Structural Polarity and Functional Bile Canaliculi in Rat Hepatocyte Spheroids

Susan Fugett Abu-Absi,* Julie R. Friend,* Linda K. Hansen,† and Wei-Shou Hu*,†

*Department of Chemical Engineering and Materials Science and †Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

Primary hepatocytes self-assemble into spheroids that possess tight junctions and microvilli-lined channels. We hypothesized that polarity develops gradually and that the channels structurally and functionally resemble bile canaliculi. Immunofluorescence labeling of apical and basolateral proteins demonstrated reorganization of the membrane proteins into a polarized distribution during spheroid culture. By means of fluorescent dextran diffusion and confocal microscopy, an extensive network of channels was revealed in the interior of the spheroids. These channels connected over several planes and opened to pores on the surface. To examine the content of apical proteins in the channel membranes, the bile canalicular enzyme dipeptidyl peptidase IV (DPPIV) was localized using a fluorogenic substrate, Ala-Pro-cresyl violet. The results show that DPPIV activity is heterogeneously distributed in spheroids and localized in part to channels. Bile acid excretion was then investigated to demonstrate functional polarity. A fluorescent bile acid analogue, fluorescein isothiocyanate-labeled glycocholate, was taken up into the spheroids and excreted into bile canalicular channels. Due to the structural polarity of spheroids and their ability to excrete bile into channels, they are a unique three-dimensional model of in vitro liver tissue self-assembly. (Video animations of some results are available at http://hugroup.cems.umn.edu/research_movies).

Key Words: 3D culture; multicellular aggregates; tissue engineering; bile acid excretion; dipeptidyl peptidase IV.

INTRODUCTION

Primary hepatocytes, when cultured on moderately adhesive surfaces or in suspension, self-assemble into three-dimensional, compacted aggregates called spheroids [1–4]. Hepatocyte spheroids sustain viability for extended culture periods and maintain high levels of liver-specific functions including albumin production, urea synthesis, and cytochrome P450 activity [2, 4–7]. In addition, they bear a high degree of structural similarity to native liver tissue. As shown by scanning electron microscopy, the surface of the spheroid is smooth and cell–cell borders are barely distinguishable [2, 4, 8, 9]. Typically, a number of pore-like openings are also present on the surface of each spheroid. Examination by transmission electron microscopy revealed that adjacent hepatocytes inside spheroids often share microvilli-lined channels that are delimited by tight junctions [4, 8, 9]. The organized structure of the spheroids suggests that they may be polarized and capable of forming channels that resemble bile canalicular [10, 11].

Hepatocytes in their native environment possess structural polarity [11]. They have a cuboidal shape, with two to three basal surfaces facing the sinusoid. A polygonal network of bile canaliculi divides the lateral domain between adjacent cells. Formed by membranes contributed from contiguous cells, microvilli-lined bile canaliculi comprise the apical domain of hepatocytes. Specific tasks related to position in the cell are carried out in each domain. For example, proteins involved in the trafficking of metabolites from the bloodstream are localized to the basal surface, whereas bile acid transporters are confined to the apical domain.

Freshly isolated hepatocytes lose this polarized structure after dissociation from liver tissue [12]. Monolayer cultures of hepatocytes on extracellular matrix proteins adopt a flat morphology [13, 14], and the development of bile canaliculi structures in these cultures is rare, heterogeneous, and short-lived [12, 15]. Several investigators have reported improved maintenance of archetypal polarity [13, 16] and bile canaliculi formation in extracellular matrix sandwich cultures [14, 15, 17], on collagen-coated porous membranes [18], and in hepatoma-derived hybrid cell lines [19–21]. However, the restraints of such two-dimensional systems hinder the development of anastomosing channels among many cells. With hybrid cells and hepatoma cell lines, the bile canaliculi developed in culture...
are often spherical rather than tubular as seen in vivo [20, 22]. Recently, Yumoto et al. [23] investigated the presence of bile canaliculi-like structures in rat hepatocyte spheroids. They showed by rhodamine-phalloidin staining that spheroids possess tubular structures encircled with actin.

To examine the presence of bile canalicular activity in the channels in spheroids and further identify their molecular composition, we examined both structural and functional polarity. Using immunofluorescence labeling, the localization of apical and basolateral membrane proteins was investigated. We then used the diffusion of a fluorescent tracer through surface pores to reveal an interconnected network of channels. A fluorogenic substrate of the apical protein dipeptidyl peptidase IV (DPP IV) was used to localize this enzyme relative to the channels. Finally, functional polarity was demonstrated through fluorescent bile acid excretion into the channels.

MATERIALS AND METHODS

All study protocols were reviewed and approved by the University of Minnesota Research Animal Resources Animal Care Committee and met the institutional and national guidelines for the humane care and use of research animals.

