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Primary Hepatocytes Outperform Hep G2 Cells as the Source of Biotransformation Functions in a Bioartificial Liver

Scott L. Nyberg, M.D., Ph.D.,*,† Rory P. Remmel, Ph.D.,‡ Henry J. Mann, Pharm.D.,§ Madhusudan V. Peshwa, Ph.D.,† Wei-Shou Hu, Ph.D.,† and Frank B. Cerra, M.D.*

From the Departments of Surgery, Chemical Engineering and Materials Science,† Medicinal Chemistry,‡ and Pharmacy Practice,§ University of Minnesota, Minneapolis, Minnesota*

Objective

Metabolic activity of transformed human liver (Hep G2) cells and primary rat hepatocytes were compared during *in vitro* application of a gel entrapment bioartificial liver.

Background

Clinical trials of bioartificial liver devices containing either transformed liver cells or primary hepatocytes have been initiated. A study comparing transformed liver cells and primary hepatocytes in a bioartificial liver under similar conditions has not been reported previously.

Methods

Gel entrapment bioartificial liver devices were inoculated with 100 million cells, Hep G2 cell line ($n = 4$), or rat hepatocytes ($n = 16$), and studied for up to 60 days of *in vitro* cultivation.

Results

Hep G2 cells grew to confluence within the gel entrapment configuration with a doubling time of 20 ± 3 hours. Rat hepatocytes significantly outperformed Hep G2 cells at confluence in all categories of biotransformation, including ureagenesis (3.5 ± 0.7 *vs.* 0.3 ± 0.1 μmol/hr, $p < 0.05$), glucuronidation (630 ± 75 *vs.* 21 ± 2 nmol/hr, $p < 0.005$), sulfation (59 ± 13 *vs.* 5 ± 2 nmol/hr, $p < 0.05$), and oxidation (233 ± 38 *vs.* < 1 nmol/hr, $p < 0.005$). At the conclusion of one experiment, Hep G2 cells were found in the extracapillary compartment of the bioartificial liver, analogous to the patient's compartment during clinical application.

Conclusions

Primary rat hepatocytes were superior to the Hep G2 cell line as the source of hepatic function in a bioartificial liver and avoided the potential risk of tumor transmigration from the bioartificial liver into the patient's circulation.

Several designs of a bioartificial liver (BAL) currently are being tested in animal[1–7] and human[3, 8–10] trials. In all cases, the BAL devices contain metabolically active liver cells, either primary (normal) hepatocytes or a transformed (tumor) cell line. Pros and cons exist for the clinical application of primary or transformed liver cells

in a BAL.[11-16] Primary liver cells perform many important hepatic functions, but have a limited life span *in vitro.* Primary liver cells usually are obtained from animal sources, and can be associated with the immunological consequences of infusing animal proteins into human patients. Transformed liver cells, on the other hand, grow to high cell density in a BAL[17] and may be obtained from human sources,[18] thus avoiding the potential xenogeneic immune response. A potential risk of the use of tumor cells in a BAL relates to the transmigration of tumor cells or tumorigenic products from the device into the patient's circulation. Recently, use of a bioartificial liver device containing transformed hepatocytes from a human hepatoblastoma was associated with successful recovery in a patient with syncytial giant-cell hepatitis[9] and clinical improvement in another patient with fulminant hepatic failure of unclear etiology.[10] Similarly, use of a device containing primary porcine hepatocytes was associated with clinical improvement of one patient[3] and successful recovery of another patient with alcoholic liver disease.[8] A study comparing transformed and primary liver cells as the source of hepatic function in a BAL has not been reported.

Based on their higher state of differentiation, we hypothesized that primary hepatocytes would provide more biotransformation activity than transformed liver cells in a bioartificial liver. Transformed liver cells (Hep G2 cell line) and normal rat hepatocytes were compared during *in vitro* application of a gel entrapment BAL.[19,20] The Hep G2 cell line was derived from a well differentiated, human hepatoblastoma,[18,21,22] similar to the C3 line of hepatoblastoma in clinical use.[10] Biochemical parameters that were measured included oxygen consumption, lactate production, glucose consumption, albumin production, and ureagenesis. Drug metabolism was evaluated by the oxidative metabolism of lidocaine (phase I, P450 activity), along with the sulfation and glucuronidation of 4-methylumbelliferone (phase II, conjugation activity). Biotransformation activity was determined from ureagenesis and drug metabolism. Bioartificial liver devices were examined grossly and microscopically after each experiment.

