

Primary Human Hepatocytes on Biodegradable Poly(L-Lactic acid) Matrices: A Promising Model for Improving Transplantation Efficiency With Tissue Engineering

Eva Török,^{1*} Marc Lutgehetmann,^{2,3*} Jeanette Bierwolf,¹ Stefan Melbeck,¹ Jochen Düllmann,⁴ Bjoern Nashan,¹ Peter X. Ma,⁵⁻⁷ and Joerg M. Pollok¹

Departments of ¹Hepatobiliary and Transplant Surgery, ²Internal Medicine I, ³Medical Microbiology, Virology, and Hygiene, and ⁴Anatomy II: Experimental Morphology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; and ⁵Department of Biologic and Materials Sciences, ⁶Macromolecular Science and Engineering Center, and ⁷Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI

Liver transplantation is an established treatment for acute and chronic liver disease. However, because of the shortage of donor organs, it does not fulfill the needs of all patients. Hepatocyte transplantation is promising as an alternative method for the treatment of end-stage liver disease and as bridging therapy until liver transplantation. Our group has been working on the optimization of matrix-based hepatocyte transplantation. In order to increase cell survival after transplantation, freshly isolated human hepatocytes were seeded onto biodegradable poly(L-lactic acid) (PLLA) polymer scaffolds and were cultured in a flow bioreactor. PLLA discs were seeded with human hepatocytes and exposed to a recirculated medium flow for 6 days. Human hepatocytes formed spheroidal aggregates with a liver-like morphology and active metabolic function. Phase contrast microscopy showed increasing numbers of spheroids of increasing diameter during the culture period. Hematoxylin and eosin histology showed viable and intact hepatocytes inside the spheroids. Immunohistochemistry confirmed sustained hepatocyte function and a preserved hepatocyte-specific cytoskeleton. Albumin, alpha-1-antitrypsin, and urea assays showed continued production during the culture period. Northern blot analysis demonstrated increasing albumin signals. Scanning electron micrographs showed hepatocyte spheroids with relatively smooth undulating surfaces and numerous microvilli. Transmission electron micrographs revealed intact hepatocytes and junctional complexes with coated pits and vesicles inside the spheroids. Therefore, we conclude that primary human hepatocytes, precultured in a flow bioreactor on a PLLA scaffold, reorganize to form morphologically intact liver neotissue, and this might offer an optimized method for hepatocyte transplantation because of the expected reduction of the initial cell loss, the high regenerative potential in vivo, and the preformed functional integrity. *Liver Transpl* 17:104-114, 2011. © 2011 AASLD.

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Abbreviations: bc, bile canaliculi; CK18, cytokeratin 18; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IgG, immunoglobulin G; mRNA, messenger RNA; NADH, reduced nicotinamide adenine dinucleotide; PCNA, proliferating cell nuclear antigen; PLLA, poly(L-lactic acid).

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*These authors contributed equally to this work.

Address reprint requests to Joerg M. Pollok, M.D., Ph.D., Department of Hepatobiliary and Transplant Surgery, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. Telephone: +49-40-7410-58572; FAX: +49-40-7410-45311; E-mail: pollok@uke.de

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Human hepatocyte transplantation could be a possible solution to the organ shortage. The excellent regenerative potential of liver cells has been the motivation for numerous researchers who have focused on hepatocyte transplantation in the last decades.¹⁻¹⁰ Next to liver transplantation, hepatocyte transplantation may offer a valuable therapeutic approach for patients with liver-based metabolic defects or fulminant hepatic failure whose liver dysfunction may resolve as regeneration occurs.¹¹⁻¹⁵ In comparison with liver transplantation, hepatocyte transplantation has the following advantages: the native liver is preserved, and it continues to perform most of its functions in the case of metabolic defects. The native liver has the potential to regain all function in the case of regenerating hepatic failure. Thus, several problems of liver transplantation, such as biliary complications and long-term side effects of immunosuppressive medications, could be avoided once the failed liver has fully recovered. More patients could receive treatment through the better use of available donor tissue or through the use of a patient's own liver as a cell source. If it is necessary, liver transplantation remains an option. As bridging therapy, hepatocyte transplantation could also complement liver transplantation until a suitable donor organ is available for a patient with acute liver failure.^{16,17} The most frequently applied hepatocyte transplantation method, the injection of a hepatocyte solution into the portal blood stream, has the problems of insufficient cell survival and engraftment^{11,18-20} and a restriction on the number of transplantable cells due to the potential complication of portal venous embolization.²¹

In previous studies, we demonstrated that hepatocytes regenerate *in vivo*^{22,23} within a polymer scaffold (up to 10 times the number of the initially surviving 1%-2% of cells²⁴). We further showed that tissue-engineered rat hepatocytes cultivated in a pulsatile flow bioreactor on biodegradable poly(L-lactic acid) (PLLA) polymers²⁵⁻²⁷ form spheroidal aggregates with preserved hepatocyte function and morphology.^{26,28} Such precultured hepatocytes, when they were transplanted on a PLLA polymer scaffold, experienced less initial cell loss. They regained almost 100% of their cell mass 6 months after implantation into pockets of mesenteric leaves in a syngeneic rat model because of strong regeneration activity, which was supported by portocaval shunt stimulation (M.L., unpublished data, 2010).

Encouraged by these findings, we established in this study a method of isolating human hepatocytes from healthy liver resection margins or from human donor livers not used for transplantation. We determined the optimal flow bioreactor culture conditions for human hepatocytes on polymer scaffolds before implantation with the intention of improving the posttransplantation survival rate, regeneration potential, and tissue formation of heterotopically transplanted human hepatocytes.

MATERIALS AND METHODS

Polymer Scaffold Fabrication

Porous PLLA discs were fabricated as previously described.^{27,29} After fabrication, the PLLA discs were

coated with a poly(vinyl alcohol) solution to increase the hydrophilicity. The dried PLLA discs were sterilized with ethylene oxide (H. W. Anderson Products, Haw River, NC). The discs were approximately 18 mm in diameter and 1 mm thick.