Reagents. Rabbit polyclonal antibodies to the apical membrane protein HA4 [24] and the basolateral marker HA321 [25] were kindly provided by Dr. Ann Hubbard (Johns Hopkins University, Baltimore, MD). Alamar- cresyl violet [26], a fluorogenic substrate of DPPIV (E.C. 3.4.14.5), was a generous gift from Dr. C. J. Van Noorden (University of Amsterdam). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Isolation and culture of primary hepatocytes. Rat hepatocytes were harvested from 4- to 6-week-old male Sprague-Dawley rats weighing 200–250 by a two-step in situ collagenase perfusion technique modified from Seglen [27] and described in more detail elsewhere [28]. Following the perfusion, the liver was removed from the rat and placed in a petri dish containing Williams medium E supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, and 15 mM 2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes), all from Life Technologies (Rockville, MD), and 2.0 U/mL insulin (Eli Lilly, Indianapolis, IN). The liver was combed to release the hepatocytes and the cell suspension was filtered through a 100-μm sterile nylon mesh. To remove dead cells and debris, the cell suspension was spun in a centrifuge at 50g for 2 min and resuspended in fresh medium a total of three times. Each harvest routinely yielded hepatocytes with viability greater than 89% based on trypan blue exclusion. Hepatocytes were cultured in Williams medium E supplemented with 0.2 U/mL insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.0 mM L-glutamine, 500 μg/mL bovine albumin, 150 mM NaCl, 15 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, 2 mM L-glutamine, 5 mg/mL mouse epidermal growth factor, 1 μM dexamethasone, 4 mM MgCl2, 10 mM porcine glucagon, 6 μM human transferrin, 20 mM Gly-His-Lys (formerly called liver cell growth factor), 15.0 mM Hepes, 0.1 μM CuSO4, 5H2O, 30 mM Na2SO4, and 50 μM ZnSO4. Spheroid formation was achieved by culturing the hepatocytes in suspension as described previously [4]. Briefly, hepatocytes were inoculated at a concentration of 3.0 × 106 cells/mL into a 250-ml spinner vessel (Wilbur Scientific, Boston, MA) containing 100 mL of suspension medium at 37°C. The cells were pelleted by centrifugation at 105 g for 5 min, rinsed with phosphate-buffered saline (PBS; Life Technologies), pelleted again, and resuspended in lysis buffer. The lysis buffer consisted of 10 mM Tris, 0.1% Triton X-100 (Fisher Scientific, Pittsburgh, PA), 1% Triton X-100, 0.5% Nonidet-P-40, 150 mM NaCl (Malinckrodt Baker, Paris, KY), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N',N'-tetraacetic acid, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin (Roche Molecular Biochemicals, Indianapolis, IN), 1 μg/mL pepstatin (Roche), and 25 μg/mL leupeptin (Roche). The lysed samples were spun at 16,000g and the supernatant was stored at -20°C. Freshly thawed lysates were separated in a 7.5% SDS–polyacrylamide gel run overnight at 4°C and then transferred to nitrocellulose. Thirty micrograms of total protein, as determined by Bradford assay (BioRad Laboratories, Hercules, CA), was loaded into each lane, and concurrent gels were run in both reducing and nonreducing sample buffer. The blots were labeled with rabbit polyclonal antibodies to HA4 and HA321 (nonreducing) followed by horseradish peroxidase conjugated goat anti-rabbit IgG antibody (NEL Life Sciences, Boston, MA). Bands were visualized by chemiluminescence detection using an ECL Western blotting detection kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The blots were exposed to Kodak XAR-5 film, which was developed in a Konica SRX-101 medical imaging film processor (Konica Medical Imaging, Inc., Wayne, NJ).

Immunofluorescence labeling of early stage aggregates. Two hours after seeding the spinner culture, single cells were clustered together in small agglomerates containing between 2 and 10 cells. These early stage aggregates were removed from the spinner flask and placed into the wells of an eight-chamber LabTek slide (Nalge Nunc, Naperville, IL). Parafomaldehyde was added directly to the medium (2% final concentration) to fix the cells in place. After 15 min the medium and paraformaldehyde were aspirated and the cells were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 30 min at room temperature. The cells were subsequently rinsed two times with PBS and permeabilized in 0.2% Triton X-100 and 0.1% bovine serum albumin (BSA) in PBS for 15 min. Permeabilized cells were incubated in a 1:200 dilution of primary antibody followed by a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Zymed Corp., South San Francisco, CA). Incubation of the primary and secondary antibodies was performed in the permeabilization buffer. Fluorescence images were acquired on a Zeiss Axioskop 20 upright microscope (Carl Zeiss, Thornwood, NY) equipped with a 35-mm MC 80 camera.

