated secondary antibodies and 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF) diacetate were purchased from Molecular Probes (Eugene, OR, USA). All other chemicals and reagents were of analytical grade and were available from commercial sources.

Rat and Human Liver Tissue

Male Wistar rats (250–300 g) from Charles River (Raleigh, NC, USA) were used as liver donors for hepatocyte isolation. Rats were housed in an alternating 12-h light and dark cycle with rat chow and water provided *ad libitum*. All research involving animals adhered to the *Principles of Laboratory Animal Care* (NIH Publication No. 85-23, revised 1985), and the Institutional Animal Care and Use Committee at the University of North Carolina approved all animal procedures. Human liver tissues were obtained by qualified medical staff from the University of North Carolina School of Medicine as waste from surgical resection or rejected donor livers. Donor consent and IRB approval (Committee on the Protection of the Rights of Human Subjects) were obtained for all studies involving human liver tissue. All studies involving human tissue followed the tenets of the Declaration of Helsinki promulgated in 1964.

Hepatocyte Isolation and Culture

Rat and human hepatocytes were isolated by a two-step collagenase perfusion method as previously described (16–18). Hepatocyte suspensions were plated in gelled collagen coated 60-mm permanox or polystyrene dishes at a density of ~3×10^6 cells/dish in supplemented DMEM containing fetal bovine serum and 0.1 μM dexamethasone. Viability, as determined by trypan blue exclusion, ranged from 88% to 98% (mean = 92%). Post plating (rat, 1–3 h; human, 2–6 h), hepatocyte cultures were shaken lightly, and nonadherent cells were aspirated. Following ~24 h of incubation at 37°C, cells were overlaid with gelled collagen and cultures were maintained in fetal bovine serum-free DMEM containing insulin/transferrin/selenium (ITS^{+}) with 0.1 μM dexamethasone for up to 10 days. Culture medium was replaced every 24 h.

Light, Fluorescence, and Confocal Laser Scanning Immunofluorescence Microscopy

Sandwich-cultured hepatocytes were visualized daily by phase contrast microscopy (Nikon TMS, Melville, NY, USA), and images were captured with a Nikon 35-mm camera. Fluorescent images for CDF studies were acquired with a Zeiss Axiovert 100TV inverted fluorescent microscope (Carl Zeiss Inc., Thornwood, NY, USA). For confocal laser scanning immunofluorescence microscopy, SC rat hepatocytes and SC human hepatocytes (n = 2 dishes/liver, in triplicate) were fixed in ice-cold acetone for 10 min at 4°C and allowed to air dry quickly. Dishes were cut into 1-cm^2 pieces and stored at –80°C until analysis. Frozen samples were rehydrated in ice-cold blocking buffer [0.01 M phosphate buffered saline (PBS) containing 5% goat serum and 1% bovine serum albumin]. Blocking buffer was removed, and samples were incubated immediately with anti-MDR1 (1:100), anti-DPPIV (1:200), and/or anti-MRP2 (1:100) primary antibodies. Samples were washed 3× with 0.01 M PBS for 15 min and subsequently incubated with fluorochrome-conjugated secondary antibodies. Hepatocytes were washed again with 0.01 M PBS and rinsed briefly with distilled water. Hepatocytes were mounted onto glass cover slips and slides with 15 μl Permount (Fisher Scientific, Atlanta, GA, USA). Immunofluorescent images were detected using an inverted LeicaTCS-NT Laser Scanning Confocal Microscope (Bannockburn, IL, USA). Confocal scanning was performed in the x-y field with a pinhole setting of 1.00 airy disk units. Laser power and PMT gain were held constant for each sample. Scan averaging was set to 6 and the 40× objective lens was used for all image acquisitions. The image zoom factor for hepatocyte images was set to 1 or 2 as appropriate.

Western Blotting

Hepatocyte cultures were lysed with Complete Protease Inhibitor (Roche Diagnostics, Indianapolis, IN, USA) in PBS with 1% SDS/1 mM EDTA and stored at –80°C until analysis. Protein (~30 μg) was loaded onto a NuPage 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) without heat denaturation. Proteins were separated by electrophoresis and transferred onto a PVDF membrane. Membranes were

blocked overnight at 4°C with 5% nonfat milk in tris-buffered saline with 0.3% Tween-20 (TBS-T) (pH = 7.4) and then incubated with primary antibody for 1 h [anti-Oatp1a4 (1:1000), anti-OATP1B3 (1:2500), anti-OATP1B1 (1:2500), anti-MRP2 (1:2000), anti-Actin (1:10,000)] or 2 h [anti-Oatp1a1 (1:2000), anti-Mdr1 (1:2000)]. Membranes were washed 2× with TBS-T for 15 min, 2× with TBS-T with 0.5% nonfat milk, probed with HRP-conjugated secondary antibodies (Amersham, Piscataway, NJ, USA), and washed again as above before detection by chemiluminescence with SuperSignal West Dura (Pierce Biotechnology, Rockford, IL, USA). Immunoreactive protein was visualized using a Versa-Doc 1000 molecular imager (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were quantified by densitometry using Quantity One v.4.1 (Bio-Rad Laboratories).

