Plasma membrane polarity of cultured skate (*Leucoraja erinacea*) hepatocytes undergoing *in vitro* morphogenesis

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Isolated liver parenchymal cells in culture have proven to be an invaluable system for studying a multitude of biochemical, physiological, and toxicological questions [1]. Hepatocytes have been isolated from many different species, including marine and freshwater fish [2]. Cultured hepatocytes offer a number of advantages over the *in vivo* or isolated perfused liver models, including elimination of systemic effects, better control of environmental conditions, reduced variability between experiments, simultaneous and repeated sampling in a single experiment, and more efficient use of reagents and time. Short-term cultured hepatocytes (<5 h) closely mimic the functions of cells in the intact liver and provide a useful model for studying acute effects of drugs and xenobiotics. Moreover, primary monolayer cultures allow for measurement of long-term effects (over days to weeks).

However, one drawback of most hepatocyte culture models is that the cells begin to dedifferentiate and lose transport polarity immediately after isolation [1,6]. The functions that are diminished or lost in culture include some drug-metabolizing enzyme activities, membrane transport functions, specialized hepatic endocrine functions, and the ability to form bile. The changes in transporter expression closely resemble the altered transporter phenotypes of cholestatic and proliferating hepatocytes *in vivo*, thus indicating that primary cultured hepatocytes acquire a cholestatic phenotype [6]. In contrast, our previous experience with short-term cultures of hepatocytes isolated from the little skate indicates that these cells largely retain hepatobiliary polarity for at least 8 h [4,5,7]. When compared with mammalian hepatocytes, skate hepatocytes are easier to isolate and maintain in short-term culture, and they exhibit high viability, stability, and maintenance of normal hepatocyte functions. Skate hepatocytes have been used to evaluate mechanisms of hepatic metabolism, detoxification, membrane transport, and cell volume regulation [3-5,7].

The objective of the present study was to examine whether long-term cultures of skate hepatocytes retain functional membrane polarity, and if so to characterize this model system. Skate hepatocytes were isolated as previously described [8], except that all solutions were prepared using tissue culture quality chemicals and water. Solutions were filtered through sterile 0.22 μm filters and all glassware was autoclaved. Immediately after isolation, cells were resuspended, washed, then plated in ice-chilled sterile elasmobranch Ringer solution (containing in mM, 270 NaCl, 4 KCl, 2.5 $CaCl_2$, 3 $MgCl_2$, 0.5 Na_2SO_4, 1 KH_2PO_4, 8 $NaHCO_3$, 350 urea, 5 D-glucose and 5 Hepes/Tris, pH 7.5). In most experiments, 0.1 μM dexamethasone was added to the culture medium. Cells were maintained at 12°C in an incubator that was gassed with room air. The cells were analyzed over a seven-day period using a variety of morphologic and biochemical analyses.

When examined shortly after their isolation, skate hepatocytes were present largely as clusters of 3-20 hepatocytes, although there were also many individual hepatocytes, and all hepatocytes contained numerous lipid-filled vesicles. The cell clusters normally contain a bile canaliculus (4). These cells and cell clusters readily attached to untreated plastic culture dishes in the presence or absence of dexamethasone; however, dexamethasone accelerated both the attachment process, and the formation of cell-cell contacts and of a network-type architecture. The cell clusters gradually annealed to one another and formed a loose, network-type three-dimensional structure. During this morphogenesis, cell margins of individual hepatocytes became less and less obvious and definitive chords of cells developed. On

occasion, these hepatocyte networks were so robust that they could be lifted intact as a thin sheet from the bottom of the plastic dishes. Trypan blue and propidium iodide exclusion was found to be >98%, and the cells maintained high intracellular concentrations of K^+, ATP, and reduced glutathione (GSH), and high ATP/ADP and GSH/GSSG ratios. Glutathione *S*-transferase activity remained constant, whereas cytochrome P450 activity declined to 16% of initial levels after 7 days. Quantitative RT-PCR analysis revealed that the mRNA levels of several genes remained constant over the 7-day period, whereas for Bsep, the canalicular bile salt export pump, levels declined slowly to 30% of initial values. The cells underwent a morphogenesis in which the clusters reannealed into a three-dimensional network of chords. During this morphogenesis, skate hepatocytes clusters maintained a polarized distribution of actin filaments and microtubules, as well as apical and basolateral membrane domains. Polarity of membrane transport systems was confirmed both morphologically, using antibodies raised against Bsep (Abcb11), and against the canalicular multispecific organic anion transporter Mrp2 (Abcc2), and functionally, by monitoring secretion of the fluorescent organic anions NBD-taurocholate, a Bsep substrate, and fluorescein-methotrexate, an Mrp2 substrate, into the bile canalicular spaces. Both of the organic anions showed a time dependent accumulation in the bile canalicular spaces of hepatocytes that were in culture for 6 days, indicating that the clusters maintain not only structural polarity, but also functional transport polarity.

Overall, the results indicate that in contrast with mammalian hepatocytes, isolated skate hepatocyte clusters retain polarity in culture, and provide an excellent system for investigating long-term effects of drugs and xenobiotics on hepatobiliary functions and for studying *in vitro* morphogenesis. (Supported by NIH/NIEHS ES03828, ES01247, and ES07026, NIH/NIDDK DK34989, DK25636, DK48823, and DK067214, and by NSF DBI-0453391).

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