Immunofluorescence labeling of embedded spheroids and liver tissue. Mature spheroids and a portion of rat liver for control were fixed, embedded in glycol methacrylate polymer, and sectioned using a microtome before immunofluorescence labeling. Spheroids were removed from the spinner flask 12 h, 24 h, 48 h, 3 days, and 5 days after inoculation and fixed in 4% paraformaldehyde for 30 min on ice. The fixative was then removed by pipetting, and the spheroids were rinsed twice with PBS. The liver was excised from a Sprague-Dawley rat, cut into 1-mm3 cubes, fixed for 4 h in 4% paraformaldehyde, and rinsed twice with PBS. Fixed spheroids and liver slices were stored in 0.05% paraformaldehyde at 4°C until embedding. After all of the samples had been collected (7 days) the sections were dehydrated in graded ethanol (75–80–95–100%) and embedded in J B4 glycol methacrylate (Polysciences, Inc., Warrington, PA) following the manufacturer’s directions for immunofluorescence. After submerging the samples in the J B4 monomer, catalyst was added and polymerization was carried out overnight at 4°C in a desiccator. The polymerization was accomplished in the cold to limit the rise in temperature caused by the exothermic reaction. Next, using a 12-mm glass knife on a Sorvall J B-4A microtome, the samples were
cut into 2-μm sections and collected on the surface of a water bath. The sections were maneuvered onto glass slides (three sections per slide) and dried at 40°C on a hot plate.

Etching and trypsinization steps were added to unmask the antigen sites hidden by the JB4 polymer. The sections were exposed to a 1:1 mixture of toluene 2 M NaOH in ethanol for 5 min and then serially diluted from ethanol (100–95–80–70%) to PBS. The slides were submerged in PBS for another 30 min to wash away the etching solution. To further expose the antigen sites, the sections were subsequently incubated in a 0.05% trypsin solution in sodium EDTA (Life Technologies) for 15 min at 37°C and then rinsed for 30 min in PBS.

The etched slides were incubated in a 1:100 dilution of primary antibody in 15% goat serum (ICN Biomedicals, Aurora, OH) and 0.1% BSA in PBS overnight at 37°C. An immunoperoxidase (Calbiochem, La Jolla, CA) barrier was traced around each section to prevent the antibody solutions from mixing, and all incubations took place in a humidified chamber to reduce evaporation. Following incubation with the primary antibody, the slides were washed for 30 min by submersion in PBS and labeled with either Oregon Green 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or FITC-conjugated goat anti-rabbit IgG antibody for 30–40 min at room temperature. The slides were washed again for 30 min in PBS, sealed with PVA glycerol and a coverslip, and stored at 4°C. As a negative control, sections were labeled with normal rabbit serum (ICN Biomedicals). To validate the image processing technique, the same antibody was used to stain consecutive sections.

Stained sections were viewed on an Olympus BX-60 upright microscope equipped with an Optronics TEC-401 cooled, single-chip camera (Optronics, Inc., Goleta, CA). Acquisition of digital grayscale images was performed using Metamorph software version 2.5 (Universal Imaging Corp., West Chester, PA). Using Adobe Photoshop (version 4.0, Adobe Systems Inc., San Jose, CA), the images were given red or green pseudocolor and superimposed to compare the staining patterns of HA4 and HA321 on serial sections.

Channel localization using fluorescent dextran. FITC–dextran (4400 Da) was diluted to 10 mg/mL in Williams' medium E without phenol red (Life Technologies). Spheroids were withdrawn from the spinner and allowed to settle by gravity. The medium was aspirated and the spheroids were resuspended in the FITC–dextran solution. Within 10–15 min the spheroids were scanned at room temperature on an epifluorescence microscope (Nikon Diaphot) linked to an argon laser confocal scanning system (Multiprobe 2001; Molecular Dynamics, Mountain View, CA) with 10-mW laser power and an excitation of 488 nm. Emitted light was collected through a 530-nm band pass filter. Confocal images, 512 × 512 pixels in size, were generated, viewed, and processed with an Iris Indigo workstation (Silicon Graphics, Mountain View, CA) using ImageSpace software version 3.11 (Molecular Dynamics).

Reconstruction of three-dimensional images was accomplished using IBM Visualization Data Explorer (DX) (Version 3.1.4; IBM Corp., Yorktown Heights, NY) on an IBM SP supercomputer at the University of Minnesota Supercomputing Institute. Raw data taken with the ImageSpace software were stored as 512 × 512 matrices of pixel intensities ranging from 0 to 255. Matrices of data from serial scans were collected into an array and imported into the DX program. Selected pixel intensities from a 30-μm slab through the dataset were then connected by shaded isosurfaces to create a three-dimensional rendering of the original confocal dataset. To achieve smooth isosurfaces, the step size between serial scans was constrained to less than twice the pixel size of the confocal images. Because the medium was saturated with FITC–dextran, the highest pixel intensities (100–255) were shaded black to create a backdrop. The fluorescence in the channels produced intensities ranging from 75 to 100; these values were assigned red isosurfaces. The fluorescence in the cells was between 10 and 70, and the isosurfaces connecting pixels in that range were shaded blue. To produce a look-through reconstruction wherein red surfaces are clearly visualized, the blue isosurfaces were created to be transparent.

Localize of DPPIV activity with a fluorescent substrate. DPPIV activity was visualized in living spheroids using the fluorogenic substrate Ala-Pro-cresyl violet. A 1.3 mM stock solution of Ala-Pro-cresyl violet in dimethyl sulfoxide (DMSO) was kept at 4°C in the dark. Spheroids were removed from the spinner, collected by centrifugation, resuspended in ice-cold phenol red-free Williams' medium E (pH 7.4), and placed into the wells of a LabTek eight-well chambered coverglass (Nalgae Nunc). The temperature of the solutions was kept cold to minimize the diffusion of the fluorescent cleavage product. The stock solution of substrate was diluted 1:10 in cold Williams' E without phenol red and added to the spheroid suspension to a final concentration of 13 μM. Cresyl violet fluorescence was detected as it formed in situ. Confocal images were acquired with ImageSpace software using an excitation of 514 nm and collecting emitted light above 595 nm.