SC Hepatocyte Transport Studies

Cells were rinsed with 2 ml Hank's Balanced Salt Solution (HBSS) at 37°C and pre-incubated for 10 min at 37°C in 3 ml standard or Ca^{2+}-free HBSS. Hepatocytes were incubated with 15 μM ^{3}H-DPDPE or 10 μM CDF-diacetate for up to 10 min in standard HBSS, rinsed vigorously 3× with ice-cold HBSS, and lysed with 2 ml 0.5% Triton-X 100. Nonspecific binding was accounted for by including a blank dish (collagen only) in each group. Cell lysates from ^{3}H-DPDPE accumulation were analyzed by liquid scintillation spectroscopy (Packard Tricarb, Packard Corp., Meriden, CT, USA). CDF concentrations were determined as reported previously (19). Total protein in each dish was determined by the BCA method (20). The biliary excretion index (BEI; the percentage of accumulated substrate in canalicular networks) was calculated as the difference in substrate accumulation in the presence and absence of intact canalicular networks normalized for the substrate accumulation in the presence of intact canalicular networks (17). Differences in accumulation between experimental groups were analyzed with Student's *t* test. A p value of <0.05 was considered significant. For CDF microscopy studies, SC human hepatocytes were rinsed 3 times with 37°C HBSS (3 ml) and incubated with CDF-diacetate at a final concentration of 2 μM for 10 min at 37°C. Subsequently, cells were rinsed 3 times with 37°C HBSS (4 ml) and 2 ml HBSS was added immediately prior to fluorescent image acquisition.

RESULTS

Evaluation of SC Rat Hepatocytes and SC Human Hepatocytes by Light Microscopy

Previous studies demonstrated that rat hepatocytes cultured between two layers of collagen repolarize and form canalicular networks (7). This observation was confirmed as extensive canalicular network formation (arrows) was observed in SC rat hepatocytes by Day 4 (Fig. 1, top right) relative to Day 1 (Fig. 1, top left). By Day 4, extensive network formation (arrows) was observed in SC rat hepatocytes (Fig. 1, top right). SC human hepatocytes appeared slightly smaller and more cuboidal than SC rat hepatocytes. Canalicular network formation in SC human hepatocytes was not apparent on Day 1 (Fig. 1, bottom left). Even on Day 6, the small cuboidal nature of the SC human hepatocytes limited the visualization of extensive canalicular network formation. However, some canalicular networks (arrows) were evident in SC human hepatocytes on Day 6 (Fig. 1, bottom right).

P-gp Expression in SC Rat Hepatocytes and SC Human Hepatocytes

The effect of culture time on P-gp expression in SC rat hepatocytes and SC human hepatocytes was determined by western blot analysis. P-gp expression in SC rat hepatocytes increased modestly with time in culture (Fig. 2, left). When normalized to actin, expression levels were ~2-fold higher by

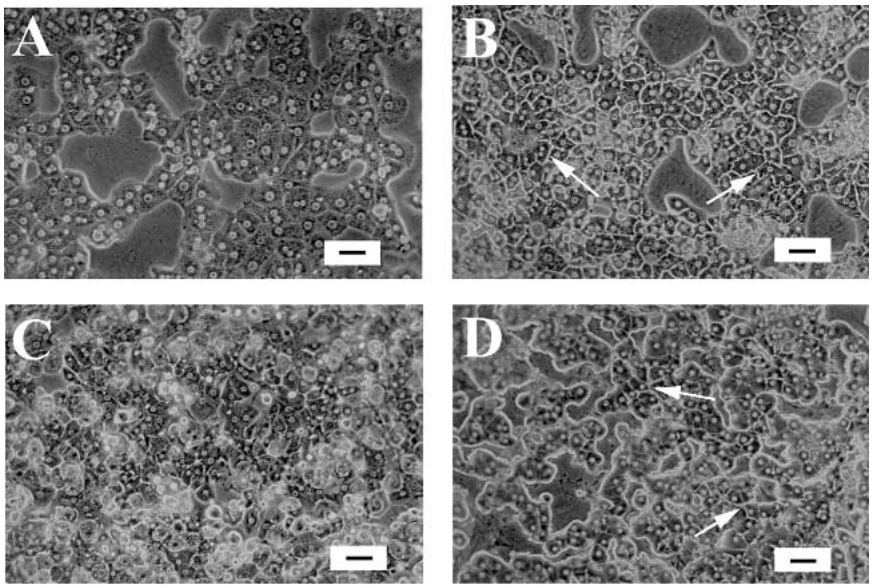

Fig. 1. Phase contrast images of SC rat hepatocytes on Day 1 (A) and Day 4 (B) in culture. Phase contrast images of SC human hepatocytes on Day 1 (C) and Day 6 (D) in culture. Arrows in panels B and D represent canalicular network formation, indicated by the bright, white belt-like structures between cells. Bar = 20 μm.