Human Hepatocyte Isolation

Human hepatocytes were isolated (1) from human donor livers not suitable for transplantation because of mechanical lesions during organ harvesting, a long ischemic time, or the diagnosis of fatty degeneration or (2) from the discarded parts of donor livers after size reduction for pediatric transplantation. The other source of human hepatocytes was healthy resection margins after partial hepatectomy due to hemangioma or colorectal metastasis. In this case, we used healthy resection margins with a 3-cm security distance to isolate human hepatocytes. This protocol was approved *a priori* by Deutsche Stiftung Organtransplantation and the ethics committee of Ärztekammer Hamburg in accordance with national guidelines and the 1975 Declaration of Helsinki and after informed consent in writing from each patient.

Immediately after resection, the liver tissue was perfused with a cold (4°C) histidine tryptophan ketoglutarate solution.^{30,31} Human hepatocytes were isolated with a 2-step collagenase digestion method, as previously reported.³² A branch of the vena portae was cannulated, and the liver tissue was perfused at 37°C with a 0.05% collagenase solution (Worthington collagenase type I) at a flow speed of 40 to 100 mL/minute (this depended on the size of the liver specimen). The digested liver tissue was placed in a cold buffer with 5 mmol/L calcium dichloride, and the liver capsule was incised. The hepatocytes were mobilized by gentle shaking of the tissue, and the suspension was filtered through a nylon mesh with a pore size of 100 µm. The cell suspension was centrifuged 3 times at 50g for 5 minutes in order to separate the hepatocytes from the nonparenchymal cells. The hepatocyte viability and concentration were determined with the trypan blue test.

Polymer Seeding

The PLLA scaffolds were homogeneously seeded on ice with a 400-µL hepatocyte suspension with a concentration of 10×10^6 cells/mL. This meant that 4×10^6 cells were seeded per polymer. Twenty minutes after seeding, the cell-seeded polymer discs were transferred into the culture chamber of the flow bioreactor system, as previously reported.^{26,27}

Culture Medium and Conditions

Hepatocytes on a PLLA polymer matrix were cultured in serum-free Williams' E medium (without L-glutamine) supplemented with 2 mM *N*-acetyl-L-alanyl-L-glutamine (Biochrom, Berlin, Germany), a 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer

(Biochrom), 4 $\mu\text{g}/\text{mL}$ insulin (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 5 nM dexamethasone (Sigma, St. Louis, MO), 10 ng/mL epidermal growth factor (Gibco BRL), and 1% penicillin/streptomycin (Biochrom). Polymer constructs with 4×10^6 hepatocytes were secured perpendicularly to the flow vector in a Cellmax Quad flow module modified to house the constructs (Cellco Spektrum, Germantown, MD). Four constructs fixed in a flow chamber were exposed to a pulsatile flow of the culture medium (24 mL/minute). The medium (265 mL) was pumped from a 500-mL reservoir bottle with a Cellmax Quad pump apparatus (Cellco), as previously reported.^{26,27} Silicon tubing was used to connect the reservoir to the flow chamber to allow oxygenation and gas exchange. The medium was recirculated back to the reservoir. Two-thirds of the volume of the recirculated culture medium was exchanged every other day during the entire culture period. Every day, medium samples were taken for analysis, and we especially focused on the albumin concentration. On the day of the medium change, the medium samples were taken before and 15 minutes after the medium exchange. The cell polymer constructs were cultured for 1, 2, 4, and 6 days. All cultures were kept in an incubator at 37°C with 5% carbon dioxide supplementation.

Specimen Analysis

Medium samples (5 mL) were taken every day from the flow bioreactor and kept at -80°C until further analysis. After the medium flow was stopped, cell-polymer specimens were extracted from the flow chamber of the bioreactor and transferred into a polystyrene culture dish for further analysis.

Phase Contrast Microscopy

All specimens were viewed by phase contrast microscopy (IX 50, Olympus, Hamburg, Germany) prior to fixation. The density of the hepatocyte spheroids was evaluated as follows. Two investigators counted the number and measured the diameter of the spheroids per 3.8-mm² visual field (20 evenly distributed areas of the specimen were examined) at a magnification of $\times 25$.

Human Albumin Detection

The amount of human albumin secreted into the medium was measured by enzyme-linked immunosorbent assay (ELISA). Standard purified human albumin, goat anti-human albumin immunoglobulin G (IgG) antibodies, and peroxidase-conjugated goat anti-human albumin IgG antibodies were purchased from ICN (Eschwege, Germany). Briefly, 96-well plates (Nunc, Wiesbaden, Germany) were coated with 100 μL (10 $\mu\text{g}/\text{mL}$) of goat anti-rat albumin antibodies in a phosphate-buffered saline buffer. Then, the plate was blocked by the addition of 1% gelatin (Sigma) in distilled water. After 1 hour of incubation at 37°C, the plate was washed, and 100 μL of the appropriately

diluted sample was added to the wells. After 1 hour of incubation, the wells were washed, and 100 μL of a phosphate-buffered saline buffer containing peroxidase-conjugated goat anti-rat albumin antibodies (1:500) was added. After 1 hour, the wells were washed, and a (2,2)-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma) was added. All incubation steps were performed at 37°C. After 45 minutes of incubation in the dark, the reaction was stopped, and the color reaction was measured at 405 nm with a photometer (MR 5000, DynaTech, Overath, Germany). To establish a standard curve, known amounts of purified human albumin (ICN) were used.

Alpha-1-Antitrypsin Assay

The amount of human alpha-1-antitrypsin secreted into the medium was measured by ELISA with an alpha-1-antitrypsin kit (Immundiagnostik AG, Bernsheim, Germany) according to the manufacturer's recommendations; as samples, 100 μL or appropriate dilutions of the cell culture medium were used. The color reaction was measured at 450 nm with a photometer. To establish a 4-parameter standard curve, known amounts of alpha-1-antitrypsin (included in the ELISA kit) were used.