To simultaneously visualize DPPIV activity and the location of the channels, FITC–dextran labeling was performed subsequent to the Ala-Pro-cresyl violet assay. In these experiments, the spheroids were first immobilized on collagen so that the addition of the FITC–dextran solution would not change their position or orientation. The wells of the chambered coverglass were coated with 8 μg/cm² type I collagen as described previously [6]. The spheroids were removed from the spinner and allowed to attach to the collagen-coated wells for approximately 1.5 h. After the spheroids were immobilized, they were rinsed twice with ice-cold phenol Williams' E without phenol red and then incubated with 13 μM substrate for 15 min at 4°C. After scanning for cresyl violet fluorescence, FITC–dextran was added to a final concentration of 10 mg/mL. The confocal settings to detect the FITC–dextran were the same as described previously. As a precaution, the amount of cresyl violet fluorescence detected using the FITC filter set was determined beforehand and found to be negligible.

Representative data were collected and imported into the DX program as described above to prepare three-dimensional reconstructions. The projection of a 3.5-μm thick cross section through a spheroid (Fig. 5A) was produced by layering 10 confocal images taken at a step size of 0.35 μm. Pixels with intensities above 140 were connected by red isosurfaces, whereas values between 50 and 140 were shaded blue. The background (intensities less than 50) was left black. Figure 5B was produced in a similar fashion. The image included 62 confocal sections representing a total thickness of approximately 20 μm. Only the highest intensities (200–255) were represented by red isosurfaces, whereas values less than 200 were shaded transparent blue. Figure 6C is a 4-μm-thick cross section of a spheroid showing the localization of DPPIV activity in reference to the channels. It was constructed by superimposing Ala-Pro-cresyl violet section scans and FITC–dextran scans using DX. Due to the low intensity of the FITC–dextran fluorescence, isosurfaces for values ranging from 12 to 16 were displayed and shaded blue. The highest values of cresyl violet fluorescence (150–250) were represented in red since those areas were nearest to the site of DPPIV activity. The perspective of Figure 6C was changed to be viewed from the side and above, to more clearly demonstrate the thickness of the channels. In addition, the image is zoomed in on an area of the spheroid that received the most uniform illumination by the confocal laser.

Bile acid excitation. A fluorescently tagged bile acid, fluorescein isothiocyanate–glycocholate (FITC–GC) was prepared according to the method of Sherman and Fish [29]. The product was stored as a 2 mM stock solution in DMSO at 4°C in the dark.

The FITC–GC medium was prepared by diluting the stock solution to 2 μM in culture medium, resulting in a final concentration of 0.1% DMSO. An aliquot of spheroid suspension was withdrawn from the spinner, and supernatant medium was removed and replaced with the FITC–GC medium. Spheroids were incubated in the FITC–GC medium at 37°C in the dark for 1 h before being examined with the confocal laser scanning microscope using an excitation wavelength of...
spheroid formation and the amount of each decreases slightly by day 5.

Membrane Protein Localization

To examine structural polarity in hepatocyte spheroids, the presence and localization of two membrane-bound proteins, HA4 and HA321, were investigated. HA4, a single transmembrane protein, is present at the apical domain of hepatocyte membranes in vivo [11, 30] and has many possible functions, including acting as a cell adhesion molecule [31], an ecto-ATPase [32], and a bile acid transporter [33]. HA321 is localized to the basolateral domain of hepatocytes and is thought to be a cell adhesion molecule [34].

Hepatocytes were cultivated in suspension to form spheroids as described in detail previously [4]. Within 2 h after inoculation, the cells aggregated into clusters containing between 2 and 10 rounded cells. About 12 h after inoculation, the small aggregates began to rearrange, changing from irregular shapes into a more compacted form. By 24 h the culture consisted of mostly spherical aggregates. The aggregates continued to merge and compact, eventually becoming spheroids of approximately 100 μm in diameter by 3 days. The increase in size of the spheroids is predominantly due to aggregation, as no evidence of significant hepatocyte proliferation has been found in spheroid culture conditions.

The mature spheroids had tightly packed structures and smooth surfaces. Very few changes to the morphology of the spheroids, including size, smoothness, and degree of compaction, were observed during microscopic examination from day 3 to day 5 (data not shown). The viability of the spheroids was also routinely assessed at 4 days using ethidium bromide and fluorescein diacetate as described previously [4]. Virtually no dead cells were observed with confocal microscopy, even in the center of the spheroids (not shown). In addition, viability stains and transmission electron microscopy (TEM) examination of spheroids cultured for up to 8 days revealed high viability and maintenance of tight junctions and microvilli-lined channels between hepatocytes [35, 4]. This stable viability and preservation of structure facilitated studies of polarity and channel formation in spheroids.