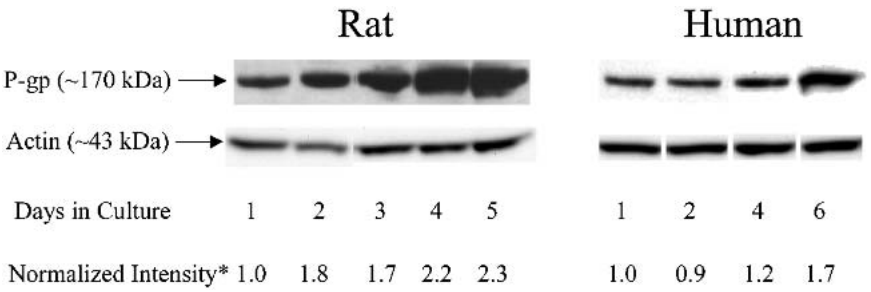

Fig. 2. Expression of P-gp and actin in SC rat hepatocytes (left) and SC human hepatocytes (right). *Optical density expressed as a ratio normalized to actin. Expression ratio on Day 1 was set to 1.0.

Day 4. P-gp expression in SC human hepatocytes was constant through Day 4, but increased slightly by Day 6 (Fig. 2, right).

P-gp and DPPIV Localization in SC Rat Hepatocytes and SC Human Hepatocytes

Incubation with anti-P-gp or anti-DPPIV primary antibodies followed by conjugation with fluorochrome-labeled secondary antibodies permitted protein detection and cellular localization by laser scanning confocal microscopy. To determine the membrane localization of P-gp, the protein was co-localized with the canalicular marker DPPIV. In both SC rat hepatocytes and SC human hepatocytes a distinct pattern of P-gp immunofluorescence was observed, similar to the pattern of canalicular networks observed by light microscopy (Fig. 3, panels A and E). Localization of DPPIV (Fig. 3, panels B and F) in the same samples showed similar localization to P-gp. To confirm specificity of the secondary antibody, and as a negative control, cells were incubated with rabbit IgG from non-P-gp immunized rabbits followed by fluorochrome-conjugated anti-rabbit secondary antibodies. Limited background signal from rabbit IgG (acting as the primary antibody) contributed to the signal for P-gp (Fig. 3, panels C and G). Furthermore, when panels A and B were overlaid (SC rat hepatocytes) and panels E and F were overlaid (SC human hepatocytes) a distinct co-localization (indicated by yellow fluorescence; Fig. 3, panels D and H) of P-gp with the canalicular marker protein DPPIV was observed, indicating repolarization and proper localization of P-gp by Day 4 and Day 6 in SC rat hepatocytes and SC human hepatocytes, respectively. Because light microscopy data indicated a lack of net-