METHODS

Bioartificial liver devices were inoculated with freshly harvested rat hepatocytes (n = 16) or the Hep G2 cell line (n = 4), and cultivated under *in vitro* conditions. During each experiment, culture media was sampled for determination of rates of biochemical activity and drug metabolism. All BAL systems were terminated electively, at which time they were disassembled for gross and microscopic examination. All animal experimental protocols were reviewed and approved by the Committee on Animal Usage in Research at the University of Minnesota.

Hepatocyte Harvest

Hepatocytes were harvested from 4- to 6-week-old male Sprague-Dawley rats, weighing 200 to 250 g by a two-step *in situ* collagenase perfusion technique modified from the method described by Seglen.[23] Hepatocyte viability at the time of inoculation ranged from 85% to 93%, based on trypan blue exclusion.

Hep G2 Cell Line

Hep G2 cells were grown until confluent in polystyrene tissue culture flasks in Williams' E medium supplemented with 5% fetal calf serum, 200 units/L of insulin, 8.0 mM/L of glutamine, 40,000 units/L of penicillin G, and 400 mg/L of streptomycin sulfate. Cells were harvested with 0.25% trypsin in saline before entrapment in collagen gel.

Cell Entrapment

Rat hepatocytes or Hep G2 cells were suspended in collagen solution (3:1 mixture of type 1 collagen [Vitrogen 100, Collagen Corp., Palo Alto, CA] and fourfold concentrated Williams' E medium) at 0.5 to 1.0 $\times$ 10^7 cells/mL. The collagen-cell suspension then was inoculated into the lumina of the hollow fiber cartridge, and incubated at 37 C for 10 minutes to accelerate gel formation.

BAL Apparatus

Hollow fiber cartridges (H1P100, Amicon, Danvers, MA) were inoculated with 100 million cells—Hep G2 cells or freshly harvested rat hepatocytes—and studied in a microprocessor-controlled BAL system (Cellex Biosciences, Inc, Minneapolis, MN) as described previously.[19] A schematic of the three-dimensional, gel entrapment configuration is shown in Figure 1B. Temperature (37 C), pH (7.2), and inlet pO_2 (140 mmHg) were controlled by the BAL system. The hollow fibers used in these experiments were polysulfone with a 100-kd nominal molecular weight cut-off. Williams' E medium, supplemented with 5% fetal calf serum, 200 units/L of insulin,

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Address reprint requests to Frank B. Cerra, M.D., 406 Harvard Street S.E., Box 42 UMHC, Minneapolis, MN 55455.