 SDS-PAGE was performed on total cell lysates collected at various time points during the culture. Western blot analysis revealed that the HA4 and HA321 antigens were present at all time points (Fig. 1). Immunofluorescence labeling was then used to study the polarity of hepatocytes during spheroid formation, particularly the arrangement of the HA4 (apical) and HA321 (basolateral) proteins. Early aggregates from the culture were composed of only a few cells, and visualization of the staining was performed using confocal microscopy.

FIG. 1. Western blot analysis of membrane-specific proteins during spheroid formation. SDS-PAGE was performed to separate protein from total cell lysates taken at various points during spheroid formation; 30 μg of total protein was loaded in each lane. Western blots were then carried out using antibodies against the apical marker HA4 and basolateral protein HA321. Both proteins are present throughout spheroid formation and the amount of each decreases slightly by day 5.

488 nm. The emitted fluorescence above 510 nm was collected. Confocal images, 512 x 512 pixels in size, were generated, viewed, and processed with an Iris Indigo workstation using ImageSpace software.

RESULTS

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FIG. 5. DPPIV activity in mature spheroids. Spheroids from 5 days in culture were placed in medium containing 13 μM Ala-Pro-cresyl violet and imaged using laser excitation at 514 nm. (A) Thin cross section slab through the bottom of a spheroid. (B) Reconstruction of another spheroid showing activity localized to a channel (arrow). Red represents areas of highest activity, while blue represents background fluorescence. The black circles in A are present at cell nuclei, where fluorescence is very low. Results indicate heterogeneous localization of DPPIV activity. Scale bars indicate 10 μm.

FIG. 6. DPPIV activity relative to channel position. Spheroids were analyzed for DPPIV activity as in Fig. 5 and then incubated with FITC–dextran to localize channels. (A) Pseudocolor image of cresyl violet fluorescence representing active DPPIV. (B) Pseudocolor image of FITC–dextran in channels. (C) Reconstruction of both the cresyl violet (red) and the channels (blue). Areas of overlap between channels and DPPIV activity appear pink. Only the highest intensities from A, corresponding to the proximity of cleavage, are represented in C; 60% of the DPPIV was localized adjacent to channels. Scale bars indicate 2 μm.

FIG. 7. FITC–glycocholate excretion in spheroids after 3 days in culture. Images are single sections taken with the confocal microscope and are shown in pseudocolor. Distances from the top of the spheroid are specified. Arrows indicate fluorescence accumulation in bile canalicular channels. Scale bars represent 10 μm.
ventional fluorescence microscopy. The spheroids were embedded in glycol methacrylate and cut into thin 2-μm sections using a microtome. Consecutive sections were then labeled with different antibodies to allow the comparison of antigen location.

The results of staining with the HA4 antibody are shown in Fig. 2. At 2 h, the cells were arranged in small clusters but retained their rounded morphology. HA4 labeling was localized to all areas of cell–cell contact (Fig. 2A). The pattern of HA321 staining was in a comparable lateral configuration (not shown). By 3 days, the culture consisted of larger, more compacted spheroids, and HA4 began to localize to discrete portions of the membrane domains (Fig. 2B). As a positive control, staining was also performed on slices of rat liver (Fig. 2C), and fluorescence was clearly localized to the bile canaliculi between adjacent hepatocytes. Comparing the 2-h and 3-day samples, HA4 had redistributed from all areas of cell contact to localized regions of the membrane. The pattern of staining observed in the spheroid section was very similar to that in the liver.

To investigate the state of hepatocyte polarity in mature spheroids, the localization of HA4 and HA321 were compared. Since the antibodies were both raised in rabbits, it was not feasible to perform dual immunofluorescence labeling using secondary antibodies. Instead, consecutive sections were labeled with each of the two antibodies. Grayscale images of the stains were acquired, given pseudocolor, and digitally superimposed. Negative controls using normal rabbit serum were included and serial sections were stained with the same antibody (not shown). Nonspecific staining was negligible in all cases. Furthermore, when consecutive sections were stained with the same antibody, the localization of antigen in the two sections was virtually identical, indicating that physical aberration in sectioning was minimal.

Shown in Fig. 3 are the results of labeling consecutive sections. The HA4 staining is shown in green, while HA321 labeling is represented in red. Spheroids from 12- and 24-h cultures showed little difference in the distribution of HA4 and HA321 compared to the 2-h sample (not shown). At 2 days, both HA4 and HA321 were distributed diffusely and the staining patterns often overlapped. It appears that a significant quantity of HA321 and, to a lesser extent HA4, was intracellular (Fig. 3A). By 3 days, HA4 had segregated to specific regions of the cell membrane. HA321 was more localized to the membranes than at 2 days, but the staining pattern remained diffuse (Fig. 3B). By 5 days, however, both HA321 and HA4 were localized to distinct regions of the cell membrane, indicating that the spheroids had adopted a form of polarization (Fig. 3C).