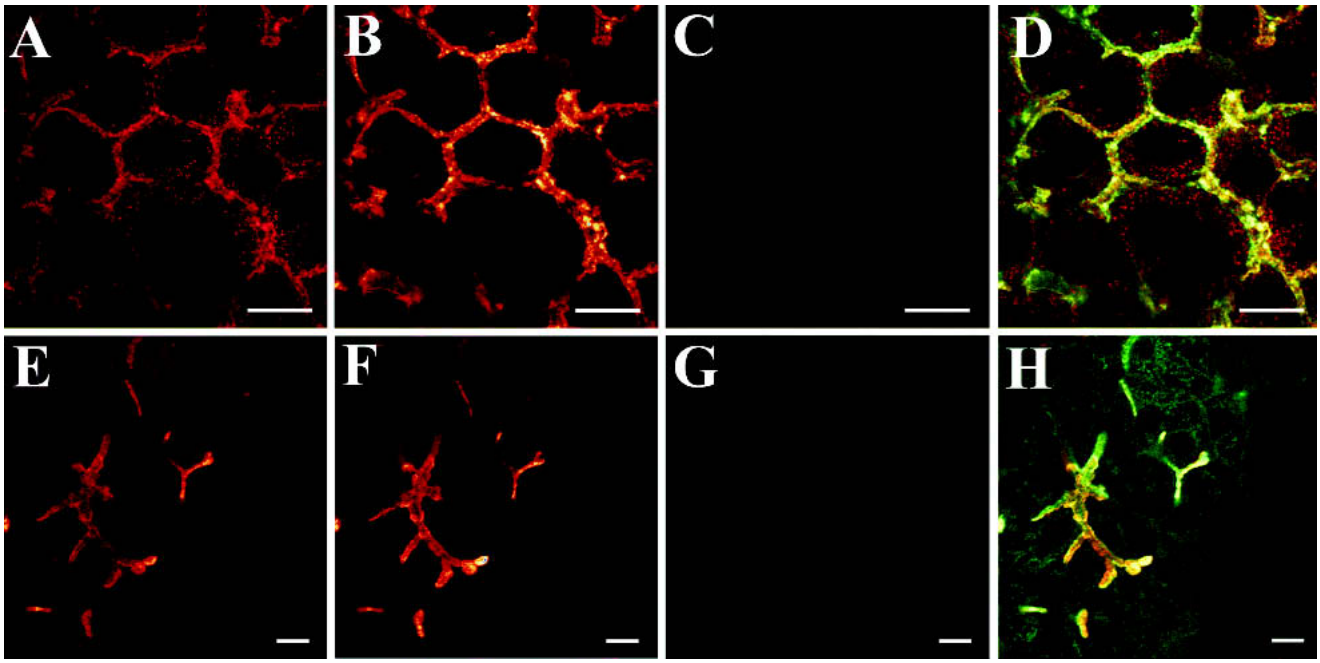

Fig. 3. Confocal images of Day 4 SC rat hepatocytes incubated with anti-Mdr1a/b antibodies (A), anti-rat DPPIV antibodies (B), or rabbit IgG (C). Panel D shows the overlay of panels A and B where Mdr1a/b is represented by the red channel and rat DPPIV is represented by the green channel. Co-localization is indicated where green and red overlap (indicated by the yellow color). Confocal images of Day 6 SC human hepatocytes incubated with anti-MDR antibodies (E), anti-human DPPIV antibodies (F), or control rabbit serum (G). Panel H shows the overlay of panels E and F where MDR1 is represented by the green channel and human DPPIV is represented by the red channel. Co-localization is indicated where green and red overlap (indicated by the yellow color). Images were acquired with a 40× objective oil-immersion lens; zoom factor = 2 for SC rat hepatocytes, zoom factor = 1 for SC human hepatocytes. Bar = 10 μm.

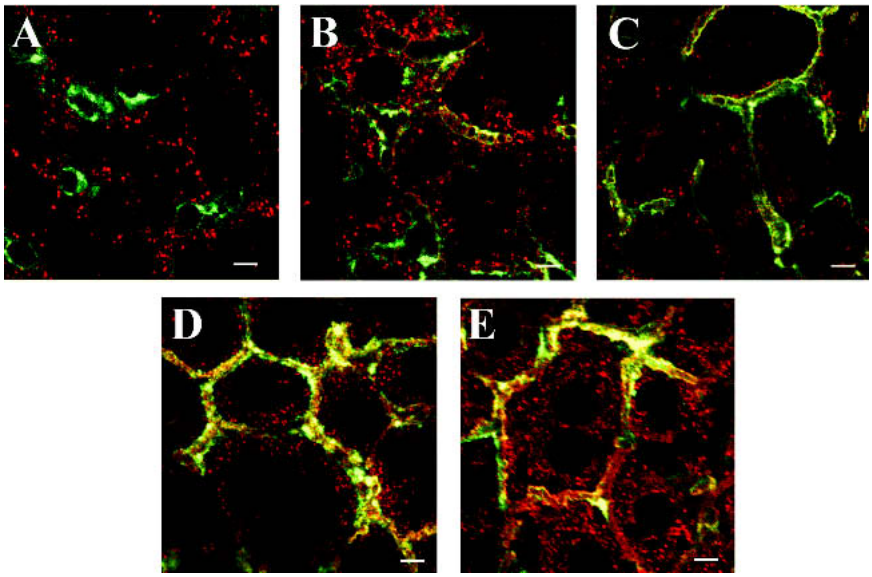

Fig. 4. Representative confocal images of Mdr1a/b (red) and rat DPPIV (green) in SC rat hepatocytes from a single liver on Day 1 (A), Day 2 (B), Day 3 (C), Day 4 (D), and Day 5 (E) in culture. Co-localization was evident by Day 3 in culture, indicated by the yellow color in panels C, D, and E. Images were acquired with a 40× objective oil-immersion lens, zoom factor = 2. Bar = 5 μm.

work formation on Day 1, the time course of localization of P-gp and DPPIV in SC rat hepatocytes and SC human hepatocytes was investigated. In SC rat hepatocytes, P-gp (red) and rat DPPIV (green) immunofluorescence showed diffuse patterns throughout the cytoplasm on Day 1 (Fig. 4A). Canalicular network formation and co-localization (yellow) were noticeable as early as Day 2 (Fig. 4B) with extensive fluorescent canalicular networks visible by Day 3 (Fig. 4C). Network formation, as determined by P-gp and DPPIV localization, was maintained for at least 5 days in culture (Figs. 4A–4E). Similar to SC rat hepatocytes, MDR1 (green) and human DPPIV (red) immunofluorescence in SC human hepatocytes showed diffuse patterns throughout the cytoplasm on Day 1 (Fig. 5A). By Day 3, both proteins were co-localized (yellow) and concentrated at the canalicular domains of hepatocytes (Fig. 5B). A canalicular network of co-localized P-gp and DPPIV was observed by Days 6–7 (Figs. 5D–5E; Fig. 3H); networks were maintained through 10 days in culture (Figs. 5A–5F).

