0273-2289/95/5401-03—0065$09.40

A Stereotypic, Transplantable Liver Tissue-Culture System

BRIAN A. NAUGHTON,* BENSON SIBANDA, JORY P. WEINTRAUB, JULIA SAN ROMÁN, AND VAFA KAMALI

Hematology Laboratory, Advanced Tissue Sciences Inc., LaJolla CA 92037

ABSTRACT

A method of coculturing adult rat hepatic parenchymal cells (PC) and stromal cells in a three-dimensional framework of nylon filtration screens or biodegradable polymer meshes was developed in our laboratory. Rat liver stroma, which includes vascular and bile duct endothelial cells, fat-storing cells, fibroblasts, and Kupffer cells, were isolated by gradient centrifugation after *in situ* liver perfusion and expanded in monolayer culture prior to seeding onto nylon screens or bioresorbable polyglycolic acid (PGA) polymers oriented into a felt-like construct. A second inoculum of freshly isolated PC was applied after the stromal cells became established. Histological analyses revealed that PC proliferation occurred until all available space for expansion within the template was exhausted. These cells retained their rounded morphology, and after 4–5 wk 7–9 "layers" of PC filled the 140-μm deep template. Dioxin-inducible cytochrome P450 activity was detected for up to 58 d in culture, and albumin, fibrinogen, transferrin, and soluble fibronectin were detected in the medium by enzyme-linked immunosorbent assay (ELISA) for 48 d in vitro. Immunohistochemical analysis of sections through the cultures confirmed the presence of these proteins as well as cytokeratin at the cellular level; the extracellular matrix stained for both collagen type III and laminin. Long-term PC proliferation and function were enhanced by the presence of stromal cells as well as by a meshwork template whose geometry allows the interaction of PC with stroma and matrix on several different planes. To permit transplantation, cocultures of hepatic PC and stromal cells were established on PGA felt constructs instead of nylon screens. After ~24 d in vitro, these con-

*Author to whom all correspondence and reprint requests should be addressed.

structs were grafted into sites in the mesentery, omentum, and subcutaneous tissues of adult Long-Evans rats. The growth of hepatocytes after 30 d *in situ* was evident by histological analysis; grafts of cocultures regenerated a liver-like architecture consisting of sinusoids and putative biliary structures. In addition, PC at these extrahepatic graft sites were positive for albumin, transferrin, and fibrinogen synthesis by immunohistochemistry. Graft survival was enhanced when recipients were subjected to ~40% hepatectomy. Hepatic PC:stromal cell cocultures may prove useful in the restoration of liver function either by direct transplantation using PGA or similar templates, or as extracorporeal devices, using nylon screens.

Index Entries: Albumin; cytochrome P450; fibrinogen; hepatic parenchymal cells; stromal cells; three-dimensional culture; bioresorbable polymers.

Abbreviations: cP450, cytochrome P450; ECM, extracellular matrix EFEE, ethoxyfluorescein ethyl ester; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FLS, forward-angle light scatter; PBS, phosphate-buffered saline; PC, parenchymal cells; PGA, polyglycolic acid; PTFE, polytetrafluoroethylene; SEM, standard error of the mean; SFM, serum-free medium; SS, side or 90° light scatter; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

INTRODUCTION

Hepatic parenchymal cells (PC) function in vitro only in association with extracellular matrix (ECM) substances or with stromal cells of hepatic or nonhepatic origin. Liver-specific activities, such as albumin synthesis and cytochrome P450 (cP450) enzyme activity, are evident for only short periods in suspension or monolayer cultures of hepatocytes, but can be prolonged if PC are inoculated into flasks or membranes coated with type I collagen (*1,2*), homogenized liver matrix (*3*), type IV collagen, laminin (*4*), fibronectin (*5*), or other ECM proteins. A similar enhancement of hepatocyte function in vitro was observed when PC were cocultured with nonparenchymal hepatic cells or various types of nonhepatic stromal cells. In this regard, both human and rat hepatocytes that were cocultured with liver endothelial cells of the same species displayed active albumin synthesis for several weeks in culture (*6,7*). In addition, rat hepatocytes that were cocultured with human fibroblasts (*8*) or endothelial cells (*7*) were reported to sustain cP450 activity for more than 10 d. Even though hepatic PC function in vitro has been improved by these methods (*1–8*), the gains have been, for the most part, marginal. Thus, hepatocytes cultured on plates coated with collagen failed to express cP450 activity by the fifth day in vitro (*9*), and albumin synthesis in a similar system was quantifiable for <7 d (*14*). In contrast, when hepatocytes were cultured between two layers of collagen, albumin production

was still evident by 6 wk, although at levels essentially identical to those quantities secreted by input cells (*2*). Likewise, Sudhakaran et al. (*10*) reported that albumin production by hepatocytes declined by 86, 55, and 48%, respectively, over a 4 d period when cultured in dishes coated with laminin, fibronectin, or collagen. When cP450 activity was the basis of measurement, cocultures of rat liver cells and human fibroblasts were superior to hepatocyte culture in collagen-coated flasks although expression of this enzyme system declined by >80% within 5 d of culture (*9*). Similarly, measurement of protein synthesis in cocultures of rat hepatocytes and human 3T3 cells by ^{35}S pulse chase followed by immunoprecipitation revealed that albumin synthesis declined by 65% by day 8 and by >80% after 28 d of culture (*8*). When Guguen-Guillouzo et al. cultured hepatocytes on a pre-established monolayer of adherent liver-derived cells, albumin synthesis peaked by ~300% over that of input cells by day 10, but was basically identical to input levels after 6 wk of culture (*6*). Although this experiment clearly demonstrated the superiority of hepatic-derived feeder cells over nonhepatic cells to prolong PC function in vitro, it was not clear to what extent the pre-established adherent layer directly contributed to albumin production.

The decline in protein production/enzyme activity with increasing time of culture and the general failure of PC to proliferate beyond the first few days in vitro may be attributed in part to the template on which cells are inoculated. In monolayer culture, hepatocytes that adhere to plastic spread rapidly, proliferate for several days, and lose their ability to divide, manufacture albumin and other proteins, and to metabolize drugs via cP450 pathways (*1,4,5*). These cells also retain their flattened configurations and may display dysplastic growth patterns that have been described by some investigators as dedifferentiation (*11*). In contrast, if hepatocytes are seeded onto ECM-coated flasks or onto established feeder cells, they retain their rounded shape and continue to function as liver cells, but proliferation is limited or absent in most of these culture systems for reasons that remain obscure. Adult liver is a mitotically quiescent organ under normal conditions, but has the potential to regenerate after subtotal hepatectomy or partial chemical destruction (*12,13*). The rapidity of this response (rats recover most of the liver mass lost to ~85% hepatectomy within 96 h [*13*]) indicates that the liver contains a population(s) of cells with a high degree of proliferative capacity if the appropriate conditions are met. These conditions have clearly not been mimicked by existing culture systems for hepatocytes. We chose nylon filtration screens as templates for our liver coculture system for several reasons: the surface area for cell growth is approximately seven times that for flask-adherent monolayer cultures, stromal cell deposition of matrix is favored over stromal cell proliferation on this template, PC attach to stromal cell processes and grow in a stereotypic manner, and since the culture is suspended in liquid medium, nutrient access is maximized. The

characterization of this culture system, its adaptation to bioresorbable PGA templates, and the direct grafting of liver tissue that was bioengineered in vitro are described in this article.

MATERIALS AND METHODS

Hepatic Cell Isolation and Culture

Male Long-Evans rats (6–9 wk of age) were anesthetized with sodium pentobarbital and subjected to a series of prograde perfusions through the portal vein using: Ca^{2+}-free buffer (500 mL) (*14*), buffer containing Ca^{2+} and 0.05 g/dL type IV collagenase (100 mL) (Sigma Chemical Co., St. Louis, MO), and buffer conditioned with 10% fetal bovine serum (FBS) (50 mL). Medium was perfused at a flow rate of 50 mL/min using a Harvard Instruments (MA) peristaltic pump. Hepatocytes were liberated into suspension, filtered through a 185-μm nylon sieve, collected by centrifugation, and resuspended in complete medium.

Hepatic cells were separated into various subpopulations using either a two-step Percoll gradient centrifugation (*15*) or a preformed continuous gradient. Briefly, neat Percoll stock solution was diluted to 70% (v/v) with 1X Dulbecco's phosphate-buffered saline (DPBS). The cell suspension was overlaid and spun at 1200*g* for 15 min at 10°C in an IEC swinging bucket centrifuge to remove cellular debris and erythrocytes. The remaining cells were resuspended in DPBS, layered atop a 25:50% discontinuous Percoll gradient, and centrifuged as in the first step. Alternatively, a 30% Percoll solution was centrifuged at 30,000*g* for 30 min in a Sorvall RC-5B centrifuge with a 20° fixed-angle rotor to form a continuous gradient. Suspensions of hepatic cells were overlaid and centrifuged at 200*g* for 15 min at 10°C. The densities of the various isolation zones were determined using density marker beads, and cytosmear preparations of cells of each zone were stained with Diff-Quik (Baxter, IL).