Channel Formation

Previously it was shown that tight junctions and microvillli-lined channels are present in hepatocyte spheroids and that the surfaces contain pores [4, 9]. We hypothesized that the pores on the surface of the spheroids open into an interconnected network of bile canalicular channels. To examine the channels, spheroids were incubated in medium containing FITC-dextran. Dextran was chosen as a tracer because it is relatively inert and nonlethal to the cells. In initial experiments, FITC-dextran molecules ranging from 4 to 500 kDa in molecular weight were tested for their suitability to channel visualization (not shown). The FITC-dextran molecule used in our studies was also used previously as an extracellular reporter molecule [20] and easily diffuses through the pores into the channels in spheroids.

Figure 4A shows a series of optical sections through a single 4-day spheroid after incubation in FITC-dextran. Vertical distances from the bottom of the spheroid are shown for each section. These images are represented by pseudocolor with increasing fluorescence intensities indicated by orange, red, and white. Black and blue represent background levels of fluorescence. FITC-dextran showed thin lines of bright fluorescence penetrating into the spheroids. This result strongly suggests the existence of a network of channels continuous with the surface and interconnected in the interior. These lines were estimated to be 1-3 μm in diameter, only slightly larger than the size of bile canaliculi in vivo.

A 30-μm slab through the center of another spheroid incubated with FITC-dextran was reconstructed into three-dimensional form (Fig. 4B). During the reconstruction process, internal areas with the highest pixel intensities were selected and shaded red against the blue background of the cells. A few large circular areas of fluorescence were present within the spheroids. These objects (typically about 20 μm in diameter) are believed to be dead cells that were unable to exclude the dextran molecule. However, channels of fluorescence clearly penetrated into the spheroid. This figure also demonstrates that many of the channels originated at the surface and connected over more than one plane. The portion enclosed in the square was rotated to a different viewing angle and shown in the inset to give a closer view of several channels intersecting from different planes. Thus, an interconnected network of channels was formed within hepatocyte spheroids.

Time course studies revealed that channels form gradually during the process of spheroid formation (not shown). At 10 h, fluorescent dextran penetrated between the loosely aggregated cells. FITC-dextran was able to infiltrate into channel-like structures in 24-h spheroids, but a bright network of pronounced fluores-
censure was not detected until 2 days. This indicates that
the development of the channel network progresses as
the spheroids form and compact over the first 2 days in
culture.

Localization of DPPIV Activity

The results shown above provide a few lines of evi-
dence that cell polarization occurs in spheroids and
interconnected channels similar to bile canaliculi exist.
To further demonstrate that the channels are structur-
ally similar to bile canaliculi, we investigated the dis-
tribution of an apical protein localized to canaliculi,
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tribution of an apical protein localized to canaliculi in
vivo. DPPIV deaves N-terminal peptides with a pen-
ultimate prolyl residue from polypeptides [36] and has
been shown to bind to extracellular matrix molecules
[37, 38]. A fluorogenic substrate to DPPIV consisting of
two pairs of alanine and proline residues flanking a
cresyl violet molecule has been developed [26]. The
cresyl violet fluoresces when DPPIV deaves at the
proline residue, permitting localization of the active
DPPIV enzyme. Using confocal microscopy, areas of
DPPIV activity within spheroids were identified.

Spheroids were incubated with the substrate and
optically sectioned using confocal microscopy. After-
ward, the raw data were reconstructed into three-di-

mensional images. The images in Fig. 5 show typical
results obtained with 5-day spheroids. The blue areas
represent background fluorescence in the cytoplasm,
and the black round structures are cell nuclei where
fluorescence is lowest. The areas in red, corresponding
to bright cresyl violet fluorescence, represent DPPIV
activity. Figure 5A shows a cross section slab through
the bottom of a spheroid, which appears oblong due to
its proximity to the surface. The DPPIV activity is
heterogeneously distributed throughout the spheroid,
with some fluorescence localized to specific regions of
the cells in the interior. To better visualize the staining
pattern, three-dimensional reconstruction of the bot-
tom hemisphere of another spheroid was performed
(Fig. 5B). Very high activity can be seen at the periph-
ery. Interestingly, areas of high activity were also lo-
calized in a channel-like configuration (white arrow in
Fig. 5B). Due to rigorous examination of the spheroids
using viability staining and TEM, it is unlikely that
this pattern was due to fluorescence accumulation in
dead spaces within the spheroids.

To further investigate the possibility of DPPIV
localization in the channels, areas of cresyl violet
fluorescence were compared to channel location.
Spheroids were immobilized on a thin film of colla-
gen to prevent any movement or rotation during
imaging, and the Ala-Pro-cresyl violet assay was per-
formed to localize DPPIV activity. Next, FITC–
dextran was carefully added to the medium to allow for
detection of the channels. Figure 6A displays the
DPPIV activity and Fig. 6B shows the location of the
channels as revealed by FITC–dextran diffusion.