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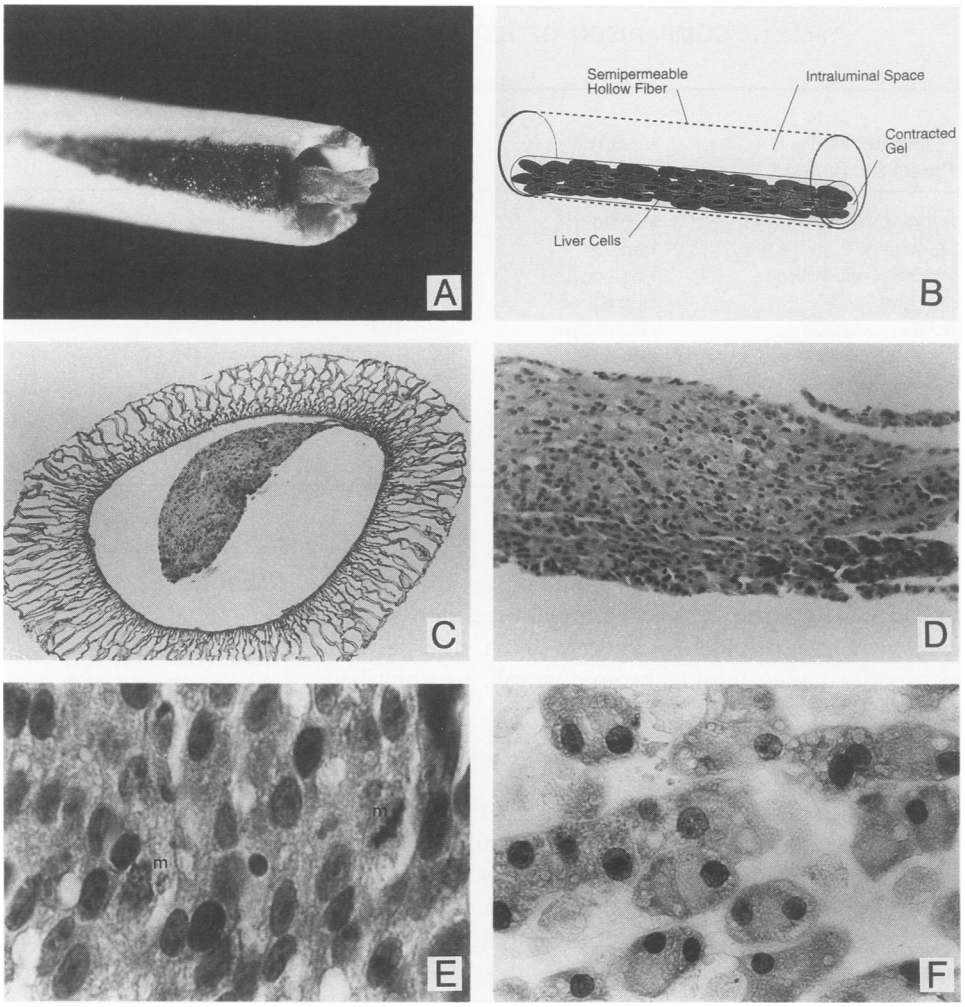

Figure 1. Photographic enlargement (A), schematic representation (B), and cross sectional view (C) of a single hollow fiber containing a contracted gel (D, E) 60 days after inoculation of Hep G2 cells into the bioartificial liver device. Gel-entrapped rat hepatocytes removed from the bioartificial liver 10 days after inoculation are shown for comparison (F). Mitotic activity (m) was only observed in gels containing the Hep G2 cell line. Hollow fiber dimensions: internal diameter 1.1 mm, outer diameter 2.0 mm. (Magnification: A × 20, C × 75, D × 200, E × 900, F × 900).

8.0 mM/L of glutamine, 40,000 units/L of penicillin G, and 400 mg/L of streptomycin sulfate and two markers of drug metabolism (5 μg/mL of lidocaine and 60 nmol/mL of 4-methylumbelliferone [4-MU]) was recirculated in the shell space at 30 mL/hr. Culture media in the shell space was replaced with fresh media 24 hours after cell inoculation and every 7 days thereafter. All bioreactor devices received intraluminal perfusion at 9 mL/hr, initiated 24 hours after cell inoculation. Medium from the intraluminal outflow was discarded after one pass.

Oxygen Measurements

Oxygen consumption (ΔO_2) by cells in the BAL was determined from the following equation:

$$\Delta O_2 = S_{O_2} \cdot [P_{in} - P_{out}] \cdot Q_E \text{ mol } O_2/h \text{ (units)}$$

where P_{in} and P_{out} were the oxygen tensions (mmHg) in the shell inlet and outlet, respectively. Oxygen tensions were measured continuously with a commercial (Ingold Electrode, Wilmington, MA) dissolved oxygen probe. Q_E was the medium flow rate (mL/hr) in the shell space. Solubility of oxygen (S_{O2}) in aqueous medium was 1.29×10^{-9} mol O_2/mL/mmHg at 37 C.