Nylon filtration screens (3-210/36, Tetko, NJ) were cut into 15 × 60 mm pieces, treated with 1.0*M* acetic acid, washed in distilled water, and soaked in FBS to enhance cellular attachment. The screen itself was composed of nylon fibers that were 90 μm in diameter and arranged into a square-weave pattern with openings of 140 × 140 μm so that the total volume for cell growth within each of these spaces was $\sim 1.8 \times 10^6$ μm^3. Screens were placed in Tissue Tek slide chambers (Nunc, Inc., IL) and inoculated with 10^7 liver stromal cells that had been expanded in monolayer culture through three to four subcultivations. Screens were transferred to 25-cm^2 flasks 18–24 h later. Within 10 d, projections of developing stromal cells extended across three to four of every five mesh openings. Screen cultures were placed in slide chambers, inoculated with 2–5 × 10^6 hepatic PC or acidophilic cells, and transferred to 25-cm^2 flasks after 18–24 h.

Cells were cultured in an incubator (5% CO_2/35–37°C/ > 90% humidity) in DMEM conditioned with 6% FBS and 10% equine serum and supplemented with 10 ng/mL glucagon, 10 μg/mL insulin (Sigma Chemical Co.), and $10^{-7}M$ hydrocortisone hemisuccinate. Glucose concentration was adjusted to 4.5 mg/mL. Complete medium replacement was performed 4–5 times/wk.

Analytical Methods

Medium collected at each feeding was assayed for rat albumin using an enzyme-linked immunosorbent assay (ELISA). Reagents were purchased from Cappel Inc. (NC). One hundred microliters of spent medium were added to 96-well plates and stored at 4°C for 12–14 h. The wells were washed with 0.05% Tween-20 in PBS, and nonspecific binding sites were blocked with 0.5% bovine serum albumin (BSA) (Miles Inc., IL) in PBS. After washing with 0.05% Tween-20, 100 μL of peroxidase-conjugated sheep antirat albumin were added to each well and incubated (1 h at 22°C). The wells were washed with 0.05% Tween-20 and incubated for 15 min with *o*-phenylenediamine substrate. The reaction was stopped, and absorbance at 490 nm was measured with a kinetic microplate reader (Molecular Devices Inc., CA). Results were calculated from a standard curve constructed using chromatographically pure rat albumin. Because of the lack of suitable species-specific antibodies for rat fibrinogen, fibronectin, and transferrin, liver cultures were transferred to serum-free medium (SFM) for 24 h prior to collection of supernatant samples. We modified the SFM formulation of Enat et al. (*16*) by supplementing with hydrocortisone hemisuccinate (50 μg/mL), fungizone (0.5 μg/mL), penicillin (5 U/mL), and streptomycin (5 μg/mL). After 24 h in SFM, cultures were returned to complete medium, and samples were stored at −20°C until assayed. SFM was used as a negative control for these ELISAs, since it lacked proteins that could potentially crossreact with the monoclonal antibodies (MAb). Standard curves were established with affinity-purified proteins (rat fibrinogen [Sigma], rat fibronectin [Chemicon], or rat transferrin [Cappel Inc.]) at a concentration range of 7.8125 ng/mL to 1 μg/mL. All supernatant samples were tested undiluted, in quadruplicate. Fifty microliters of control, standard, or supernatant were added to each well of a 96-well plate and incubated 15 h at 4°C. Wells were blocked with 0.5% BSA in PBS for 1 h at 37°C and washed with 0.05% Tween-20 in PBS. Fifty microliters of peroxidase-conjugated antibodies (antihuman fibrinogen, antihuman fibronectin, or antirat transferrin [The Binding Site, Ltd., CA]) were added to each well, incubated for 1 h at 37°C, and washed with PBS/Tween-20. Fifty microliters of K-Blue peroxidase substrate (Elisa Technologies, KY) were added to each well. Plates were incubated at ~35°C for 10 min, in the dark, and read on a Dynatech plate reader at 650 nm.

Phenotypic analysis was accomplished by flow cytometry. Freshly isolated liver cells and cells derived from liver cultures were reacted on ice with 100 μL mouse monoclonal IgG_1 polymorphic antibodies to either rat MHC I or MHC II antigens, which were conjugated to fluorescein isothiocyanate (FITC) (Serotec Inc., UK). The samples were analyzed using an EPICS C flow cytometer (Coulter Electronics, FL) tuned to a wavelength of 488 nm. The fluorescence gain was adjusted so that the lower threshold excluded ≥98% of control cells that were treated with mouse IgG_1-FITC alone. Positive fluorescence was defined as the difference between the percentages of experimental and control cells exceeding this lower threshold. Windows were established around the various cell populations using the forward-angle light scatter (FLS) vs side-scatter (SS) two-parameter histogram, and the percentage of positively fluorescent events was determined.

Flow cytometry was also employed to measure cP450 enzyme activity in cultured cells by quantifying incremental fluorescein fluorescence in cells accumulating ethoxyfluorescein ethyl ester (EFEE) (*17,18*). EFEE-to-fluorescein conversion occurs via the specific cleavage of an ether linkage by a polycyclic aromatic hydrocarbon (PAH)-induced cP450 (*17*). As with other cP450-catalyzed reactions, EFEE metabolism requires NADPH, and can be inhibited by carbon monoxide or MAb that decrease PAH or benzo (a)pyrene metabolism (*17*). At 18 h prior to cP450 assay, cells were induced with 1-n*M* solution derived from a 1-μ*M* stock solution of the nonfluorescent compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Chemical Carcinogen Repository, National Cancer Institute, Kansas City, MO) in dimethylsulfoxide (DMSO) (Sigma Chemical Co.). Cultured cells were lifted using a dispase–collagenase mixture, centrifuged, and resuspended in PBS at a density of $\sim 5 \times 10^5$ cells/mL, stored on ice for 1 h, and gradually warmed to 37°C. Cells were incubated with 50 n*M* EFEE (Molecular Probes, OR) in PBS for 5 min at 37°C, and green fluorescence was quantified on an EPICS C flow cytometer equipped with a 515-nm long-pass filter and tuned to the 488-nm band. Fluorescence resulting from EFEE-to-fluorescein conversion was gated on various populations of cells based on differences in FLS vs SS characteristics, and data were collected once/min for up to 25 min in samples maintained at 37°C. Flow cytometry measurements were taken in triplicate on sample sizes of 5000 (phenotypic analysis) events. EFEE-to-fluorescein conversion was measured as a function of time with 3000–5000 events being sampled/min for up to 20 min. All results are expressed as $x \pm 1$ SEM. Levels of significance (P) were determined using Student's *t*-test. Data were considered significant at the 5% level.

Total nonadherent and adherent-zone cell counts were determined using a Coulter Model ZM cell counter. Differential cell counts were based strictly on the morphology of cells stained using Diff-Quik (Baxter, SP, IL). Phagocytosis of colloidal carbon and reaction with FITC-conjugated antibodies to the vW factor VIII segment were used initially to identify

Kupffer cells and vascular endothelial cells, respectively. Radiothymidine incorporation into DNA was determined by incubating the cultures with tritiated thymidine (1 μCi/mL) for 24 h at 37°C, 5% CO_2, and >90% humidity for 24 h. Cells were removed from the template by digestion with a collagenase/dispase solution (as described earlier in this section), and trichloroacetate-precipitated DNA/protein was entrapped in glass fibers. Radiolabeling was quantified using a BetaPlate scintillation counter (Beckman Instruments, CA).

Specimens for immunochemical evaluation were fixed with 10% formalin in PBS, dehydrated in a graded series of ethanols, cleared with Hemo-De (Fisher Scientific, NJ), embedded in paraffin at 56°C, and sectioned at 5 μm. Sections were cleared in xylene, rehydrated in a series of ethanols, and predigested with 0.5 mg/mL bovine testicular hyaluronidase in 0.1*N* sodium acetate-acetic acid buffer at pH = 6.0 (Sigma Chemical Co.). Slides were blocked with a solution of 4% goat serum, 0.1% BSA, and 0.1% Tween-20 in 0.1*M* NaCl. Polyclonal rabbit primary antibodies to human fibronectin, collagen type III, laminin (Telios Pharmaceuticals, CA), transferrin, fibrinogen, or rat albumin (Cappel Inc.) were reacted with the sections for 1–3 h at 22°C, washed in 0.1% Tween-20 in 0.1*M* NaCl, placed in Tris buffer for 10 min, and labeled with goat antirabbit IgG-FITC for 30 min at 22°C prior to viewing on a Nikon Epifluorescence microscope. Enzyme-digested serial sections also were reacted with MoAB to rat cytokeratin 19 (Chemicon International Inc., CA) for 90 min at 37°C, washed, and incubated with sheep antimouse IgG-FITC for 30 min. Photographs were obtained using a Nikon Optiphot fluorescence microscope.

In Vivo Grafting of Liver Cocultures Grown on PGA Felt Templates

PGA felt material (10 × 60 × 5 mm) (Davis & Geck, CT) was placed into a Tissue Tek slide chamber (Nunc, Inc.), inoculated with ~10^7 liver stromal cells whose numbers had been expanded by several passages in monolayer culture, and suspended in liquid medium. This culture was transferred to a fresh slide chamber, and a second inoculum of 5 × 10^6 PC was applied 7 d later. The coculture was grown for an additional 2 wk and grafted into the omentum, mesentery, at the excision point of a left hepatic lobectomy, or at subcutaneous sites of Long-Evans rats anesthetized with sodium pentobarbital (104 mg/kg body wt). After 21 d *in situ*, the grafts were excised, fixed in 10% formaldehyde in PBS, and processed for histological evaluation. Seven-micrometer serial sections through graft and control sites were cleared in Hemo-De (Fisher Scientific) and rehydrated through a graded series of ethanols (100, 90, 70%) to dH_2O. Alternating sections were stained with hematoxylin and eosin, or processed for immunocytochemistry as described in the previous paragraph.