Figure 6C is a three-dimensional slab 4 μm thick
showing DPPIV activity (red) and the channels
(blue) simultaneously in an enlarged portion of the
spheroid. The regions in which the DPPIV activity
and channels overlap appear pink. Only the highest
pixel intensities from Figs. 6A and 6B were repre-
sented so that the areas closest to the cleavage site
were visualized. In addition, the slab was rotated to
produce a viewing angle from above and the side to
show the channels more clearly. A large amount of
overlap occurs between the channels and the DPPIV,
as represented by the pink color.

Quantitative comparison of the red and blue pixel
locations in Fig. 6C indicated that 60% of the highest
DPPIV activity was in the channels. The majority of
the remaining 40% of the cresyl violet fluorescence was
in the hepatocytes on the outer layer of the spheroid.
The amount of substrate to penetrate into the interior
of the spheroids during the assay was limited by diffu-
sion; therefore, higher fluorescence levels in the outer
cells may be the result of exposure to an elevated
concentration of DPPIV substrate. Dipeptidyl pepti-
dase II in lysosomes also shares some substrate affinity
with external DPPIV [39] and may therefore be pro-
ducing cresyl violet intracellularly. In addition, after
cleavage by DPPIV, cresyl violet diffuses away from
the cleavage site [26]. The combination of these factors
may have given rise to brighter fluorescence inside the
cells on the periphery.

Bile Acid Excretion into Channels

The polarization of hepatocytes in spheroids may
also result in functional similarity between the chan-
nels and bile canaliculi in vivo. To investigate the abil-
ity of hepatocytes in spheroids to transport bile acids
into the channels, a fluorescent bile acid analogue was
used. Fluorescein isothiocyanate-labeled glycocholate
was synthesized and tested in polarized monolayers of
hepatocytes on collagen gel. The polarized hepatocytes
in the monolayer were able to excrete the FITC-GC
into bile canalicular spaces between them (not shown).

FITC-GC excretion in a 3-day spheroid is shown in
Fig. 7. Each image is a cross section through the spher-
oid, and the vertical distances from the surface of the
spheroid are indicated for each section. The blue back-
ground surrounding the spheroids represents the
fluorescence intensity level of the FITC-GC concen-
tration in the medium. Thin lines of fluorescence were detected
within spheroids, particularly in the deeper sections
(arrows in Fig. 7B), and the pattern is reminiscent
of the FITC–dextran results described earlier. The fluo-
rescence intensity inside the channels was higher than
the background surrounding the spheroid, indicating a
higher concentration of bile acid in the channels. The result suggests that the presence of the bile acid analogue in channels was not merely due to simple diffusion through the pores (in which case, the concentration would not be higher than background). The accumulation in the channels was more likely the result of transport and excretion.

In addition to the fluorescence in the channels, there were several very bright hepatocytes typically near the periphery of the spheroid. These cells were clearly able to take up the FITC-GC but were not able to excrete it efficiently or at all, causing intracellular accumulation. This observation suggests that the cells on the periphery lacked the polarity or differentiation of the cells inside the spheroid. We cannot exclude the possibility, however, that some of the bright cells were nonviable.

**DISCUSSION**

Freshly isolated hepatocytes self-assemble into multicellular spheroids under suitable conditions. Mature spheroids have a tissue-like morphology that includes smooth surfaces, exterior pores, and microvilli-lined channels demarcated by tight junctions [4, 8]. Spheroids also display higher levels of liver specific activity than monolayer cultures. Free-floating spheroids have high cytochrome P450 activity [6], albumin production [1, 2, 4, 5, 9], transferrin secretion [5], ureagenesis [4], and tyrosine aminotransferase induction [1]. Sustained liver specific activity has been demonstrated in spheroids cultured for up to 60 days, perhaps due to their mimicry of liver architecture [5].

Since polarity is an important characteristic of hepatocytes in vivo and necessary for proper hepatic function, the first goal of this study was to investigate the relative location of apical and basolateral proteins in hepatocyte spheroids. Specifically, we examined the distribution of the HA4 and HA321 antigens, since antibodies to these proteins have been utilized previously to examine polarity in primary sandwich cultures [16] and hepatoma-derived hybrid cell lines [20, 34, 40]. The arrangement of cells in spheroids, with extensive cell-cell contact in all directions, is very different from the plate structure in the liver. Therefore, we did not expect an exact duplication of in vivo polarity, but anticipated segregation of the proteins to discrete locations. The immunofluorescence labeling confirmed that the aggregates at the very initial stage of spheroid formation (2 h) were not polarized, but between 3 and 5 days, the apical and lateral marker proteins segregated to discrete regions of the membrane domain. This polarization of HA4 and HA321 distribution occurred gradually, and each reorganized on a different time scale. Gradual polarity development and bile canaliculi formation has also been discovered during studies of hepatocytes cultured in a collagen sandwich [14, 16].

Microvilli-lined channels, often identified as bile canaliculi, were discovered during previous studies of spheroids. Our second aim was to visualize the channels and investigate the extent to which they are interconnected. Fluorescent dextran (FITC-dextran) was placed into the medium and allowed to diffuse through the exterior pores into the channels. FITC-dextran was previously used in studies to investigate the boundaries between apical and basolateral domains of WIF-B cells [20] and is therefore a suitable extracellular reporter molecule. The studies reported here revealed that the channels formed an anastomosing network and time course studies indicated that the process of channel formation spanned several days. The results also show that a few cells were highly fluorescent, possibly due to a loss in viability. Some cell death may have occurred during optical sectioning by the confocal microscope. Spheroid cultures typically have very high viability out to at least 8 days [4]; however, during the laser scanning, the spheroids experience a decrease in available oxygen and a slight rise in pH.