Urea Assay

To monitor the enzymatic activity of the human hepatocytes within the flow culture, the urea production was quantified with the Enzytec Fluid Harnstoff UV quantification kit (R-Biopharm, Darmstadt, Germany). This test is based on the photometric measurement of reduced nicotinamide adenine dinucleotide (NADH) before and after the enzymatic conversion of urea by exogen-added urease. The assay was performed according to the manufacturer's recommendations. Briefly, 2 mL of the frozen cell culture sample was added to a 1-mL buffer containing 0.4 mg of NADH, heated for 15 minutes at 80°C to inactivate potential contamination enzymes, and centrifuged at 5000 rpm to remove cell debris. A urease solution (20 μL) was added, and after the sample was mixed, the first photometric measurement (blank value; 340 nm) was performed. The addition of 20 μL of the glutamate dehydrogenase solution then started the conversion of nicotinamide adenine dinucleotide to NADH. After 20 minutes of incubation at 24°C, a second photometric measurement (340 nm) was performed to determine the amount of NADH and to indirectly determine the amount of urea in the samples.

Northern Blot Analysis for Human Albumin Messenger RNA (mRNA)

Total RNA was extracted from the hepatocytes within the scaffolds with the TriPure isolation reagent (Boehringer, Mannheim, Germany) according to the manufacturer's recommendations. After extraction, the total RNA was size-fractionated by formaldehyde/agarose gel

(1%) electrophoresis (10 µg of RNA per lane) and transferred to a nylon membrane (Hybond-N, Amersham, Little Chalfont, United Kingdom). After the fixation of the RNA to the membrane with UV light, the membrane was prehybridized for 60 minutes at 42°C with DIG Easy Hyp (Boehringer). For the detection of human albumin mRNA, the membrane was hybridized for 14 hours at 42°C with a solution of a labeled 32P complementary DNA probe (50 ng) and Readiprime (Amersham). After hybridization, the membrane was washed 3 times with a 0.1 M sodium chloride citrate buffer and 0.1% sodium dodecyl sulfate at 68°C. A signal was obtained with a phosphor imager (Fujix BAS 2000 TR, Fuji, Japan).

Histology

Specimens were cut into 2 parts, fixed in Bouin's solution, paraffin-embedded, and cross-sectioned. Sections of the specimens were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy. The viability and morphological integrity of the hepatocytes inside the spheroids were evaluated.

Immunohistochemistry and Immunofluorescence

For immunohistochemical analysis, 6-µm cryosections were obtained. The specimens were fixed in Bouin's solution overnight (immunohistochemistry), embedded in a 2% agarose solution (SeaKem LE, FMC Bio-Products, Rockland, ME), kept for 4 hours in an incubator at 60°C, and then frozen in Tissue-Tech optimal cutting temperature compound (Sakura, Zoelerwoude, the Netherlands) at -20°C or directly snap-frozen in the optimal cutting temperature compound at -80°C (immunofluorescence). Albumin and alpha-1-antitrypsin staining was based on the avidin-biotin complex method and the HistoMouse-SP kit (Zymed Laboratories, Inc., South San Francisco, CA). The substrate chromogen was converted by the peroxidase in a positive reaction to a red metabolite. Counterstaining of the nuclei was performed with Gill's hemalaun.

Albumin immunohistochemistry was performed with a monoclonal mouse anti-human albumin IgG antibody as the primary antibody (Sigma; 1:100 dilution and 1-hour incubation). Alpha-1-antitrypsin immunohistochemistry was performed with a prediluted monoclonal anti-human alpha-1-antitrypsin antibody as the primary antibody (Biotrend Chemikalien, Cologne, Germany; 1-hour incubation). The double labeling of proliferating cell nuclear antigen (PCNA; Dako, Glostrup, Denmark; 1:500 dilution and 1-hour incubation) and cytokeratin 18 (CK18; Antibodies-Online, Aachen, Germany; 1:100 dilution and 1-hour incubation) was performed with the EnVision G2 double-staining system (Dako) according to the manufacturer's recommendations. For immunofluorescent staining of cytochrome P450, a primary antibody from MBL International (Woburn, MA; 1:100 dilution and 1-hour incubation) was used. The P450-

specific signal was visualized with an Alexa 555-labeled secondary antibody from Invitrogen (Carlsbad, CA; 1:800 dilution). For immunofluorescent staining of CK18, the primary antibody from Antibodies-Online (1:100 dilution and 1-hour incubation) was used in combination with an Alexa 488-labeled secondary antibody (Invitrogen; 1:400 dilution). For the detection of actin filaments in bile canaliculi (bc), Alexa 488-labeled phalloidin (Invitrogen; 1:50 dilution) was used. Hoechst 33258 (Invitrogen) was applied as a counterstain for viable cell nuclei (1:20,000 dilution and 1-minute incubation) in all of our immunofluorescent staining procedures.

Electron Microscopy

Both for scanning and transmission electron microscopy, the polymer-spheroid specimens were fixed at 4°C in 3% glutaraldehyde in a 0.1 M phosphate buffer. The specimens were washed with 0.1 M phosphate saccharose overnight and postfixed with 1% osmium tetroxide in 0.1 M phosphate saccharose. The specimens were dehydrated through graded series (35%-100%) of ethanol.

For scanning electron microscopy, the prepared specimens were dried with a critical point dryer and placed onto mounting studs. The mounted specimens were coated with gold on a sputter coater (scanning electron microscopy coating system, Bio-Rad, Richmond, CA). The coated specimens were stored in a desiccator until the time of viewing under a scanning electron microscope (DSM 940, Zeiss, Oberkochen, Germany).