RESULTS

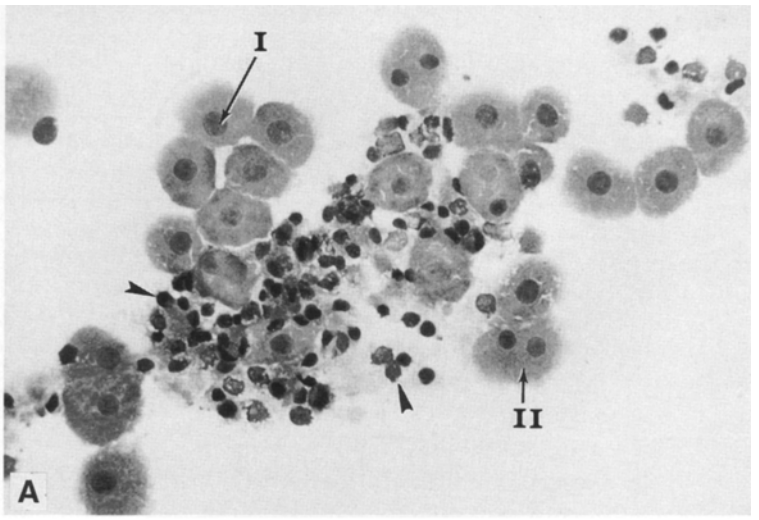
Characteristics of Isolated Cells

The various types of hepatic cells, their relative densities, and method of isolation are listed in Table 1. The two major cell populations of the liver are parenchymal and stromal. Three types of PC were distinguished based on morphological characteristics, such as size, number and density of nuclei, and presence of nucleoli, as well as their reaction to standard acid/base or immunochemical stains. Type I PC were small (15–20 μm) mononuclear hepatocytes with deep cytoplasmic basophilia. These were relatively dense, attached poorly to pre-established stromal cells, did not adhere directly to plastic, and did not proliferate in vitro in association with either substratum. Type II PC were moderately sized (20–30 μm) binuclear cells with one to two nucleoli and moderate-deep cytoplasmic basophilia (Fig. 1A). Some of these cells adhered to plastic, and some Type I PC attached to them. They proliferated for 2–3 d after plating in monolayer culture and then became mitotically inactive. Type III PC were large (25–35 μm), ghost-like cells (Fig. 1B). They were usually binuclear with 2–3 very prominant nucleoli/nucleus, accumulated little or no basic stain in the cytoplasm, and displayed buoyant densities similar to hepatic stromal cells, although their volume was substantially greater than these cells. These "acidophilic" PC adhered rapidly and firmly to plastic, had a much higher mitotic activity than other liver cells, and retained this mitotic activity for 10–14 d in monolayer culture. Although virtually all of these cells adhered, all gradually detached from the plastic within 2–3 wk and assumed the appearance of type II cells. In addition, Type I and Type II PC attached to pre-established monolayers of acidophilic cells. Although some Type I, II, and III PC weakly expressed MHC I antigens, these were not detectable on most PC cells. PC did not express MHC II.

The stromal cell population of the liver included fibroblasts, vascular and biliary endothelia, adipocytes, and Kupffer cells that were coseparated by centrifuging freshly prepared cells against a 70% Percoll gradient in 10X PBS (density = 1.09 g/mL) for 10 min, forming a pellet and a central zone. Cells from the central zone were washed and centrifuged on a 25/50% Percoll column. Stromal cells were localized in the interface zone (density = 1.03625 g/mL). This isolate also contained small numbers of peripheral blood leukocytes. The small size of stromal cells as compared to PC (Fig. 1A) permits a relatively simple differentiation of these cell types. However, the individual subpopulations of liver stromal cells are less distinct from each other based purely on morphological parameters. Whereas fibroblasts and adipocytes were identified morphologically, Kupffer cells and endothelial cells were differentiated by their ability to phagocytose colloidal carbon or be recognized by an MAb to vW factor, respectively. Moderate levels of MHC I antigens were detected on all of

Table 1
Characteristics of Cells Isolated from Rat Liver Cells Using Percoll Density Centrifugation Carried Out at an Osmolality of 360–370 mosM

Cell type	Characteristics	Density, g/mL	Percentage of total
PC			
Mononuclear	17–22 μm deeply basophilic cytoplasm, nucleus: vesicular chromatin pattern with 1–2 nucleoli, MHC I negative; MHC II negative	1.0669	35
Binuclear	20–27 μm basophilic cytoplasm, nucleus: vesicular chromatin pattern with 1 and occasionally 2 nucleoli, some weakly express MHC I; MHC II negative	1.0706	24
Acidophilic	25–35 μm lightly acidophilic cytoplasm, nucleus: little condensed chromatin with 2–3 large prominent nucleoli, MHC 1 negative; MHC II negative	1.0381	5
Stroma			
Kupffer cells	12–16 μm lightly basophilic cytoplasm with few vacuoles, moderately basophilic, oval nucleus with no obvious chromatin pattern, phagocytose colloidal carbon, most express low-density MHC I; MHC II positive	1.0363	8
Endothelia	11–12 μm lightly basophilic cytoplasm without inclusions, elliptical nucleus without a remarkable chromatin pattern, MHC I positive; MHC II positive, vW factor VIII positive	1.0363	15
Adipocytes	15–18 μm pale, vacuolated cytoplasm, lightly basophilic nucleus	1.0363	3
Fibroblasts	12–15 μm lightly basophilic cytoplasm without inclusions, moderately basophilic nucleus with 0–1 nucleoli	1.0363	10



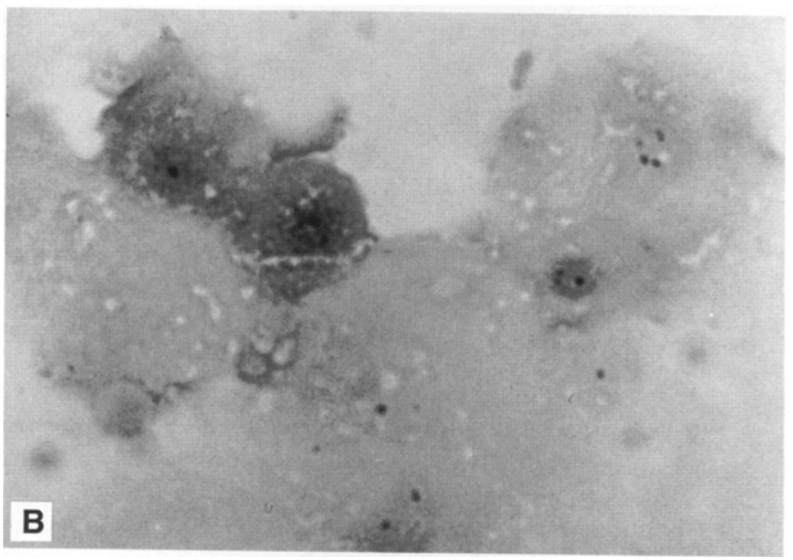


Fig. 1. (**A**) A cytosmear depicting type I (mononuclear) and type II (binuclear) PC stained with Diff-Quik and isolated by Percoll gradient centrifugation. The smaller stromal elements (arrows) are differentiated from the PC by virtue of their size, nuclear configuration, and nuclear staining density. Original magnification = 1000×. (**B**) Photomicrograph of a cytosmear of cells from the lowest of two bands forming at the discontinuous Percoll interface (acidophilic cells). This preparation was stained with Diff-Quik for longer periods than was necessary for other hepatocytes in order to visualize their uneven contours, vacuolation, nucleus, and multiple nucleoli. Original magnification = 1000×.

these stromal cell subsets; Kupffer cells and endothelia were the only stroma to express MHC II.