Functional Excretion of DPDPE in SC Rat Hepatocytes and SC Human Hepatocytes

P-gp function was determined with the P-gp substrate ^{3}H-DPDPE (9, 10). Uptake and excretion of ^{3}H-DPDPE were evaluated in SC rat hepatocytes immediately after gelled col-

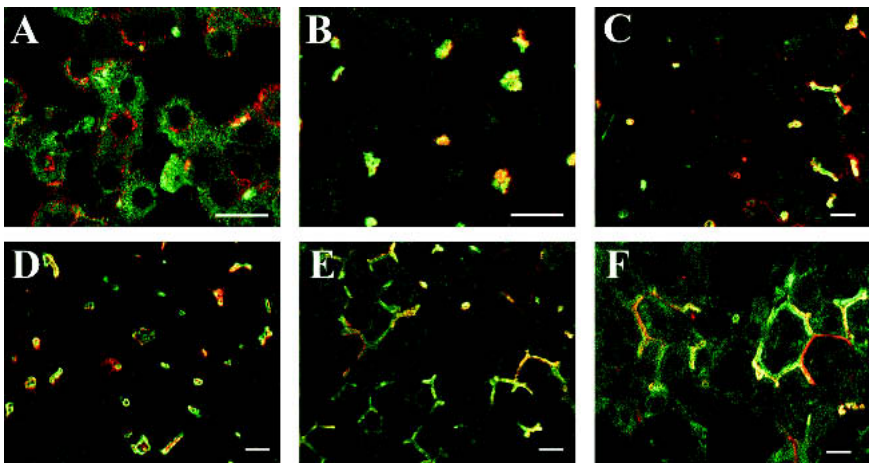

Fig. 5. Representative confocal images of MDR1 (green) and human DPPIV (red) in SC human hepatocytes from a single liver on Day 1 (A), Day 3 (B), Day 4 (C), Day 6 (D), Day 7 (E), and Day 10 (F) in culture. Co-localization, indicated by the yellow color, is evident by Day 3 in culture (B); however, co-localization did not become extensive until Day 6 in culture (D, E, and F). Images were acquired with a 40× objective oil-immersion lens, zoom factor = 2 (panels A, B), zoom factor = 1 (panels C-F). Bar = 10 μm.

lagen overlay (Day 1) and every 24 h through Day 5. Accumulation of ^{3}H-DPDPE in cells with intact bile canaliculi (BC) from SC rat hepatocytes was maximal on Day 1 and decreased as time in culture increased (Fig. 6). Concurrent with P-gp localization to the canalicular domain of the hepatocyte (Fig. 4), biliary excretion of ^{3}H-DPDPE (determined as BEI) was negligible on Day 1 and gradually increased to 44% by Day 4 in culture (Fig. 6; Table I). Due to the limited availability of human hepatocytes, the uptake and excretion of ^{3}H-DPDPE in SC human hepatocytes were evaluated only on Day 6 in culture (Fig. 7). A time course of ^{3}H-DPDPE accumulation in SC human hepatocytes indicated measurable biliary excretion at both 5 and 10 min (Fig. 7). BEI suggested functional excretion of ^{3}H-DPDPE in both species, with maximal excretion on Day 4 in SC rat hepatocytes. The BEI of ^{3}H-DPDPE was slightly higher in SC rat hepatocytes than in SC human hepatocytes (Table I).

Basolateral Transport Protein Expression in SC Rat Hepatocytes and SC Human Hepatocytes

Expression of the rat and human hepatic transport proteins likely responsible for the uptake of ^{3}H-DPDPE in SC hepatocytes over time in culture was investigated by Western blot (13, 21). Oatp1a1 expression was maintained through Day 4 in SC rat hepatocytes (Fig. 8). However, Oatp1a4 expression gradually decreased to about 50% by Day 4 in culture (Fig. 8). Expression of OATP1B1 and OATP1B3 was determined in sandwich-cultured human hepatocytes on Days 1, 3, and 6 in culture. Although expression of OATP1B1 decreased on Day 3, expression of OATP1B1 increased slightly above Day 1 levels on Day 6 in culture (Fig. 8). OATP1B3 expression was similar on Days 1, 3, and 6 in SC human hepatocytes.

Table I. Mean Biliary Excretion Index (BEI) of ^{3}H-DPDPE in SC Rat Hepatocytes and SC Human Hepatocytes at 10 min

Species	Days in culture	BEI (%)
Rat	1	<0
	2	22 ± 11
	3	38 ± 9
	4	44 ± 12
	5	35 ± 10
Human	6	21

BEI is the % of substrate taken up into the cell that is excreted into the canalicular space. Data are represented as mean ± SEM where appropriate (rat, n = 3 livers, in triplicate; human, n = 1 liver, in triplicate).

MRP2 Expression, Localization, and Function in SC Human Hepatocytes

The expression, localization, and function of MRP2 in SC human hepatocytes was evaluated. MRP2 expression increased slightly over time in culture (Fig. 9A). Localization of MRP2 in SC human hepatocytes on Day 6 showed a characteristic pattern of immunofluorescence similar to that of P-gp and DPPIV (Fig. 9B). MDR1 (red) and MRP2 (green) colocalization (yellow) on Day 6 in culture confirmed that in SC human hepatocytes, MRP2 was localized to the canalicular domain of the hepatocyte (Fig. 9B). CDF-diacetate readily diffuses into hepatocytes and is converted rapidly by intracellular esterases to CDF, a fluorescent MRP2 substrate. MRP2 function in SC human hepatocytes was confirmed by administering CDF-diacetate and measuring CDF accumulation for 10 min. On Day 1, CDF was retained inside SC human hepatocytes, whereas by Day 6, CDF fluorescence was concentrated in the canalicular networks of SC human hepatocytes (Fig. 9C), similar to the pattern observed by MRP2 antibody staining. The biliary excretion index of CDF on Day 10 was 34% (Fig. 9D).