For transmission electron microscopy, in order to extract the hepatocyte spheroids from the polymer scaffold, the following procedure was performed. The prepared specimens were incubated twice for 10 minutes in propylenoxide at 4°C, and this was followed by 5 minutes of centrifugation at 1500 rpm. Thereafter, the supernatant was removed. The pellet was incubated for 1 hour in a 1:1 vol/vol mixture of epon (Serva, Heidelberg, Germany) and propylenoxide, and this was followed by centrifugation. This procedure was repeated with incubation in 1:3 vol/vol epon/propylenoxide and then in 100% epon; this was followed by polymerization for 24 hours at 60°C. The polymerized samples underwent ultrathin sectioning with an Ultracut E apparatus (Reichert-Jung, Heidelberg, Germany), were stained with uranyl acetate and lead citrate, and then were viewed under a transmission electron microscope (CM 100, Philips, Kassel, Germany).

RESULTS

Formation of Spheroidal Hepatocyte Aggregates

Human hepatocytes were isolated from 12 human liver specimens (8 donor livers and 4 resection specimens) with a yield of 6.6×10^6 to 6.7×10^8 cells per liver specimen and a viability rate of $81.6\% \pm 11.6\%$ (Table 1). Already after 2 days, hepatocytes formed

TABLE 1. Isolation of Human Hepatocytes From Rejected Donor Livers and From Healthy Resection Margins After Partial Hepatectomy

Specimen	Cell Yield ($\times 10^6$)	Viability (%)
Donor liver	500	90
Resection margin	6.6	70
Donor liver	100	80
Donor liver	500	80
Donor liver	11	98
Donor liver	670	75
Resection margin	180	87
Donor liver	15	60
Resection margin	216	89
Donor liver	150	82
Donor liver	75	70
Resection margin	250	98
Average \pm standard deviation		81.6 \pm 11.6

tightly packed cellular aggregates with smooth boundaries. These spherical aggregates, called spheroids, had diameters ranging from 50 to 250 μm and acquired a liver-like morphology (Fig. 1A) during the culture periods of 2, 4, and 6 days. The human hepatocyte spheroids were homogeneously distributed and occupied the pores within the entire volume of the 3-dimensional porous structure of the PLLA polymer. The number and diameter of the spheroids within the polymer constructs were evaluated by the observation of 20 evenly distributed 3.8-mm² visual fields. This sampling method covered approximately 30% of the entire polymer surface. On day 1, there was no spheroid to observe. From day 2 to day 4, the average number of spheroidal cell aggregates doubled from 17.55 ± 9.27 to 40.90 ± 19.72 per visual field. Up to day 6, there was a further increase to 45.05 ± 13.80 spheroids per visual field. The average diameter of the spheroids increased from $129 \pm 43 \mu\text{m}$ on day 2 to $161 \pm 43 \mu\text{m}$ on day 6.

Active Production and Accumulation of Human Serum Albumin in Flow-Cultured Hepatocytes

On day 1, the albumin concentration in the culture medium (265 mL) was $155 \pm 62 \text{ ng/mL}$. This meant that 16×10^6 human hepatocytes, seeded on 4 polymer scaffolds, produced $41.3 \pm 16.4 \mu\text{g}$ of albumin within the first 24 hours of flow culture. This corresponded to a value of $0.11 \pm 0.04 \text{ pg/cell/hour}$ (calculated per seeded cell). The maximum daily increase of $1135 \pm 408 \text{ ng/mL}$ was observed on day 5 of the flow culture. This represented $302.4 \pm 108.7 \mu\text{g}$ per 16×10^6 seeded hepatocytes in 24 hours or $0.79 \pm 0.28 \text{ pg/cell/hour}$. The average daily increase in the albumin concentration was $418 \pm 295 \text{ ng/mL}$, $111 \pm 78 \mu\text{g}$ per 16×10^6 hepatocytes in 24 hours, or $0.29 \pm 0.20 \text{ pg/cell/hour}$ (still calculated per seeded hepatocyte; Fig. 2A). In order to verify that the albumin

detected with ELISA resembled the active production of albumin by human hepatocytes, we performed northern blot analysis for human mRNA. Signals for human albumin were detectable at every time point: right after isolation (day 0) and after 1, 2, 4, and 6 days of flow culture on the PLLA polymer. The mRNA signal decreased from day 1 to day 2 but thereafter increased continuously. On day 4, the mRNA signal was already higher than it was on day 0 at the time of cell isolation, and it continued to increase until day 6. This indicated differentiated cell function and active metabolism of the flow-cultured primary human hepatocytes on the PLLA polymer scaffolds (Fig. 2B).

Active Production of Human Alpha-1-Antitrypsin in Flow-Cultured Hepatocytes

Alpha-1-antitrypsin production, determined by ELISA, revealed a similar pattern of increasing culture medium concentrations during the entire culture period of 6 days. On day 1, the alpha-1-antitrypsin level in the culture medium (265 mL) was $73 \pm 25 \text{ ng/mL}$. The maximum daily increase of $112 \pm 84 \text{ ng/mL}$ was observed on day 6 of the flow culture. The average daily increase in the alpha-1-antitrypsin concentration was $79 \pm 45 \text{ ng/mL}$ (Fig. 3A).

Urea Production as an Indication for Hepatocellular Mitochondrial Activity

Urea production, which was measured on every other day of the flow culture, remained stable for the 6-day culture period. On day 1, the urea concentration in the culture medium (265 mL) was $1 \pm 0.2 \text{ mg/L/scaffold/day}$, and it remained almost constant at similar levels throughout the 6-day culture period. This indicated that the mitochondrial activity of the flow-cultured hepatocytes was maintained (Fig. 3B).