Biochemical Measurements

Rat and human albumin concentrations were determined from media samples by a competitive enzyme-linked immunoassay.[20] Urea concentrations were determined by high-performance liquid chromatography.[24] Lactate and glucose concentrations were determined on an automated industrial analyzer (Model 27, Yellow Springs Instruments Co., Yellow Springs, OH). Concentration of lidocaine and two metabolites, monoethylglycinexylidide (MEGX) and 3-OH-lidocaine, were determined by reverse-phase, ion-pairing, high-performance liquid chromatograph, as described previously.[25] Con-

Table 1. COMPARISON OF BIOCHEMICAL RATES (MEAN ± SEM) OF BIOARTIFICIAL LIVER (BAL) GROUPS OVER TIME

Biochemical Parameter (units)	BAL Group	Time Interval			
		0–24 hr	72–120 hr	168–336 hr	>336 hr
Oxygen consumption	Hepatocyte	17.3 ± 2.1	18.2 ± 3.4	14.5 ± 3.4	—
(μmol/hr)	Hep G2	8.5 ± 3.8	10.6 ± 3.8	12.4 ± 4.6	16.5 ± 4.9
Glucose consumption	Hepatocyte	<0.5*	<0.5*	—	—
(mg/hr)	Hep G2	2.9 ± 0.6	5.2 ± 0.5	8.1 ± 1.5	9.4 ± 1.7
Lactate production	Hepatocyte	<0.3*	<0.3*	—	—
(mg/hr)	Hep G2	1.6 ± 0.4	3.3 ± 0.4	4.7 ± 0.9	6.4 ± 1.3
Albumin production	Hepatocyte	24.2 ± 2.9	32.3 ± 5.0*	27.2 ± 1.5*	—
(μg/hr)	Hep G2	17.8 ± 6.1	78.3 ± 11.4	108.9 ± 18.5	178.9 ± 32.2

* $p < 0.005$ *vs.* Hep G2 group by unpaired t test.

centration of 4-MU and two metabolites, methylumbelliferone-glucuronide and methylumbelliferone-sulfate, were determined by reverse-phase, ion-pairing, high-performance liquid chromatograph, as described previously.[25]

Metabolic Rates and Statistical Analysis

Media samples were obtained in pairs from the shell compartment and lumen outlet on a daily basis, and more frequently as needed. These samples were used to determine overall rates of albumin production, lactate production, glucose consumption, ureagenesis, lidocaine oxidation, 4-MU glucuronidation, and 4-MU sulfation for the entire BAL system (shell compartment + lumen compartment). Metabolic rates were determined by a linear regression fit of the data. Average metabolic rates were determined from the average of single data points from individual experiments in each group. Statistical significance between groups was determined by two-sided, unpaired Student's t test. Data are reported as mean ± SEM.

Histological Methods

After each experiment, gels were removed from the hollow fiber cartridge, fixed in 10% buffered formaldehyde, processed in a routine fashion, and stained with hematoxylin-eosin for examination by light microscopy (Axioscope System, Carl Zeiss Inc., Batavia, IL). Fixation of tissue adherent to the hollow fiber cartridge also was performed with 10% buffered formaldehyde. After cutting open the housing of the hollow fiber cartridge with a band saw, individual hollow fibers were removed from the cartridge and examined grossly. Representative fibers were sectioned serially and stained with hematoxylin-eosin for examination by light microscopy.

RESULTS

Comparison was made between 4 BAL devices inoculated with Hep G2 cells (100 ± 16 million) and 16 BAL devices inoculated with rat hepatocytes (75 ± 6 million). Hep G2 devices were studied for 717 ± 202 hours (382–1441 hr). Eight rat hepatocyte devices were terminated electively at 24 hours, and eight were studied for 211 ± 30 hours (141–336 hr). There were no problems with microbial contamination in any of these experiments.

Average oxygen, glucose, lactate, and albumin metabolism rates of the rat hepatocyte and Hep G2 BAL groups are summarized in Table 1. Oxygen consumption and albumin production remained relatively stable in the rat hepatocyte BAL group, consistent with a quiescent state. Negligible rates of glucose consumption (<0.5 mg/hr) and lactate production (<0.3 mg/hr) by rat hepatocytes indicated that oxygen transport was adequate to these cells. In the Hep G2 group, the rates of glucose consumption, lactate production, and albumin production increased rapidly during the initial 200 hours of cultivation, and increased less rapidly thereafter (Table 1, Fig. 2). This pattern of biochemical activity was consistent with an early phase of exponential growth by the Hep G2 cells followed by contact inhibition of cell growth as three-dimensional confluence was approached within the gel. Doubling times of gel-entrapped Hep G2 cells were determined from a logarithmic fit of biochemical data during the initial 200 hours of growth (Table 2).