Characteristics of Suspended Nylon Screen Cocultures

When PC were inoculated onto nylon screens containing pre-established stromal cells, they grew in clusters for the first 1–2 wk of culture (Fig. 2A); patterns of cell growth were increasingly more difficult to discern after this time because of the high cell density of the template. The sections shown in Fig. 2B and C depict the field across one of these screen openings. Stromal cells and their processes are the major elements of the field at several hours after the inoculation of the stromal cell template with PC (Fig. 2B). With time, the PC will eventually fill in most of the available spaces seen in this section. The section of a 52/d-old culture (Fig. 2C) shows a screen space that is tightly packed with large, round PC. The process of filling in this template was generally complete by 4–6 wk of coculture. Although the radiothymidine incorporation data (Fig. 3A) do not discriminate PC vs stromal cells, it is apparent on comparison with the absolute adherent cell count and differential counts (Fig. 3B) that the nylon screen template has a fixed capacity for cell growth ($\sim 10^7$ for liver cells and 2–5 $\times$ 10^7 for bone marrow cells [*19*]). Tritiated thymidine incorporation into DNA decreases as the spaces fill in with growing liver cells and the absolute number of cells reaches a plateau. Differential counts of adherent zone cells revealed increases in the numbers of PC as well as stromal cells with time in vitro (Fig. 3B), but the numbers of PC remained higher than stromal cell counts for the duration of the experiment. The PC, which are considerably larger cells, occupy most of the area in the cultures. PC in the suspended nylon screen cocultures displayed a rounded, as compared to the spread/flat morphology that is typical of hepatocyte culture on plastic flasks. These round cells stained positively for albumin using the immunoperoxidase method (Fig. 4A), and for fibrinogen, transferrin, and fibronectin by immunofluorescence (pictures not shown). They also stained positively for cytokeratin 19, an epithelial cell marker that is found on PC (Fig. 4B) and also on biliary cells, which by comparison, are considerably smaller (Table 1). By comparison, the thinner stromal cells that interweave between the PC in these cultures did not express liver-specific proteins, but did stain positively for laminin, collagen type III, and fibronectin, as did the matrix that surrounded them (Table 2). As evaluated by methods described in this article, cocultures established with >85% homogeneous populations of acidophilic PC exhibited higher seeding efficiencies and rates of cluster formation than cocultures inoculated with mixed PC populations, but were not superior in terms of functional activity.

PC derived from suspended nylon screen cocultures displayed TCDD-inducible cP450 enzyme activity for up to 58 d as indicated by their ability

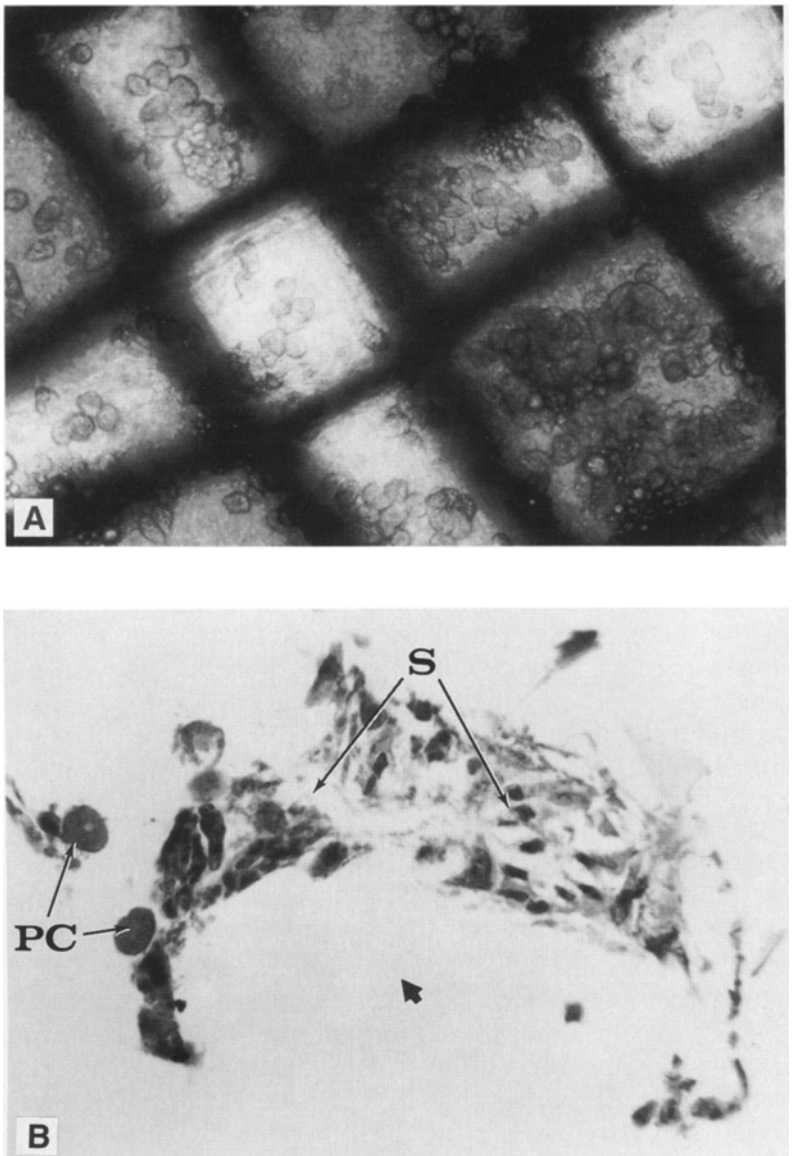


Fig. 2. (**A**) Inverted-phase photomicrograph of an 8-d-old coculture of various hepatic PC derived from the 70% Percoll pellet and hepatic stroma on a nylon screen template. The inoculation with this heterogeneous mixture of cells promotes the growth of morphologically distinct clusters. (**B**) Hematoxylin-eosin (H-E) stained section (~6–7 μm) through a liver cell coculture 24 h after inoculation of PC. 1000×. The semicircular space (arrow) denotes the location of the nylon filament. Several round PC are associated with stroma (S). (**C**) H-E stained section through a 52-d-old liver cell coculture. Round PC fill in all of the available space within the template. n = nylon fiber in cross-section.

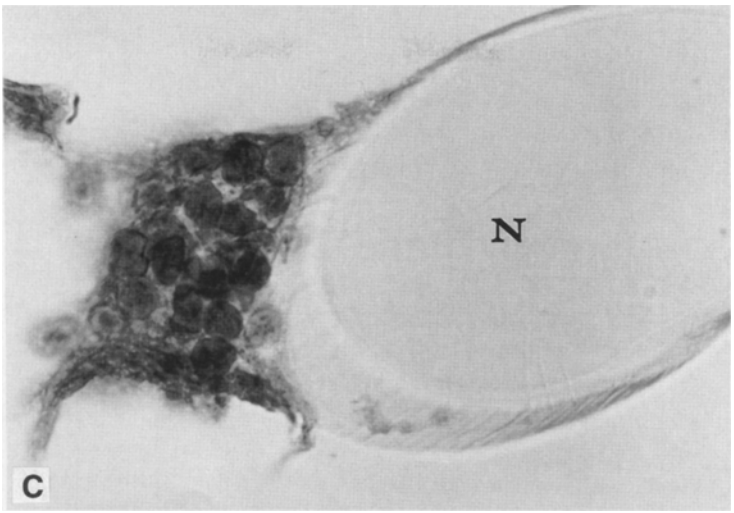
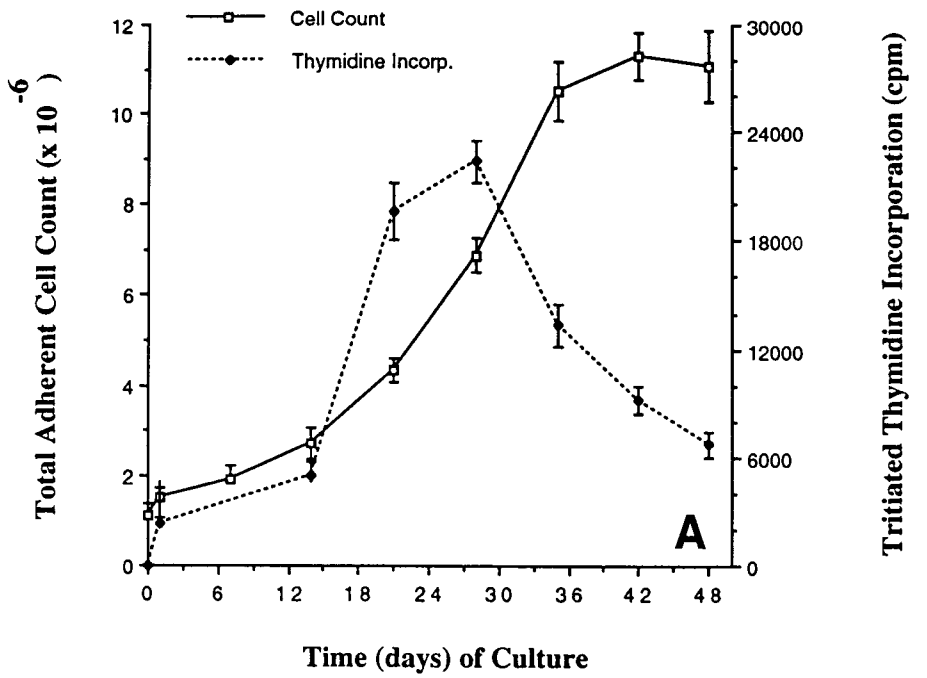


Fig. 2 (*cont'd*).

to transform EFEE to fluorescein (Fig. 5). Since the flow cytometer was gated on discrete populations of cells based on their physical characteristics (FLS vs SS), the EFEE-to-fluorescein conversion was quantified at the cellular level, and leakage of fluorescein from the cell to the medium did not influence this measurement. Peak EFEE-to-fluorescein conversion was >2 times higher in the 17, 26, 41, or 58 d cocultures than in either freshly isolated liver cells or 24-h suspension cultures of these hepatocytes (Fig. 5). Although peak fluorescence was not contingent on the age of the coculture, the rates of EFEE conversion varied between different cocultures. In this regard, peak fluorescence was achieved by cells derived from 26, 41, and 58 d cocultures at 7, 4, and 5 min after exposure to EFEE, respectively. In addition, the EFEE conversion kinetics of TCDD-primed cells varied depending on the concentration of substrate; transformation of EFEE to fluorescein and subsequent egress from the cells were complete by 14 min at EFEE levels of 2.5 and 5 μL/mL, whereas metabolism of 7.5 μL/mL of EFEE stock solution did not drop to baseline levels until 23 min after addition of substrate to 58-d-old cultures. Although cP450 activity was observed in Kupffer cells and in PC of various sizes, moderate to large PC displayed the highest EFEE-to-fluorescein conversion (data not shown). Arbitrary conversion units were calculated as the product of the percent positive fluorescence and peak channel number as described by Miller (*17*). This provided an index of the percentage of cells having cP450 activity and the strength of their activity.