Function and Architecture of Human Spheroids Determined by Immunohistochemistry, Immunofluorescence, and Electron Microscopy

H&E histology showed spheroids of human hepatocytes at every observation time point from day 2 onward. The diameter varied between 50 and 250 μm . The histological examination showed viable and intact human hepatocytes inside the spheroids (Fig. 1B). During the immunohistochemical staining process, the polymer scaffolds dissolved, and this led us to the special agarose embedding technique. The homogeneous albumin, alpha-1-antitrypsin, and CK18 contents of the spheroid-forming human hepatocytes in the flow culture were observed at every observation point. Albumin (Fig. 1C) and alpha-1-antitrypsin signals (Fig. 1D) by immunohistochemistry and CK18 (Fig. 4C) and cytochrome P450 (Fig. 4B) by immunofluorescence after 6 days of flow culture were displayed. Although the main mass of the spheroids was formed by aggregation of the hepatocytes within the PLLA polymer, PCNA staining (Fig. 4A) revealed that a small fraction of the

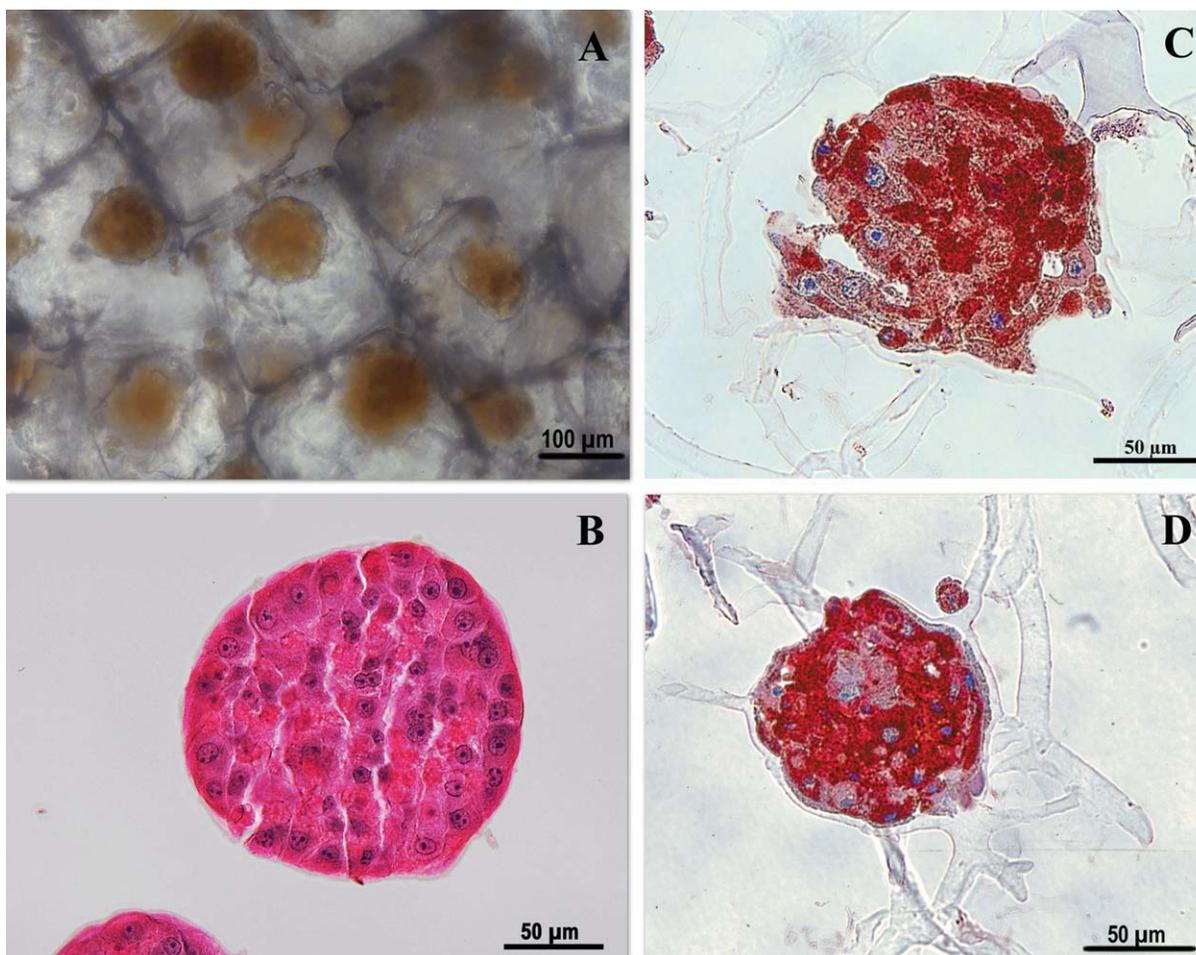


Figure 1. Morphological analysis of human hepatocyte spheroids. (A) Phase contrast microscopy image of human hepatocytes on a PLLA scaffold after 4 days of flow culture. The hepatocytes formed spheroids within the pores of the scaffold (original magnification $\times 25$). (B) Light microscopy section and H&E stain of a human hepatocyte spheroid after 4 days of flow culture (diameter = 165 μm). Vital hepatocytes appeared throughout the spheroid. Immunohistochemistry for (C) human albumin and (D) human alpha-1-antitrypsin on a cryosection of flow-cultured human hepatocytes after 6 days. The hepatocytes displayed a homogeneously positive signal (dark red) for albumin and alpha-1-antitrypsin within the entire spheroid (diameter = 100 μm). During the staining procedure with the cryo-embedding protocol, the agar structures remained intact.

hepatocytes (CK18 positive cells) within the spheroids actively divided even after 6 days of culture.

Scanning electron micrographs indicated that primary human hepatocytes cultured in the flow bioreactor formed spheroidal aggregates inside and next to the cuboidally shaped pores of the PLLA polymer scaffold already after 2 days of culture (Fig. 5A). At an even higher magnification, the boundaries of the individual hepatocytes as well as the lawn of fine microvilli on their surface could be visualized. Individual cells were distinguishable from one another. Pores and gaps between the hepatocytes on the outer surface of the spheroids could be found, especially when 3 hepatocytes were adjacent to one another.

Transmission electron micrographs revealed intact hepatocytes and extensive cell-cell contact inside the spheroids. The cellular components of the spheroid-forming hepatocytes showed typical ultrastructural characteristics of polarization of the aggregated human hepatocytes. This included a bc network with

the formation of microvilli and the detection of fully differentiated junctional complexes (Fig. 5A,B). It was difficult to distinguish whether the gaps and pores between adjoining hepatocytes on the surface of the spheroids represented bc or intercellular spaces formed by lateral cell surfaces because both structures are rich in microvilli (Fig. 5B).