Initial rates (0–24 hr) of albumin production reported in Table 1 indicate that the cellular rate of albumin production by rat hepatocytes and Hep G2 cells were similar because both systems were inoculated with about 100 million cells. Significantly more albumin was produced in the Hep G2 devices overall, because cell number increased with tumor growth in these devices. Maximum

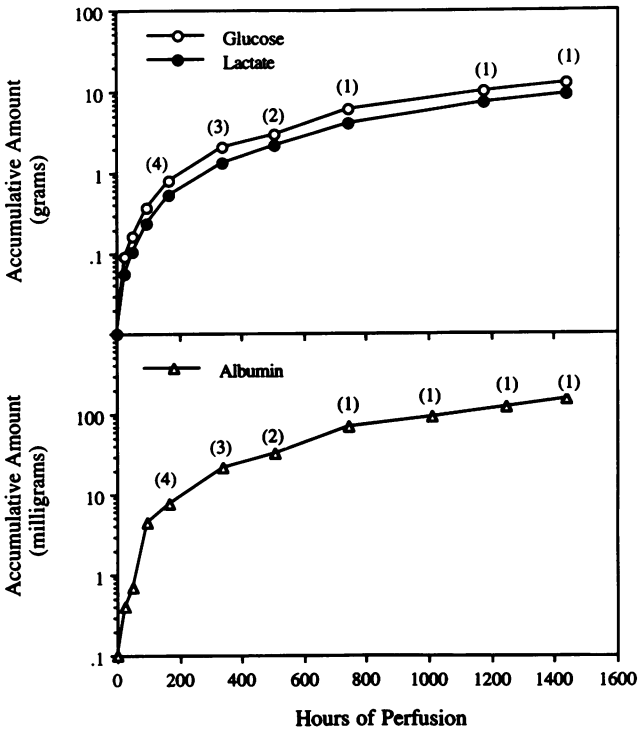

Figure 2. Accumulative amounts of glucose consumption (-o-), lactate production (-•-), and albumin production by BAL devices inoculated with Hep G2 cells at t = 0 hr. Data points were determined from the average of (n) devices.

rates of albumin production (179 ± 32 μg/hr), along with glucose consumption (9 ± 2 mg/hr) and lactate production (6 ± 1 mg/hr), were measured after 2 weeks (>336 hr) in the Hep G2 BAL group. Oxygen consumption increased less rapidly in the Hep G2 group, suggesting that anaerobic metabolism was favored by gel-entrapped Hep G2 cells at high cell density.

Biotransformation rates of ureagenesis and drug metabolism by the Hep G2 BAL group and rat hepatocyte BAL group are compared in Table 3. Rates of ureagenesis and drug metabolism were determined at confluence, at least 200 hours after inoculation, in the Hep G2 BAL group. Rates of both glucuronidation and sulfation of 4-MU were significantly greater in the rat hepatocyte group. Oxidative metabolism of lidocaine, determined from the sum of formation rates of two metabolites (MEGX, 3-OH-lidocaine), was detected only in the rat hepatocyte group. Chromatograms from the Hep G2 BAL group also were examined for minor metabolites of lidocaine, including 3-OH-MEGX and glycinexylidide. No minor metabolites of lidocaine nor abnormal metabolite peaks were identified in samples from the Hep G2 BAL group.

After each experiment, gels were removed from each bioartificial liver device and examined grossly and microscopically. Gel contraction was associated with the presence of viable Hep G2 cells (Fig. 1A, Fig. 1C). Gel-entrapped Hep G2 cells grew to confluence, as demonstrated under low magnification (Fig. 1C, Fig. 1D) and high magnification (Fig. 1E). The density of gel-entrapped Hep G2 cells at confluence (Fig. 1E) was fivefold to tenfold greater than the density of gel-entrapped rat hepatocytes (Fig. 1F), consistent with the increased rate of albumin production observed in the Hep G2 group (Table 1).