Although the levels of albumin, fibrinogen, transferrin, and free fibronectin in the medium varied, all were present in the culture medium



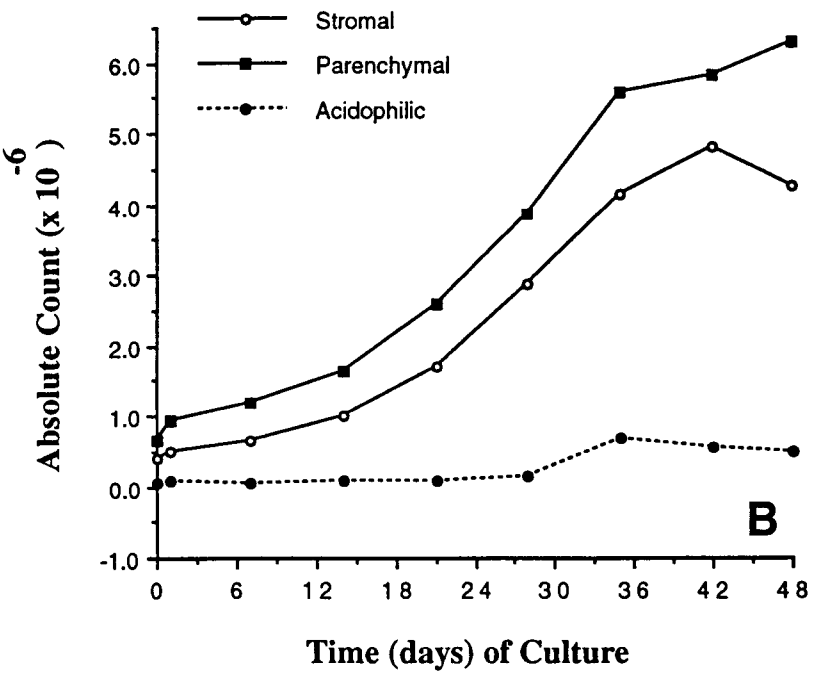


Fig. 3. (**A**) The relationship between total adherent cell count and radiothymidine incorporation in liver coculture. Vertical lines through the means indicate ± 1 SEM. (**B**) Mean differential cell counts in liver cocultures of various ages.

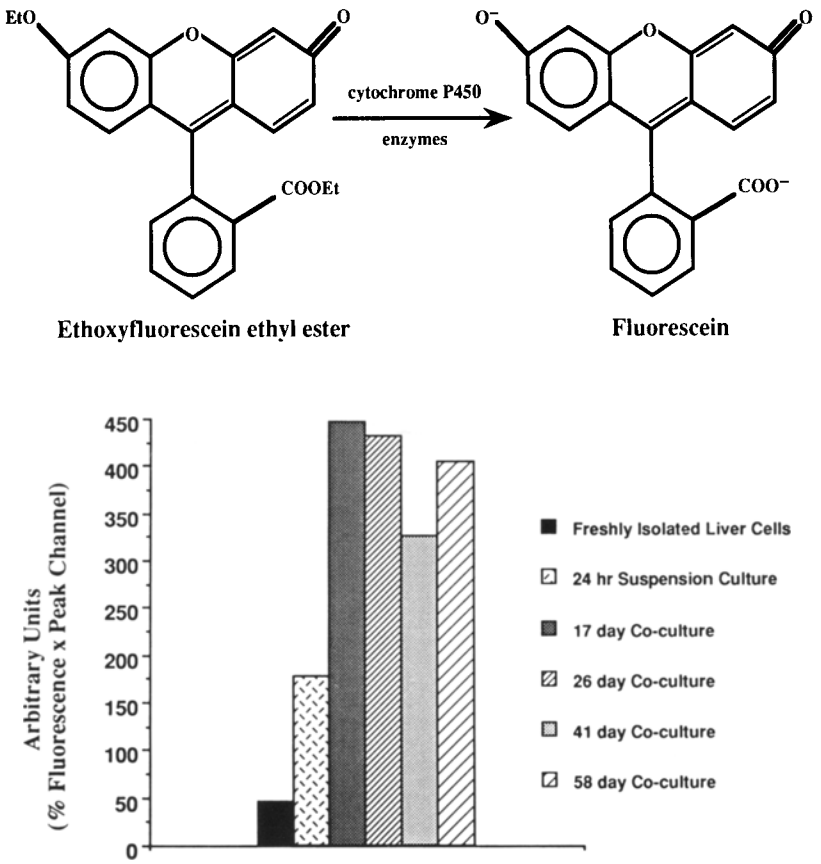


Fig. 4. Cytochrome P450 enzyme activity in hepatic cells 21 h after the introduction of TCDD. The EFEE-to-fluorescein conversion reaction was quantified from cytofluorographic distributions as the product of the percentage of positively fluorescent cells and the peak fluorescence channel number. Approximately 20,000 events were measured for each sample.

for up to 48 d (Fig. 6). Fibrinogen concentration in the culture medium actually increased over time in culture from ≤ 50 ng/mL for the first 20 d of culture to ≥ 150 ng/mL by 28 d, where it remained for the last 3 wk of the study. Fibronectin levels increased for up to 40 d, but dropped by ~75% by 48 d in vitro. A similar pattern was observed for albumin, which was present at peak level at 25 d and dropped by ~25% thereafter to a titer that was constant for the remainder of the experiment. In contrast, transferrin synthesis declined steadily for the first 2 wk of culture to a level of ~1 μg/mL, which was relatively stable over the next 4 wk in vitro. Cultured PC manifested a diminished expression of class I MHC antigens when compared to freshly isolated hepatic parenchyma (2.6 vs 4.9%, respectively). In contrast, no MHC class II antigen expression was detectable on PC, and MHC class II epitopes on macrophagic cells were substantially lower than on noncultured cells (3.5 vs 9.6%, respectively).

Table 2
Immunofluorescence Detection of Various Antigens on PC or Stromal Cells/Extracellular Matrix (ECM)[a] of Liver Cocultures or Sections of Adult Liver Tissue

	Coculture		Tissue	
Antigen	PC	Stroma/ECM	PC	Stroma/ECM
Albumin	+++	0	var	0
Collagen type III	0	+	0	+
Cytokeratin	+++	+	++	+
Fibrinogen	++	0	++	0
Fibronectin	++	+++	+	++
Laminin	0	++	0	+
Transferrin	±	++	+	++

[a]When evaluated by immunofluorescence microscopy, these proteins appear to be widely distributed on PC or stromal cells, and within extracellular matrix. Point sources of fluorescence are relatively easy to discriminate on PC, since these cells occupy the greatest volume of liver tissue in vivo and in the liver cell cocultures. Because stromal cells are embedded within the ECM substances that they secrete, these liver tissue components are grouped together in this table.

var = variable distribution among cells. 0 = no reaction. + = low fluorescence. +++ =high fluorescence.

Hepatic tissue architecture was reconstructed at most of the graft sites of liver PC:stromal cell cocultures on PGA felt (Fig. 7). This regenerating tissue contained sinusoids with lining cells that were macrophagic based on their double staining with MAb against MHC II antigens (OX-6) and the ED-1 epitope (Fig. 7; immunofluorescence picture not shown). Graft site tissues displayed the appearance of regenerating liver in that the sinusoids were somewhat wider than those in normal liver, the Kupffer cell:PC number ratio was higher, the PC were less basophilic, and mitotic figures were observed routinely (*12,13,20*). Grafts in omental and subcutaneous sites displayed a higher incidence of generation of these liver-specific structures than the mesentery, perhaps because of their relatively higher degree of vascularization. However, even when liver PC:stromal cell coculture grafts failed to develop liver-like architecture (~25% of the time), they still stained positively for the presence of albumin, transferrin, and fibrinogen, indicating that the function of synthesizing these proteins does not require patent sinusoidal structures. We did not attempt to assess cP450 activity in the grafted tissue for technical reasons. The liver grafts did not develop at ectopic sites, unless partial hepactectomy was performed at the time of grafting.