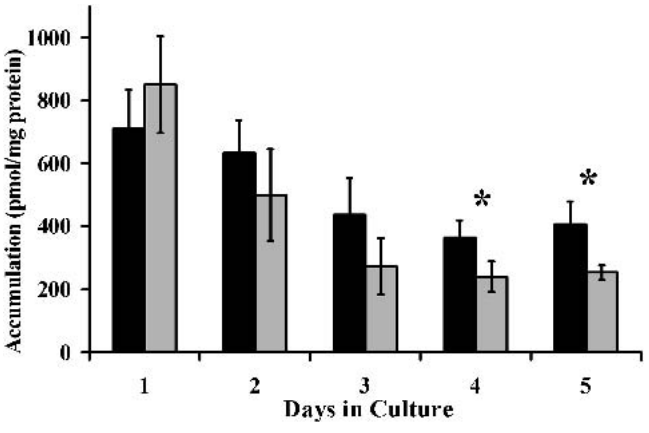

Fig. 6. Accumulation of ^{3}H-DPDPE into SC rat hepatocytes over 10 min. Solid bars represent accumulation into hepatocytes that maintain the integrity of the tight junctions and an intact canalicular space (cells + BC). Open bars represent uptake into hepatocytes where the tight junctions have been disrupted and therefore lack a canalicular space (cells). The difference between the closed and open bars represents the amount of substrate secreted into the canalicular space. Data are represented as mean ± SEM. (n = 3 to 4 livers in triplicate); *p < 0.05, cells + BC vs. cells on Day 4 and Day 5.

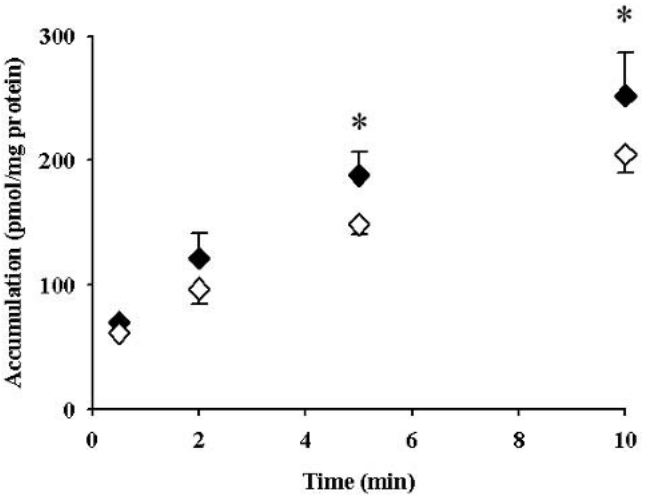

Fig. 7. Accumulation of ^{3}H-DPDPE into SC human hepatocytes over 10 min on Day 6 in culture. Solid symbols represent accumulation in cells + BC; open symbols represent accumulation in cells. The difference between the closed and open symbols represents substrate secreted into the canalicular space. Data are represented as mean ± SD (n = 1 to 2 livers, in duplicate or triplicate). *p < 0.05, cells + BC vs. cells.

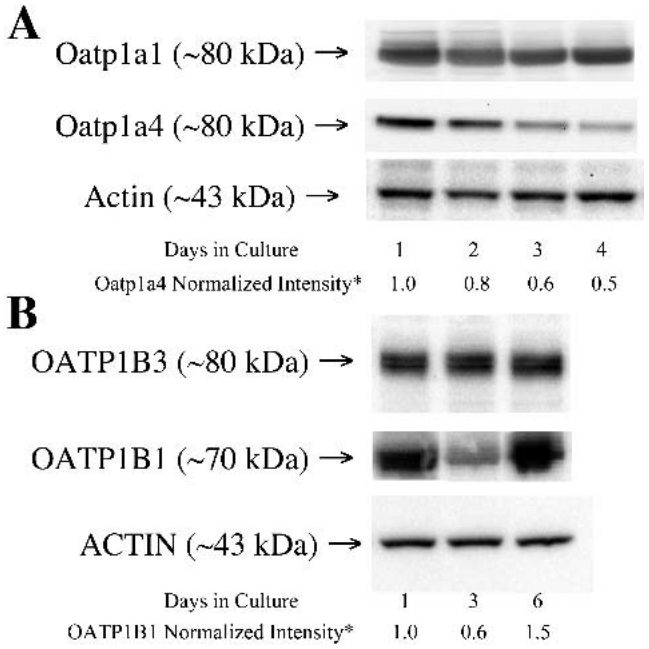

Fig. 8. Expression of Oatp1a1, Oatp1a4, and actin in SC rat hepatocytes over days in culture. Expression of OATP1B3, OATP1B1, and ACTIN in SC human hepatocytes on Day 1, Day 3, and Day 6 in culture. *Optical density expressed as a ratio normalized to actin. Expression ratio on Day 1 was set to 1.0.