At the termination of one experiment, 60 days after inoculation with Hep G2 cells, a large number (>100) of tumor nodules were found in the extracapillary compartment of the hollow fiber cartridge adherent to the external surface of the hollow fibers. All of the hollow fibers were removed from the cartridge, examined grossly (Fig. 3A) and microscopically (Figs. 3B–3D). No cracks or defects were observed in the surface of the hollow fibers.

Table 2. DOUBLING TIMES (MEAN ± SEM) OF GEL-ENTRAPPED HEP G2 CELLS BASED ON BIOCHEMICAL ACTIVITY*

Biochemical Activity	Doubling Time
Albumin production	24 ± 8 h
Lactate production	19 ± 2 h
Glucose consumption	17 ± 3 h
Overall	20 ± 3 h

* Doubling times were determined from a logarithmic fit of biochemical data during the initial 200 hours of bioreactor perfusion.

Table 3. COMPARISON OF BIOTRANSFORMATION RATES (MEAN ± SEM) BETWEEN RAT HEPATOCYTES AND HEP G2 CELLS

Biotransformation Activity	Units	Bioartificial Liver Group	
		Hepatocyte	Hep G2
Ureagenesis	μmol/hr	3.54 ± 0.67*	0.26 ± 0.08
Drug Metabolism			
†Oxidation	nmol/hr	233.2 ± 37.9‡	<1
§Glucuronidation	nmol/hr	629.8 ± 74.9‡	20.5 ± 2.0
‖Sulfation	nmol/hr	59.1 ± 12.8*	4.6 ± 1.5

* $p < 0.05$, ‡$p < 0.005$ *vs.* Hep G2 group by unpaired t test.
† Oxidative metabolism of lidocaine as the formation of MEGX and 3-OH-lidocaine.
§ Glucuronidation of 4-MU.
‖ Sulfation of 4-MU.