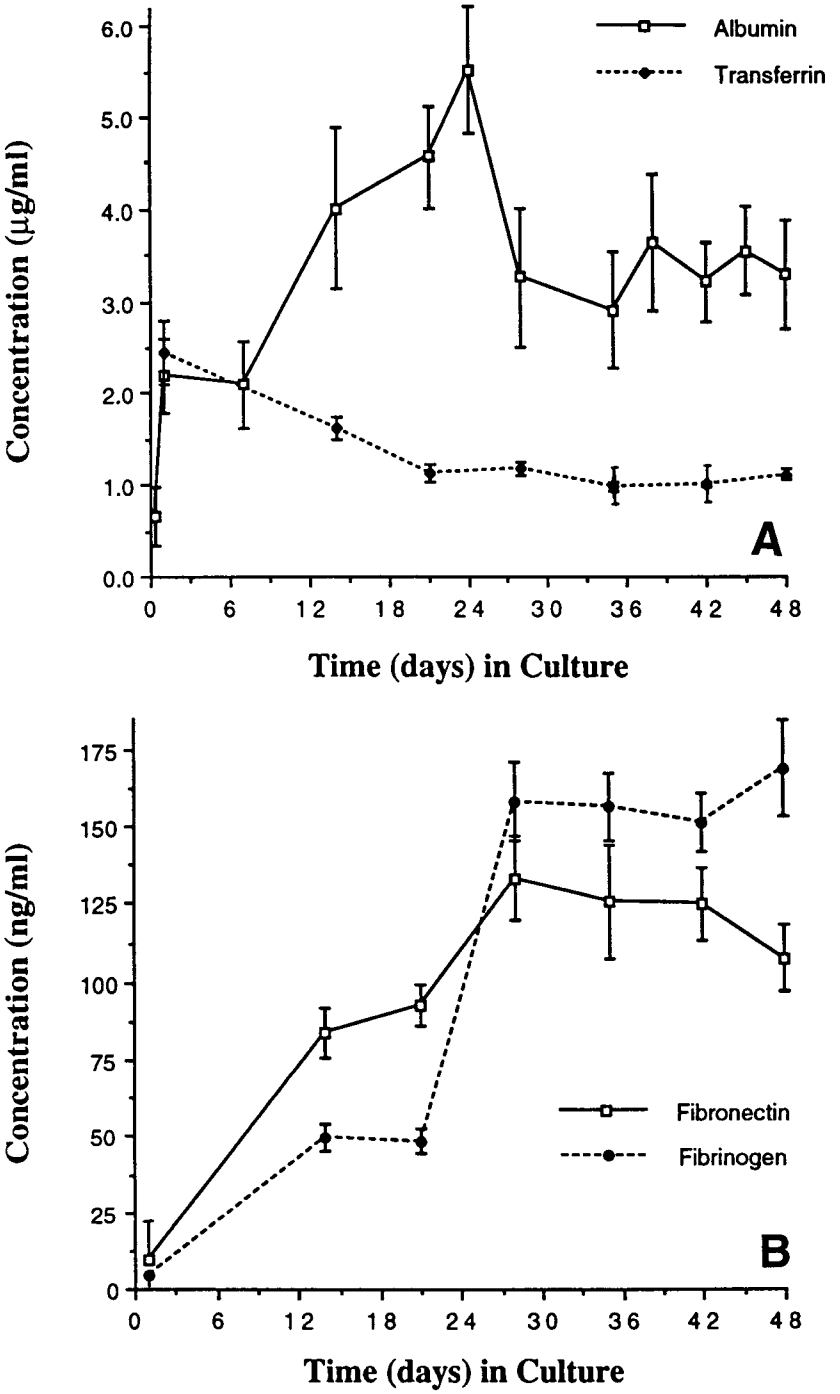


Fig. 5. Mean quantities of albumin and transferrin (top) and fibrinogen and fibronectin (bottom) present in the medium at various intervals in culture. Vertical lines through the means = ± 1 SEM.

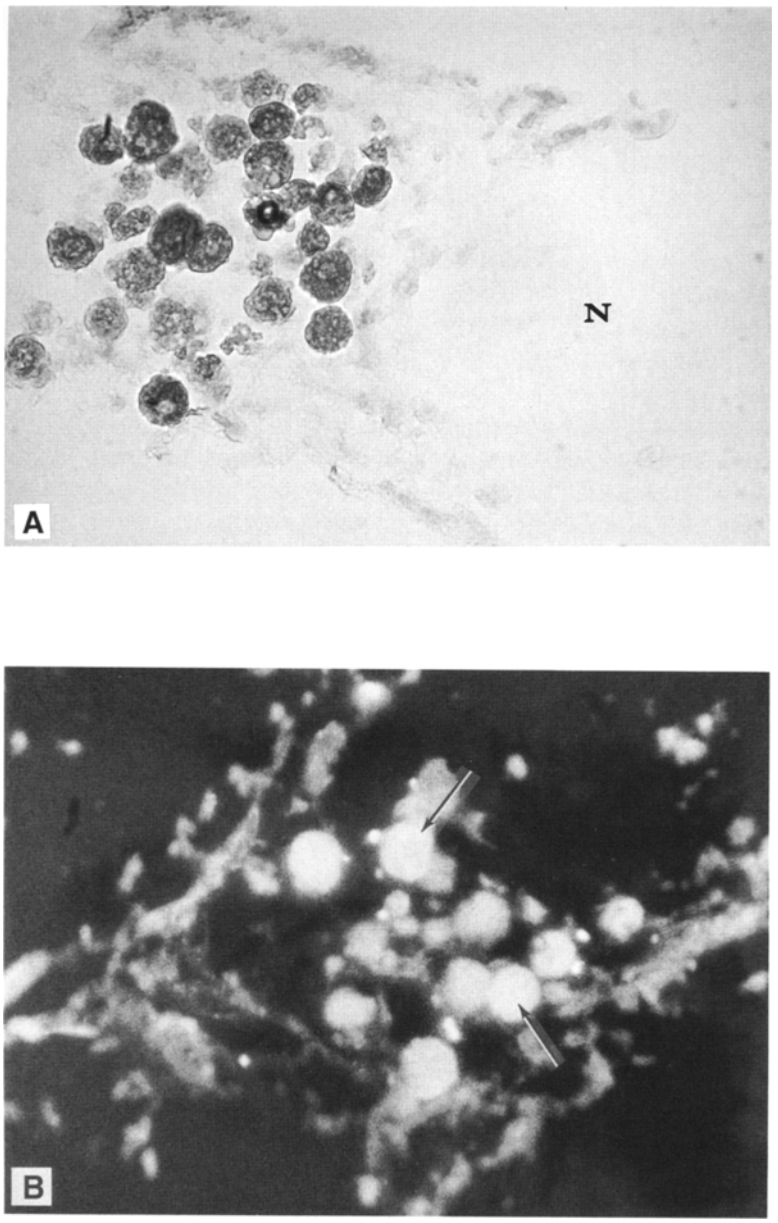


Fig. 6. Sections through a 30-d-liver coculture stained with: (**A**) the anti-albumin peroxidase method. There is a range of positivity, but albumin expression is highest in the darker cells. The interdigitating stromal cells do not express albumin and stain very faintly (background) with this technique. n = space occupied by a cross-sectioned nylon fiber of the screen. (**B**) Anti-cytokeratin immunofluorescence labeling of large, round cells (arrows) in a section through a different field of the same coculture. 500×.

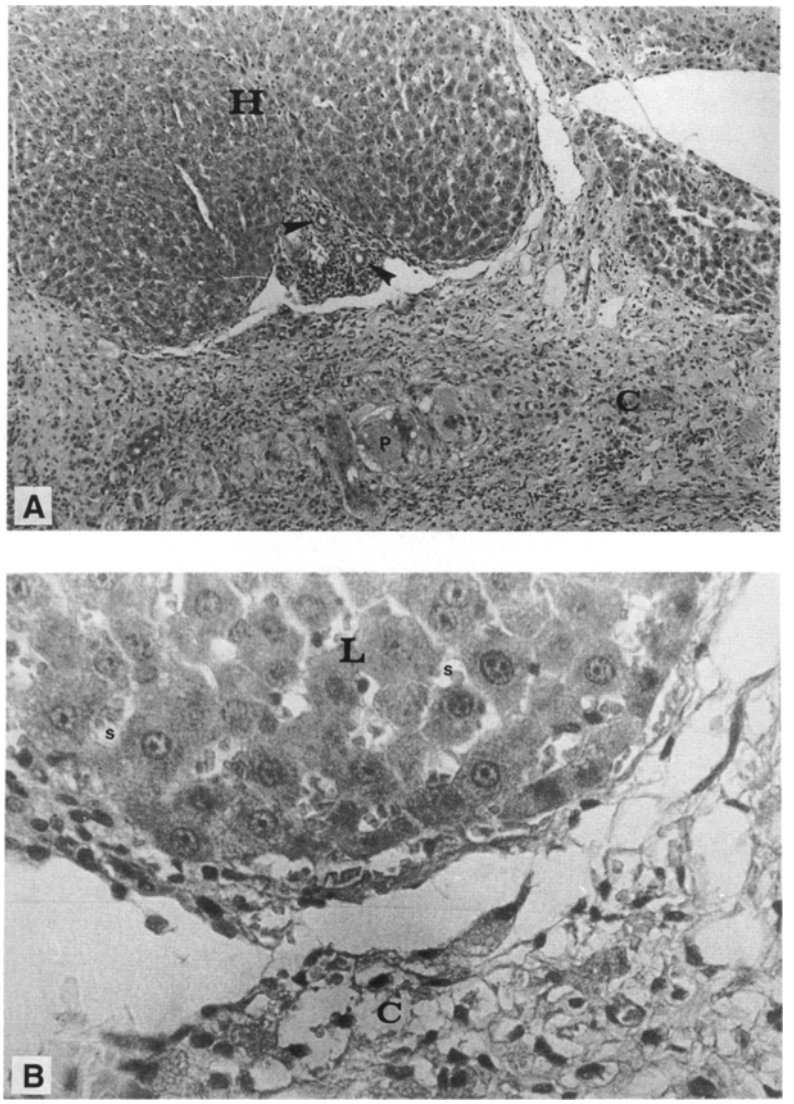


Fig. 7. Photomicrographs of cocultures of liver PC and stromal cells on PGA felt 30 d after grafting into Long-Evans rats. H-E staining. (**A**) Low-power view of a subcutaneous graft showing a focus of hepatic tissue (H) contiguous to connective tissue (C) in the process of reorganization. A tract of residual, partially hydrolyzed PGA polymers (p) is present. Arrows identify putative biliary structures associated with the regenerating hepatic tissue. 100×. (**B**) The interface between grafted liver cocultures (L) and connective tissue (C) elements of the omentum. Sinusoids (s) are evident in the hepatic area (L). The larger, open spaces in the connective tissue region (C) are characteristic of the normal histological architecture of the omentum, whereas the smaller spaces in this zone are attributed to the vacuolization of cells participating in connective tissue repair.