DISCUSSION

Although the number of liver transplants slowly increases year by year, the number of patients waiting for liver transplantation does not seem to decrease. Classic organ transplantation is not sufficient to meet the rising demand and has stimulated clinicians and researchers to focus on alternative treatments to restore lost liver function.^{9,10,33-38} Tissue engineering and the use of human hepatocyte cell implantation systems such as biodegradable polymer matrices could be possible solutions to the organ shortage.³⁹

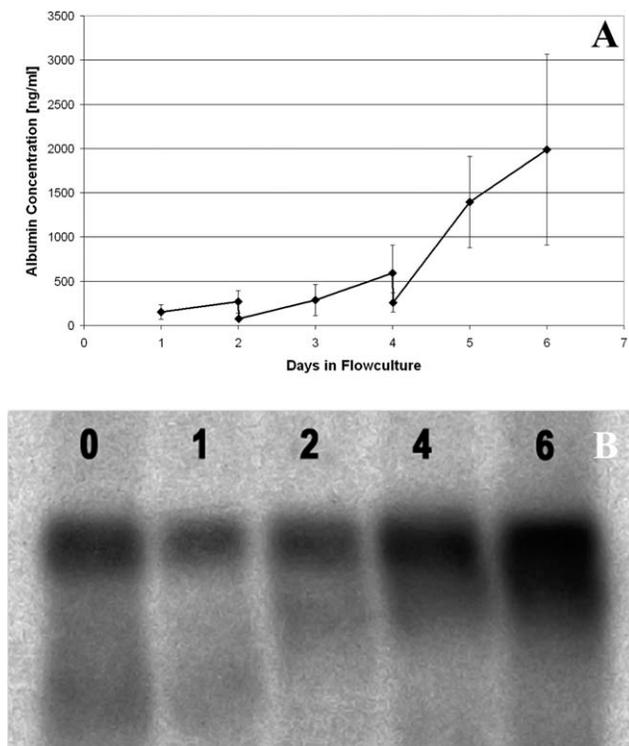


Figure 2. Albumin and albumin mRNA production. (A) On a daily basis, medium samples were taken from the flow bioreactor system. On days 2 and 4, two-thirds of the culture medium was exchanged. The lower values on these days represent the albumin concentration directly after the medium exchange. The albumin production of 16×10^6 human hepatocytes seeded on PLLA scaffolds was measured by ELISA. The rates of albumin biosynthesis increased during the entire culture period. The error bars represent 1 standard deviation. (B) Northern blotting for human albumin mRNA. The signal for human mRNA increased during the culture period. On days 4 and 6, the signal surpassed the value of freshly isolated human hepatocytes (day 0).

Hepatocyte proliferation and expression of differentiated function can be regulated by the extracellular matrix.⁴⁰⁻⁴⁵ It furthermore serves as a skeleton of the newly formed liver tissue after transplantation.

We found in our previous experiments that rat hepatocytes forming spheroidal aggregates on PLLA polymer scaffolds showed preserved hepatocyte function in flow cultures and reduced initial cell loss as well as strong cell regeneration after transplantation *in vivo*.^{26,27} Therefore, hoping for human liver neotissue formation, we were encouraged to use flow cultures for freshly isolated human hepatocytes on PLLA polymer matrices.

We successfully established an isolation method for human hepatocytes with a viability level of $81.6\% \pm 11.6\%$, which is high and comparable to the levels of other laboratories.⁴⁶⁻⁴⁸ We made an observation similar to others' observations: a greater relative yield of hepatocytes with high viability was obtained from smaller specimens of liver tissue versus whole livers or larger liver lobes.^{47,49}

During a pulsatile flow bioreactor culture, human hepatocytes formed multicellular aggregates (spher-

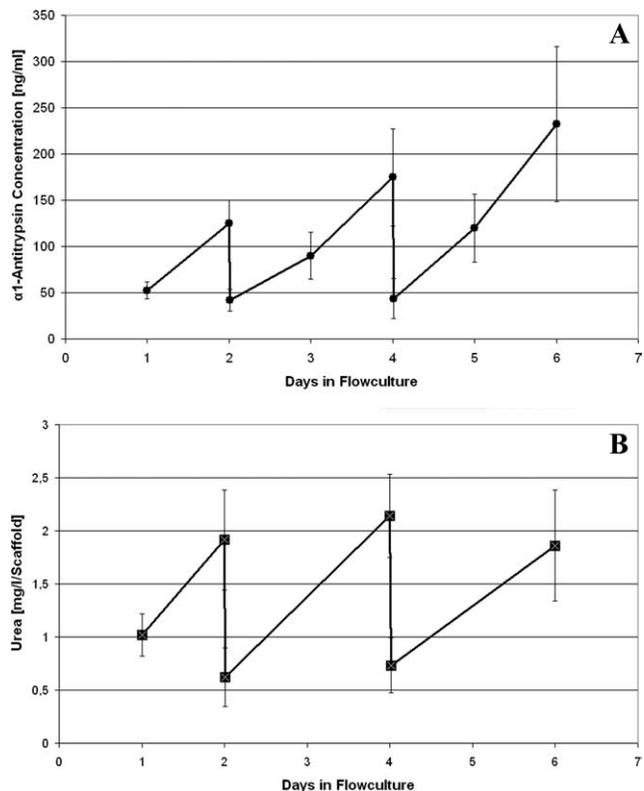


Figure 3. Alpha-1-antitrypsin secretion and urea production. (A) Alpha-1-antitrypsin secretion, determined by ELISA, increased during the entire culture period of 6 days. (B) Urea production remained stable until the last culture day. The results of both tests demonstrated the presence of highly differentiated and functional human hepatocytes on PLLA scaffolds after 6 days of flow culture.