DISCUSSION

P-gp plays an important role in the biliary excretion of xenobiotics from the body, and may be important in endogenous hepatic functions such as cholesterol flux and esterification in the liver (22). This report describes the canalicular localization of P-gp, DPPIV, and MRP2, as well as the functional repolarization of rat and human hepatocytes cultured in a sandwich configuration. As expected, Day 1 SC rat hepatocytes and SC human hepatocytes were not polarized and exhibited a diffuse pattern of immunofluorescence of both P-gp and DPPIV. Loss of hepatocyte polarization is rapid following collagenase digestion, and at 4 h in culture DPPIV has been shown to distribute asymmetrically over the cell surface (23). Interestingly, the localization of DPPIV and P-gp in both SC rat hepatocytes and SC human hepatocytes on Day 1 was somewhat different. P-gp appeared to be localized throughout the cytoplasm in small punctate compartments (more pronounced in SC rat hepatocytes), whereas DPPIV concentrated in the cytoplasm proximal to the nucleus. This pattern of P-gp localization is similar to the pattern of intracellular accumulation of the P-gp substrate daunorubicin in isolated perfused rat livers and rat hepatocyte couplets (24). Arias and colleagues have implicated cAMP in the direct intracellular sorting of P-gp and other canalicular ABC transport proteins from intracellular pools (25). Alternatively, trafficking of DPPIV in hepatocytes appears to be via indirect sorting to the basolateral membrane, followed by transcytosis to the apical domain of the cell (26). These differences in cellular trafficking pathways for proteins that both ultimately localize to the canalicular domain of the hepatocyte may explain our observations in SC rat hepatocytes and SC human hepatocytes. Differences between SC rat hepatocytes and SC human hepatocytes in the time course of

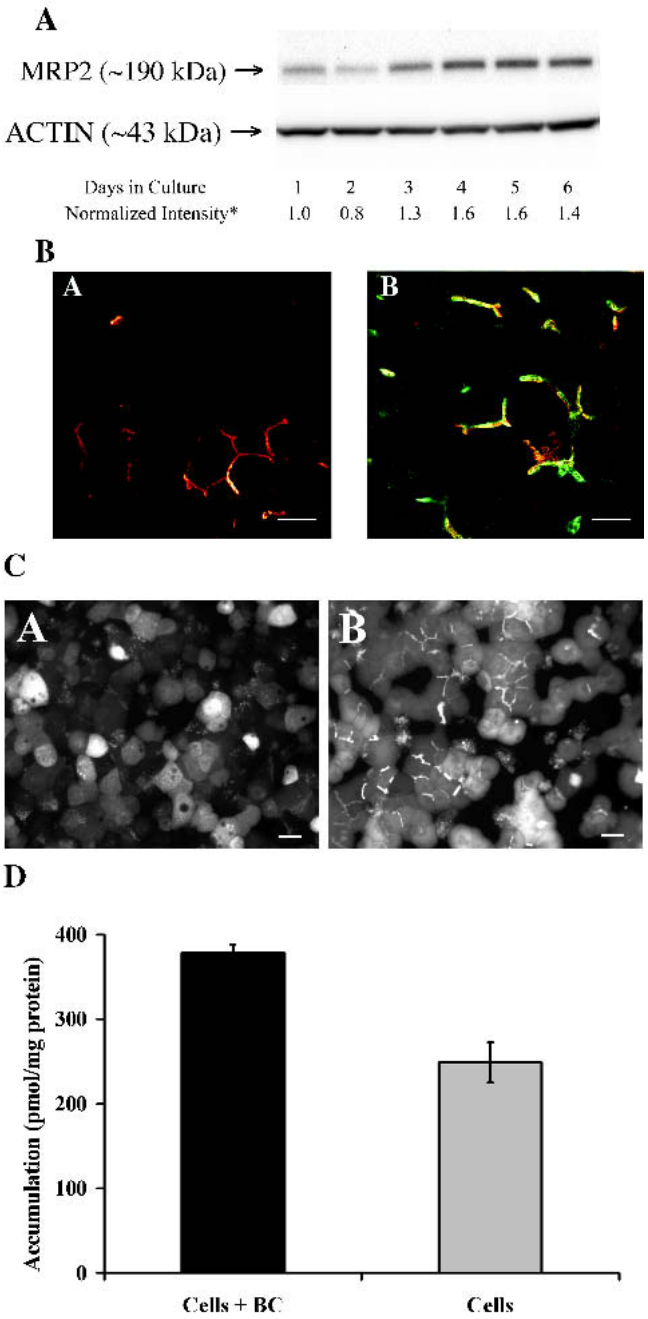

Fig. 9. Expression (A) of MRP2 in SC human hepatocytes over 6 days in culture. *Optical density expressed as a ratio normalized to actin. Expression ratio on Day 1 was set to 1.0. Localization (B, left panel) of MRP2 on Day 6 in SC human hepatocytes. Co-localization (B, right panel) of the two canilicular proteins MRP2 (green) and P-gp (red) is shown in yellow. Bar = 10 μm. CDF fluorescence following 2 μM CDF-diacetate administration for 10 min on Day 1 in SC human hepatocytes (C, panel A) and on Day 6 in SC human hepatocytes (C, panel B). Bar = 20 μm. Accumulation of CDF (D) following 10 μM CDF-diacetate administration for 10 min on Day 10 in SC human hepatocytes. Solid bars represent CDF accumulation in cells + BC; open bars represent accumulation in cells only. The biliary excretion index of CDF on Day 10 was 34%. Data are represented as mean ± SD (n = 1 liver, in triplicate) *$p < 0.05$ cells + BC vs. cells.