(*continued*)

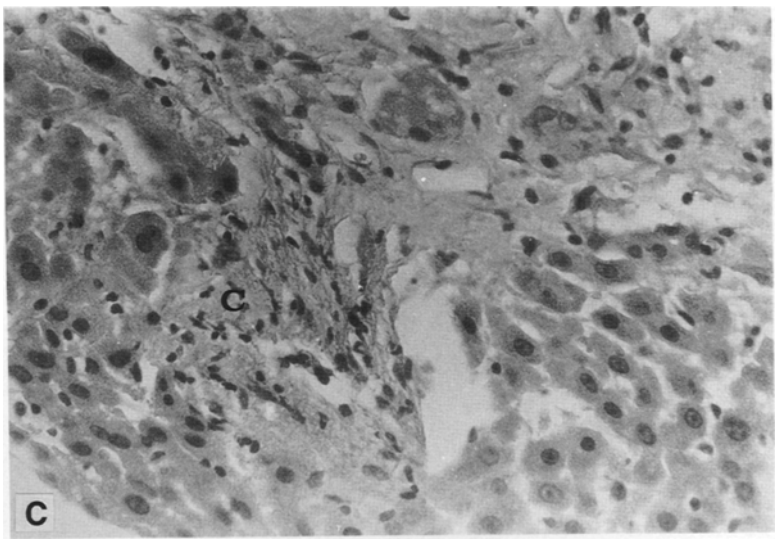


Fig. 7. (*cont'd*). (**C**) Graft site in the mesentery showing PC with sinusoids (lower center and right) and connective tissue in the process of repair (C). PC are interspersed between connective tissue elements, but do not exhibit a regular organization of hepatocytes around sinusoids (top left). 500×.

DISCUSSION

Hepatic PC function in vitro can be maintained over longer periods if they are cultured with various ECM substances or cocultured with other cell types, although proliferation is low or absent in these systems after the first several days of culture (*1–8*). Furthermore, liver cultures are intrinsically difficult to evaluate. Several reasons for this include: mitoses in cocultures of hepatic cells have been ascribed to nonparenchymal elements (*6*), nonparenchymal liver cells have been reported to express functions similar to hepatocytes, such as albumin synthesis (*21*), and hepatocyte phenotypes appear to change under some culture conditions, even though PC and hepatic stroma remain morphologically distinct (*11*). Three-dimensional constructs, such as the nylon screen, not only provide an increased surface area for cell growth, but apparently allow hepatic PC and stromal cells to form a microenvironment conducive to expression of liver-specific metabolic activity as well as PC proliferation. PC that were inoculated onto nylon screens containing the various types of liver stromal cells synthesized albumin, fibrinogen, transferrin, and other proteins, and displayed TCDD-inducible cP450 activity for >2 mo in culture. Growth of PC was evident in association with these stroma and proceeded until all available space for expansion within the template was exhausted.

Albumin secretion by PC increased by >700% over "time-zero" levels by 24 d of coculture (5.5 ± 0.70 vs 0.66 ± 0.32 μg/mL; $P \leq 0.01$), and although the levels dropped thereafter, albumin concentration was ~400% greater than input levels at 48 d of coculture (0.66 ± 0.32 vs 3.28 ± 0.59 μg/mL; $P \leq 0.025$) (Fig. 6). This compares favorably to the monolayer-based coculture system of Guguen-Guillouzo et al., who reported peak albumin levels at ~+300% of input cells after 10 d of coculture, but observed a steady decline thereafter until 42 d, when the levels of this protein were essentially equal to the quantities secreted by input cells (*6*). In the nylon screen cocultures, albumin synthesis, and possibly the production of other proteins, was related to proliferative activity and/or the cell density of the template. As the nylon screen became filled with PC, ^{3}H-thymidine incorporation and cell division dropped dramatically (Fig. 3); albumin and fibronectin synthesis also decreased as the 15 × 60 mm cultures reached their maximum capacity of ~3–5 × 10^7 total cells. That protein expression is favored in this system is not surprising; procedures that maximize cell–cell contact, such as culturing in single or double gels (*1,2,4*) or inoculating with high plating densities (*22*), have succeeded in enhancing hepatocyte protein synthesis in vitro. In addition, culture methods that temporarily preserve liver architecture or promote the re-establishment of hepatic cell–cell associations in vitro have been superior to monolayer-based techniques in terms of expression of liver-specific functions. Thus, the transcription of liver-specific genes has been reported to be higher in liver tissue slices than in single-cell suspensions of hepatocytes (*23*). Similarly, when suspensions of liver cells are treated with epidermal growth factor and other hormones, they freely associate and form aggregates of up to 10 cells. These "spheroids" were reported to display liver-specific functions for 5–6 wk in vitro (*24*).

Although PC proliferation is difficult to quantify in cocultures because of the presence of so many other types of cells, PC growth was evident in histological sections through the cultures. PC are the largest cells of the coculture, and unlike stroma, which are irregularly shaped and branched cells, are round. These large, round cells stained positively for the liver PC-associated proteins albumin, fibrinogen, transferrin, and cytokeratin 19. This finding was confirmed by differential counts of cells derived from the cocultures in which PC were distinguished from non-PC by virtue of their size, nuclear:cytoplasmic volume, nuclear characteristics, and lack of ability to phagocytose colloidal carbon and react with MoAb directed against endothelial cell epitopes. These findings, coupled with radiothymidine incorporation studies, indicate that PC proliferation occurs over a ≥4-wk period in this three-dimensional model; this phenomenon has not been demonstrated in any other liver culture system for longer than 1 wk. The limiting variable of this cell expansion appears to be space. PC grew only in association with stroma, and their rates of growth declined as the template became filled with cells.

Although it appears that most PC have the capacity to synthesize albumin in vivo, the quantity and rate of expression of this protein vary with location within the acinus (*25,26*). In contrast to normal conditions where only 15–20% of the total liver PC strongly express albumin (*27,28*), virtually all PC express this protein following hepatotoxic injury (*28*) or in response to perturbations in plasma proteins associated with nephrotic syndrome (*29*). It is tempting to speculate that initially, most PC inoculated onto the nylon screen stromal template synthesize albumin and that this model, in some respects, resembles regenerating or injured liver. As PC fill the template, their proliferation rate slows and albumin as well as fibronectin synthesis declines in the majority of the cells, presumably because of ECM or other microenvironmental factors. In this respect, ECM substances influence cell division and gene expression in hepatic cells (*9,25*), and albumin synthesis in cultured hepatocytes can be induced by certain ECM proteins (*9,30*). Likewise, PC functional heterogeneity has been described by a number of investigators and purportedly is influenced by several factors, including blood gas and nutrient gradients across the acinus (*31*), microenvironmental differences (*32*), and maturational gradients of PC from the portal region (immature) to the terminal hepatic vein (mature) (*33*). Although it is unlikely that all these microenvironmental niches can be reproduced concurrently in a cell/tissue culture, the nylon screen model displayed a number of liver-specific functions for up to 7 wk in culture, including TCDD-inducible cP450 enzyme activity. Although the three populations of hepatic cells that were resolved by flow cytometry (small, low to moderately granular cells [Kupffer cells, endothelia], medium-sized cells with low granularity [mononuclear PC], and larger, moderately granular cells [binuclear PC and mono- and binuclear acidophilic cells]) all exhibited some ability to convert EFEE to fluorescein, the highest activity was observed in the PC populations (comparative data not shown). We have not as yet measured cP450 activity in these cultures using inducing agents other than TCDD.