roids) that occupied the pores within the entire volume of the 3-dimensional porous structure of the PLLA polymer scaffolds. Several research groups have found spheroids of human hepatocytes to be an advantageous formation for the preservation of hepatocyte-specific functions and morphological features in culture.⁵⁰⁻⁵² Other groups have used human hepatoma cell lines cultured on poly(d,l-lactic-co-glycolic acid)⁵³ or chitosan-collagen textile polymer scaffolds⁵⁴ and have achieved the formation of functionally active spheroids. All functional tests performed in this study proved the preservation of hepatocyte-specific function and morphology. The albumin production ability of the hepatocytes forming spheroids was demonstrated and measured with the ELISA test. Throughout the 6-day culture period, a high level of albumin biosynthesis was detected at relatively constant rates in our serum-free culture medium (Fig. 2A). The calculated albumin production levels per cell certainly underestimated the real production because they were calculated per seeded cell. We compared these albumin production levels to the human *in vivo* situation. A normal human liver produces 2.2 mg of albumin/kg of bodyweight/hour.⁵⁵ A normal 75-kg patient has a liver of approximately 1600 g, and each gram contains 139 million hepatocytes.⁵⁶ These data allow

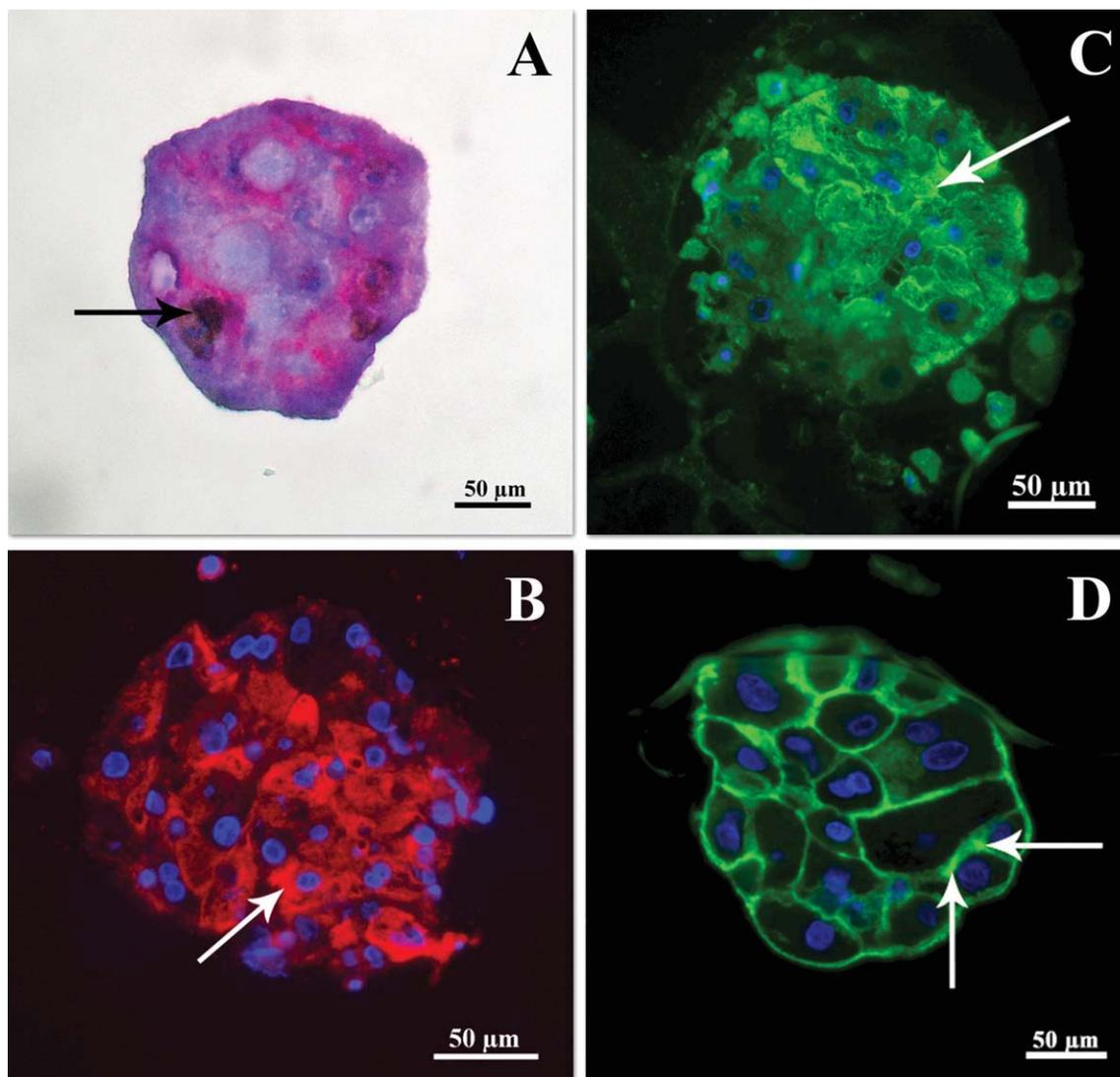


Figure 4. Morphological analysis of human hepatocyte spheroids. (A) PCNA immunohistochemistry of human hepatocytes forming spheroids after 6 days of flow culture for the detection of proliferation. Positive staining (brown) of some single cells within the spheroids was observed, but most of the cells remained negative. (B) Immunofluorescent staining of cytochrome P450 (red) revealed positive cells after 6 days of culture; this indicated the ability to metabolize toxic substances. (C) Immunofluorescent staining of CK18-positive cells (green) demonstrated an intact cytoskeleton inside the spheroids after 6 days of flow culture. (D) For the detection of actin filaments in bc, Alexa 488-labeled phalloidin (green) was used. Re-formation of bc between the adjacent hepatocytes was observed (white arrows), and a liver-like fine structure within the spheroids was displayed after 6 days.