The polarized nature of hepatocytes in spheroids suggested that the channels may contain apical membrane proteins. To investigate this possibility, a fluorogenic substrate for DPPIV, Ala-Pro-cresyl violet, was utilized to ascertain areas in spheroids that contain the functional apical ectopeptidase. This experimental design has advantages over immunofluorescence labeling for DPPIV since it identifies only active DPPIV and can be used in conjunction with confocal microscopy to explore the three-dimensional aspects of the spheroid, including the channels. The studies were complicated, however, by the rapid diffusion of the cresyl violet product. Our findings demonstrated that the staining pattern appeared to be channel-like. Comparison with FITC-dextran staining to identify channels indicated that the majority of DPPIV-produced cresyl violet fluorescence was in or near the channels.

To investigate the function of the bile canalicular channels, a fluorescent bile acid analogue was used to examine the ability of spheroids to take up and excrete bile acid. The excretion of fluorescent bile acid analogues or organic ions such as fluorescein diacetate is a common method to explore functional polarity in cultured cells [16, 20, 22, 41-43]. Our results with FITC-glycocholate indicated that bile acid was excreted from the hepatocytes and concentrated in the channels. The pattern of excretion, similar to the FITC-dextran results, provides convincing evidence that the channel network is indeed the site of FITC-GC accumulation.

Yumoto et al. [23] examined the transport of the vital dye fluorescein diacetate in spheroids. They used rhodamine-phalloidin labeling to identify tube-like structures which they deemed bile canaliculi. Disruption of
the actin network by cytochalasin B caused fluorescein to accumulate in patches in the spheroids. This was attributed to interruption of canicular peristalsis since actin-dependent contractions are important for bile acid transport in vivo [44]. The fluorescence accumulation that they detected may have been intracellular since many of the tubular structures and patches of fluorescence were very large (−10 μm). In addition, fluorescein diacetate is commonly used for viability stains and remains in the cytoplasm for extended periods of time. In contrast, the channels that we observed were closer in size to bile canaliculi in vivo, which are about 0.75 μm in diameter. Our results here, in addition to the TEM studies published previously [4], demonstrate both structural and functional similarities between the channels and the bile canaliculi and firmly establish the presence of genuine bile canaliculi in rat hepatocyte spheroids.

The abundance of microvilli in the channels [4] and the evidence that they contain DPP IV indicate that they structurally resemble bile canaliculi. In addition, FITC-GC excretion in channel-like patterns is indirect proof that the channels are functioning as a bile canicular network. Since the innermost cells in spheroids maintain high viability in culture [3, 4], it has been postulated that another function of the channels may be similar to the role of the sinusoid in vivo. The channels may help to maintain the viability of the cells on the interior by facilitating diffusion of oxygen, nutrients, and metabolites. This dual scenario could be present in single channels, or some channels may be specialized in uptake while others specialize in excretion. The formation and function of the channel network in spheroids is therefore an interesting component of their tissue-like quality.

Polarization is necessary for many of the specialized functions of the hepatocyte. Unfortunately, conventional monolayer cultures of primary hepatocytes dedifferentiate [45–47] and lose polarity [15], especially in the presence of serum [48]. Therefore, much effort has been put forth in recent years to develop appropriate in vitro models with which to study functions dependent on polarity. Promising results have been obtained with hepatoma and hybrid cell lines that maintain polarity and form bile canaliculi capable of collecting bile acid or fluorescein [22, 41, 42]. In some cases two-dimensional culture conditions, such as sandwich cultures [14, 16, 43] and collagen gel entrapment [49], are also suitable for the production and maintenance of hepatocyte polarity, bile canaliculi, and/or canicular excretion in primary cells. One drawback to these systems is that the hepatocytes do not form anastomosing bile canaliculi as they do in the liver. Although canicular networks have been reported in sandwich cultures [14], the geometry of the culture system constrains the cells from developing a three-dimensional network. The channels in spheroids, on the other hand, are highly interconnected, mimicking the three-dimensional nature of bile canaliculi in the liver more closely.

With their long-lasting viability and function in culture, hepatocyte spheroids have been proposed for in vitro studies of drug toxicity and xenobiotic metabolism [4]. The presence of a polarized structure and functional bile canaliculi adds to their value for such uses. In addition, the rearrangement that hepatocytes undergo during spheroid formation to form polarized structures is an excellent model of in vitro tissue organization. The model is ideal not only for physiological studies, but also for gene regulation investigations, since liver-specific gene transcription is known to be affected by tissue organization, cell shape, and cell–cell and cell–matrix interactions [45, 47]. Therefore, analysis of liver-specific gene expression in spheroids may help to shed light on the regulation of liver injury repair and regeneration.

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