P-gp localization were noted. These differences correlated with canalicular network formation observed by light microscopy. Culture conditions have been optimized for the formation of canalicular networks and the study of transport proteins in SC rat hepatocytes (27). Similar culture conditions were used in the current studies for SC human hepatocytes. Studies are ongoing to further optimize culture conditions to expedite the formation of canalicular networks in SC human hepatocytes. Nevertheless, under the culture conditions studied, results suggest that four days in SC rat hepatocytes and six days in SC human hepatocytes is adequate time for cellular repolarization and investigation of P-gp function. P-gp expression was increased slightly in both SC rat hepatocytes and SC human hepatocytes over days in culture, providing further evidence that these cells are capable of synthesizing new hepatic proteins in response to intracellular induction signals. SC hepatocytes continue to synthesize and excrete bile acids into the developed canalicular spaces (28). These bile acids are ligands for nuclear hormone receptors that govern the regulation of various transport proteins in the hepatocyte (29–33). The nuclear hormone receptor PXR is important in the regulation of P-gp protein levels and is functional in SC rat hepatocytes and SC human hepatocytes (34–36). Elevated levels of bile acids in SC rat hepatocytes and SC human hepatocytes may activate PXR and explain the slight induction of P-gp observed due to transcriptional activation of the *Mdr* gene. Interestingly, the increased expression of P-gp is not profound until network formation has been established, consistent with the hypothesis that accumulation of bile salts in the cell and bile canalicular networks may correlate with increased P-gp expression.

^{3}H-DPDPE biliary excretion likely is mediated by rapid Oatp uptake followed by P-gp efflux across the canalicular membrane (10, 12, 13). Several clinically relevant compounds also share this pathway for excretion, including the protease inhibitors ritonavir and saquinavir, the second generation antihistamine fexofenadine, and the antibiotic erythromycin (37). The time course of biliary excretion of ^{3}H-DPDPE in SC rat hepatocytes directly correlated with the canalicular localization of P-gp. However, total accumulation (cells + BC) of ^{3}H-DPDPE decreased in SC rat hepatocytes over days in culture, most likely due to a decrease in Oatp1a4 expression (Fig. 8A) (21). Rippin *et al.* demonstrated that when primary rat hepatocytes were cultured on collagen-coated dishes (not sandwiched), Oatp1a4 levels decreased to ~25% of basal levels (38). Trauner and colleagues have demonstrated that in primary biliary cirrhosis, hepatocytes undergo adaptive changes (e.g., downregulation of NTCP and OATP1B1 and upregulation of MDR1, MDR3, MRP3) that protect the cell against accumulation of toxic bile acids (39). The slight changes observed in transporter protein expression in SC hepatocytes may be a result of these cells adapting to the local environment by inducing cellular changes to promote hepatocyte survival. In SC human hepatocytes, similar expression changes consistent with this hypothesis were observed. Although OATP1B1 expression levels appear to change slightly over days in culture, no changes in OATP1B3 expression were observed between Day 1 and Day 6 in SC human hepatocytes. This discrepancy may reflect an independent pathway, separate from the FXR-mediated regulation of OATP1B3 responsible for the regulation of OATP1B1 (32).

Recent data generated in this laboratory suggest that in addition to P-gp, Mrp2/MRP2 may be involved in the biliary excretion of DPDPE. Expression, localization, and function of Mrp2 in SC rat hepatocytes has been demonstrated (Zhang *et al.*, submitted). Because the contribution of MRP2 in the vectorial transport of ^{3}H-DPDPE could not be ruled out, the canalicular localization, expression, and function of MRP2 was examined in SC human hepatocytes on Day 6. Excretion of CDF into canalicular networks on Day 6 and biliary excretion studies with CDF on Day 10 in SC human hepatocytes indicated that following repolarization, SC human hepatocytes are capable of excreting MRP2 substrates into the formed canalicular network.

In conclusion, the results of this study indicate that P-gp and MRP2 localize to the canalicular domain of SC rat hepatocytes and SC human hepatocytes, consistent with the localization of the marker protein DPPIV. In SC hepatocytes, these proteins maintain function, enabling the investigation of vectorial transport mechanisms. This system may have utility in understanding and predicting the functional consequences of altered regulation of hepatic transport proteins following xenobiotic insult or in various disease states. Finally, the study of hepatic transport mechanisms in SC human hepatocytes may aid in predicting clinically relevant drug transport interactions and elucidating mechanisms of such interactions.

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