us to calculate the *in vivo* albumin production per hepatocyte per hour in a normal liver as follows:

$$\begin{aligned}
 &2.2 \text{ mg of albumin/kg of body weight/hour} \\
 &\quad \times 75 \text{ kg of body weight} = 165 \text{ mg of albumin/hour} \\
 &165 \text{ mg of albumin/hour} \div 1600 \text{ g of liver} \\
 &\quad = 0.103 \text{ mg of albumin/g of liver/hour} \\
 &0.103 \text{ mg of albumin/g of liver/hour} \div 139 \\
 &\quad \times 10^6 \text{ hepatocytes/g of liver} \\
 &\quad = 0.742 \text{ pg of albumin/hepatocyte/hour}
 \end{aligned}$$

We can, therefore, postulate that our flow-cultured human hepatocytes produced similar quantities of albumin per cell per hour in comparison with the *in*

vivo situation with an average daily production of 0.29 ± 0.20 pg/cell/hour and a maximum daily production of 0.79 ± 0.28 pg/cell/hour.

Not all seeded cells will attach, aggregate, and continue to produce albumin. The increasing levels of albumin production per day with the culture period progressing were also reflected by a northern blot analysis of human mRNA, which demonstrated increasing mRNA expression levels until day 6 (Fig. 2B).

Alpha-1-antitrypsin production and urea production were investigated as additional markers for hepatocyte-specific function. They showed similar concentration patterns with continued activity levels throughout the 6-day culture period (Fig. 3A,B). Maintained urea production was a marker of sustained specific mitochondrial activity of the cultured hepatocytes.

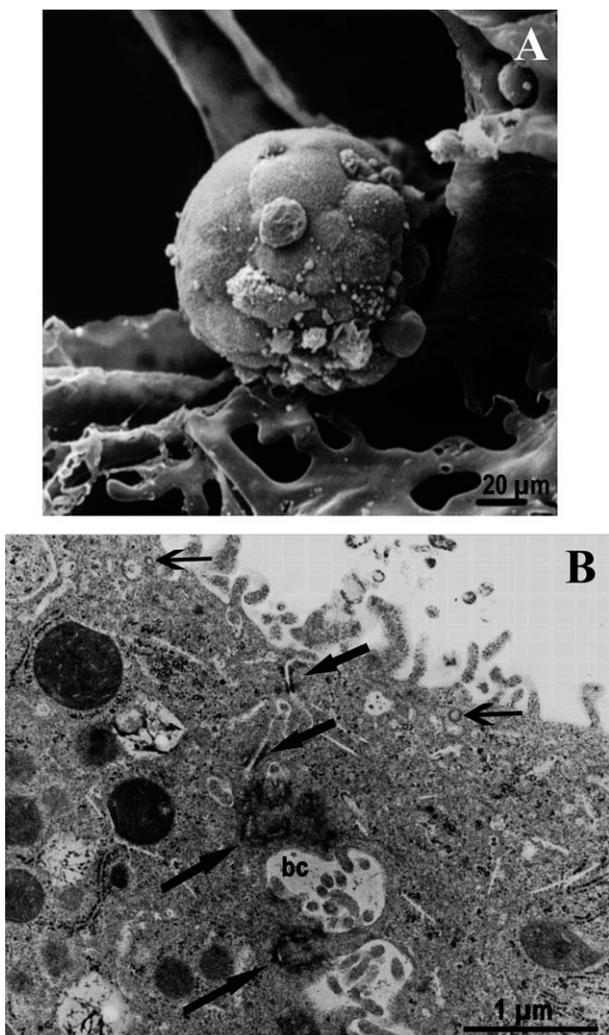


Figure 5. Electron microscopy images. (A) Scanning electron micrograph of human hepatocytes on a PLLA scaffold after 6 days of flow culture. A spheroidal aggregate of hepatocytes can be seen within a pore of the PLLA polymer scaffold (approximately 120 μm in diameter). The borders of individual hepatocytes can be discriminated. The fine stippling of the cell surface corresponds to numerous microvilli. (B) Transmission electron micrograph of a human hepatocyte spheroid on a PLLA polymer scaffold after 4 days of flow culture. Adjacent hepatocytes formed bc-like structures with numerous microvilli, which opened in pores on the outer surface of the spheroid. They were sealed off by junctional complexes (thick arrows) that formed between adjacent hepatocytes. Coated pits and vesicles (thin arrows) were signs of receptor-mediated micropinocytosis.

Immunohistochemical staining was positive not only for human albumin but also for alpha-1-antitrypsin, cytochrome P450, and CK18 (Figs. 1C,D and 4B,C), all of which are hepatocyte-specific markers; this provided further evidence of preserved human hepatocyte function and a hepatocyte-specific cytoskeleton. The positive cytochrome P450 staining indicated functioning hepatocyte-specific phase I metabolism (Fig. 4B).

Scanning and transmission electron microscopy showed evidence of preserved human hepatocyte morphology and interactive cell-cell contact. The hepato-

cytes showed a normal cell architecture and were covered by numerous microvilli. They showed typical ultrastructural characteristics of polarization. Adjacent, spheroid-forming hepatocytes displayed fully differentiated junctional complexes resembling a bc network. Interestingly, the formation of mature bc between the hepatocytes was further confirmed by positive staining of actin filaments by phalloidin (Fig. 4D). In vivo, in the human liver, actin filaments specifically surround bc and are believed to support the flow of bile (produced by the hepatocytes) in the direction to the bile ducts.

Our experimental model represents a promising tool for culturing human hepatocytes and preparing them for transplantation on a biodegradable polymer scaffold into the peritoneal cavity. With these strategies, a sufficient hepatocyte cell mass could be implanted into a recipient to support liver function and hence to correct, at least in part, his underlying liver disease. This could overcome technical problems related to cell injection procedures as well as the restricted number of transplantable cells. Hepatocytes transplanted on a polymer scaffold would already have had the chance to settle and aggregate with one another, and this might give them a survival and functional benefit in comparison with separated hepatocytes implanted via injection.

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