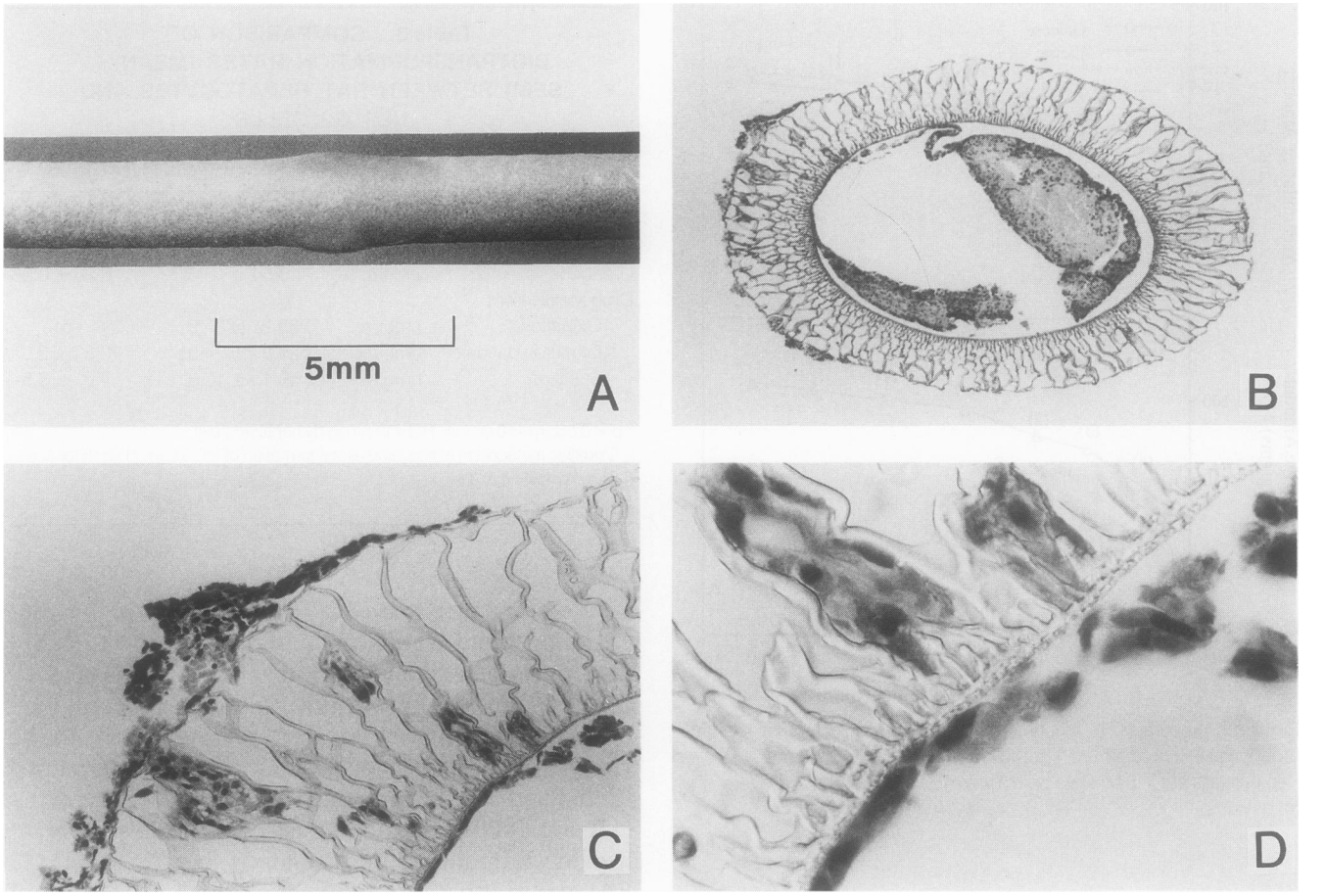

Figure 3. Growth of Hep G2 cells on the outer surface of a representative fiber from one bioartificial liver after 2 months of cultivation (A). Light microscopy demonstrated Hep G2 cells on the outer surface (B), in the wall (C), and in the lumen of the hollow fiber (D). Gel-entrapped Hep G2 cells were inoculated into the hollow fiber lumen, initially, as demonstrated in Figure 1B. (Magnification: A × 55, B × 220, C × 900).

Tumor cells were growing on both sides of the hollow fiber membrane and within the porous wall of the anisotropic polysulfone fiber (Fig. 3). Tumor nodules were not identified in the extracapillary space of the other three Hep G2 BAL devices.

DISCUSSION

This study compared a well-differentiated liver cell line, Hep G2, and normal rat hepatocytes as the source of hepatic function in a gel entrapment BAL. Multiple studies have reported the use of transformed cells[2,17] or primary hepatocytes[6,8,26–29] in a BAL; however, transformed cells and primary hepatocytes had not been compared previously in the same BAL device or in the same study. This point is relevant because both forms of artificial liver support are now in clinical trials.[8,10]

In the current study, the Hep G2 cell line grew rapidly within the three-dimensional gel and reached confluence about 100 hours after inoculation of 100 million cells. Doubling time, 20 ± 3 hours during the early phase of Hep G2 growth, was estimated from biochemical data because cell number could not be determined directly inside the BAL. This estimate of doubling time was quite similar to the doubling time reported for Hep G2 cells in low density monolayer culture,[30] suggesting that the rate of growth of Hep G2 cells at low density is similar in monolayer and three-dimensional gel cultures.

As expected from previous reports,[21,30,31] Hep G2 cells produced large quantities of protein (albumin) in the BAL. Rat hepatocytes significantly outperformed Hep G2 cells in all categories of biotransformation (ureagenesis, glucuronidation, sulfation, oxidative metabolism), however, despite the fivefold to tenfold increase in the number of cells in devices inoculated with the Hep G2 cell line. These biotransformation activities were se-

lected to evaluate metabolic activity of the BAL because of their association with the development of metabolic encephalopathy.[32] Biotransformation rates of Hep G2 cells were determined for confluent, high-density cultures because growth state has been shown to influence metabolic activity of Hep G2 cells.[30,33]

In the current study, rates of ureagenesis and both conjugation activities were negligible during the exponential phase of Hep G2 growth and became detectable after at least 200 hours of cultivation. Low ureagenesis rates were consistent with the results of Armbruster et al.,[34] who found that Hep G2 cells were unable to grow in arginine-free media. Poor conjugation activity by Hep G2 cells was reported previously.[22,35] In other studies, however, glucuronidation of naphthol and phenolphthalein were similar in Hep G2 cells and freshly isolated human hepatocytes.[36] Although not tested in our study, improved rates of conjugation may be achieved for both Hep G2 cells and primary hepatocyte cultures[37] by altering the culture medium composition[38,39] or by the induction of drug metabolizing enzymes.[36,40,41]

P450 activity, assessed by the oxidative metabolism of lidocaine, was detected only in the BAL devices that contained rat hepatocytes. Low levels of P450 activity have been reported by other groups working with hepatoblastoma cells, such as the Hep G2 cell line.[36,42–45] According to Bargetzi et al.,[46] Hep G2 cells do not express significant levels of P450 IIIA4 and thus, are unable to produce MEGX, the major lidocaine metabolite of human hepatocytes. Hep G2 cells also express low levels of P450 IA1 and P450 IA2.[47] Low expression of P450 IA2 may explain the absence of 3-OH-lidocaine formation in the Hep G2 BAL group after lidocaine administration. Improved P450 IA2 activity by Hep G2 cells[36,41] or rat hepatocytes[48,49] is expected in the BAL device after addition of an inducing agent, such as 3-methylcholanthrene. Induction experiments comparing P450 activity of rat hepatocytes and liver cell lines, such as Hep G2, are planned for future *in vitro* BAL studies.

We were startled to find a large number (>100) of tumor nodules in the extracapillary compartment of one hollow fiber cartridge 60 days after inoculation with Hep G2 cells (Fig. 3). No defects could be found in any of the hollow fibers. Two explanations are most plausible for the observation of tumor on both sides of the hollow fiber membrane, despite a 100-kd nominal molecular weight cut-off. First, tumor cells were able to pass directly through the hollow fiber membrane. Polysulfone hollow fibers are anisotropic and do not have a normally distributed molecular weight cut-off. In other words, large openings can occur in the membrane that allow passage of objects much greater than 100 kd, possibly even cells. Passage of Hep G2 cells through the inner skin of the hollow fiber membrane was suggested by light microscopy (Figs. 3B–3D). Second, Hep G2 cells may have been seeded inadvertently in the extracapillary space during the inoculation process. This second explanation is less likely because technical problems during the inoculation process usually are associated with microbial contamination of the bioreactor system, which was not observed. Whether a technical mistake or membrane failure, this example draws attention to the potential risks associated with the clinical use of tumor cells in a bioartificial liver. These potential risks were discussed in some detail in a recent communication by Muller and Jauregui.[14]

Another potential risk of using tumor cells in the BAL relates to the escape into the patient's circulation of tumorigenic products or other components associated with adverse side effects. Such risks are not studied well in the context of a bioartificial liver, and were not addressed in the current set of experiments. Nonetheless, transformed liver cell lines are known to produce abnormal patterns of soluble proteins.[50] Neoplastic transformation of liver epithelial cells has been attributed to retroviral-associated oncogenes[51] and other genetic alterations.[52] With respect to the Hep G2 cell line, two genetic lesions may be responsible for hepatocarcinogenesis,[53] although the possibility of tumorigenic products or other components associated with adverse side effects has not been ruled out entirely. So far, no adverse side effects have been reported with the C3 line of hepatoblastoma in clinical use.[9,10]

Primary rat hepatocytes and transformed liver cells (Hep G2 cell line) were studied under perfused *in vitro* conditions in a gel entrapment BAL. Hep G2 cells grew to confluence within the gel and produced large amounts of albumin. However, rat hepatocytes significantly outperformed the Hep G2 cell line in all biotransformation functions considered to be important to the clinical application of a BAL. In one Hep G2 BAL, tumor cells were found in the extracapillary compartment, which is analogous to the patient's compartment during clinical application. Based on our analysis of biochemical activity, drug metabolism, and histological material, primary rat hepatocytes were superior to the Hep G2 cell line during the *in vitro* application of a gel entrapment BAL

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