The characteristics of cell growth on nylon screens appear to be intrinsically different from those on flat-bottomed plastic flasks. For example, stromal cell matrix deposition is higher and proliferation rate is lower on nylon screens as compared to plastic flasks. This three-dimensional scaffold may also enhance the opportunity for normal cell–cell interactions and orientation, thereby permitting the various subpopulations of cells to act deterministically to form a tissue-like construct. Supportive of this hypothesis are recent findings by Sato et al. (*34*), who seeded suspensions containing mixed hepatic cell populations onto reticulated polyurethane, a three-dimensional template. They found that albumin synthesis and urea-to-ammonia conversion by liver cells cultured on polyurethane were greater and persisted longer than liver cells cultured in flasks (*35*). The presence of inhibitors in serum has also been hypothesized to influence PC proliferation in culture (*36*), and serum factors have been reported to contribute to the appearance of the large, bizarre, and puta-

tively dedifferentiated masses of PC in monolayer cultures (*11,21*). These cells do not arise in nylon screen cocultures, regardless of the length of the culture or the presence of serum in the medium. In addition, the serum conditioning that has been reported to adversely affect cP450 function in hepatocytes cultured on plastic flasks did not influence cP450 in this model system. Clearly, factors in addition to the presence of serum contribute to this phenomenon. We fed liver cultures with SFM in order to eliminate nonspecific protein binding for our ELISA assays, but this medium lacked sufficient nutrients to maintain them for long terms. The most obvious deficiency of this SFM was its inability to support stromal cells, which began to die after ~48 h, an effect that was ameliorated, but not completely abrogated by hydrocortisone supplementation.

Does the ability of PC to proliferate in stereotypic constructs imply the existence of a stem cell as the source of the PC? Although most adult liver cells in vivo are in G_0, a constant, although slow, turnover of liver cells within the acinus has been reported. Cells in the portal area of the rat liver incorporated ^{3}H-thymidine into their DNA to a greater extent than cells in other regions of the acinus (*33*); these cells appeared to migrate from the portal vein to the terminal hepatic vein over a period of ~5 mo. Such a maturational gradient should impart PC functional heterogeneity as well. Oval cells, which can be generated following treatment with ethionine and 2-acetylaminofluorene, as well as other carcinogens and hepatotoxins express enzyme activities consistent with those of fetal hepatocytes, and appear to form both PC and biliary cells in vitro (*36,37*). These cells, which have been hypothesized by several groups as potential stem cells, resemble endothelial cells more closely than PC and appear to be of bile duct origin (*38,39*). However, the role of oval cells in normal and actively regenerating liver is unclear. Since the process of liver regeneration appears to be a mitotic response of mature PC and stroma rather than a recapitulation of ontogeny (*40*), the biological requirement for liver stem cells is uncertain. PC seem to bear some similarities to mature lymphocytes in this regard; both may undergo proliferation after reaching maturity if the appropriate conditions are met, a process that does not require the contribution of stem cells. One hepatic cell type, the Kupffer cell, clearly does not arise from the *de novo* differentiation of stem cells following partial hepatectomy, but is replenished by Kupffer cells migrating from nondamaged areas of the liver, by the enhanced mitotic activity of the remaining Kupffer cells, and the seeding of liver with peripheral blood monocytes (*41*). We have identified and partially characterized a cell that displays a relatively high degree of mitotic activity in vitro, but has an ability to produce/differentiate only into PC. These acidophilic PC are physically dissimilar to oval cells and manifest some nuclear characteristics that are typical of immmature cells (lack of condensed chromatin, multiple, prominent nucleoli, and so forth), are present in noninduced normal liver, and develop into cells that display the PC-associated functions of albumin secretion and cP450 enzyme activity.

Since functional PC, but not other hepatic cell types develop from these acidophilic PC, these cells are lineage-restricted and may represent hepatic "reserve" cells rather than stem cells *per se*. Other explanations for their mitotic activity in vitro may be that acidophilic PC are inherently more sensitive to the rigors of the isolation procedure than other PC populations, so that alterations in their membranes either induce the synthesis of new receptors for trophic factors present in the culture medium or render existing receptor sites more accessible. However, the persistence of these cells in long-term cocultures implies that they are not morphological or functional artifacts of the isolation procedure. Mixed PC preparations as well as acidophilic PC can be used to populate liver stromal cell cultures with PC, but the potential benefits of the latter cells are related to their proliferative activity and thus their usefulness as targets for gene delivery using retroviral vectors or other methods. In this regard, we have experienced some success in introducing the bacterial β-galactosidase gene into acidophilic PC (Naughton et al., unpublished observations).

PGA single fibers arranged into a felt were used in the present study, since it almost entirely degrades by 30 d *in situ* (*42*). In earlier studies, we found that constructs containing fiber bundles persisted for substantially longer periods. Fibroblastic compartmentalization of these constructs in vivo presents a tangible problem; connective tissue accumulation around the graft site may inhibit the movement of metabolites, thereby limiting the functional life of the graft (*43*). The survival and function of grafts of hepatic PC:stromal cell cocultures on PGA felt at various implantation sites in rats indicate that these constructs can be surgically implanted as tissue equivalents. The generation of hepatic structures, such as sinusoids and ductules, implies that the cells are deterministic with respect to their formation of these structures; if all cell types that are normally present in a tissue are present in the culture/graft, they will re-establish their "normal" orientation in vivo. This is the first demonstration, to our knowledge, of the successful graft of liver tissue that was cultured in vitro. When bioresorbable polymers were employed as a delivery vehicle for freshly isolated, entrapped hepatocytes into Gunn rats, the normally high circulating levels of bilirubin declined significantly (*44*). Similar effects were achieved in this hyperbilirubinemic animal model using microcarrier-attached hepatocytes (*45*), liver cells encapsulated within a collagen matrix surrounded by a sodium alginate-poly-L-lysine-sodium alginate membrane (*46*), or via direct intrasplenic injection of hepatocytes (*47*). Other strategies for transplantation include the injection of hepatic cells into previously implanted, vascularized Ivalon sponges (*48*) or polytetrafluoroethylene (PTFE) fibers (*49*). The goal of these techniques is to restore deficient hepatic function resulting from single gene defects and, more broadly, to promote long-term survival from hepatic failure. Presently, in the United States, donor organs are available for less than one in ten people who require a transplant (*48*). Paradoxically, the technological gains that have been made in the field of transplantation and the general acceptance of

these methods as curative measures have widened the gap between the numbers of organs donated and those who could benefit from a transplant. One potential means to bridge this gap is to expand the amount of tissue that is available through bioengineering. A major technical issue is that these methods must deliver a sufficient mass of cells to be effective. A conservative estimate is that, in the case of the liver, 10%of the total liver cell number would be required (*44*). Microcarrier- and microencapsulation-based methods deliver PC as single cells or in small clusters. Although this limits cell-cell interactions, one benefit of the latter method is that the grafted cells are immunologically privileged. However, the limited survival time of these single cells in vivo is a potential issue. In contrast, the entrapped hepatocyte methods concentrate the grafted cells locally, and even though immunosuppression is required, close contact between cells is promoted, tissue-like structures develop (*44,49*), and the graft will persist for far longer than microencapsulated hepatocytes. These devices have relatively high surface areas and, since they are bioresorbable, they can be grafted with high efficiency into vascular sites, such as the omentum or the mesentery (*44,50,51*). However, PC survival and growth are difficult to quantify in these constructs and, in the case of the nonbiodegradable materials, gradual foreign body compartmentalization by connective tissue elements would be expected. An alternative approach to this problem is use of an extracorporeal device containing viable liver cells to overcome the liver function deficit. These have characteristically been hemoperfusion chambers where the blood is separated from the hepatocytes by porous membranes or implantable diffusion chamber-like systems (*48*). Although hepatocytes in these devices are secure from immune challenge, thrombotic problems were associated with the earlier models (*52*).

In conclusion, adult rat PC can be cultured for long terms in the presence of the full complement of stromal support cells; cells derived from the liver PC:stroma cocultures exhibit a structural and functional heterogeneity, as do liver cells in vivo; proliferation of PC occurs in vitro and appears to be contingent on the geometry of the culture template; and when established on bioresorbable templates, these liver PC:stromal cell cocultures are capable of regenerating a liver-like architecture at ectopic sites in partially hepatectomized rats, and retain their ability to synthesize proteins. This liver culture system may have applications as a substrate for hepatotoxicity testing and, when grown on a bioresorbable polymer template, to be implanted into subjects with inborn errors of metabolism.

REFERENCES

1. Michalopoulos, G. and Pitot, H. C. (1975), *Exp. Cell Res.* **94,** 70–78.
2. Dunn, J. C. Y., Yarmush, M L., Koebe, H. G., and Tompkins, R. G. (1989), *FASEB J.* **3,** 174–177.

3. Reid, L. M., Gaitmaitan, Z., Arias, I., Ponce, P., and Rojkind, M. (1980), *Ann. NY Acad. Sci.* **349,** 70–76.
4. Bissell, D. M., Arenson, D. M., Maher, J. J., and Roll, J. (1987), *J. Clin. Invest.* **79,** 801–812.
5. Deschenes, J., Valet, J. P., and Mareceau, N. (1980), *In Vitro* **16,** 722–730.
6. Guguen-Guillouzo, C., Clement, B., Baffet, G., Beaumont, C., Morel-Chany, E., Glaise, D., and Guillouzo, A. (1983), *Exp. Cell Res.* **143,** 47–54.
7. Begue, J. M., LeBigot, J. F., Guguen-Guillouzo, C., Kiechel, J. R., and Guillouzo, A. (1983), *Biochem. Pharmacol.* **32,** 1643–1646.
8. Kuri-Harcuch, W. and Mendoza-Figueroa, T. (1989), *Differentiation* **41,** 148–157.
9. Michalopoulos, G., Russell, F., and Biles, C. (1979), *In Vitro* **15,** 796–806.
10. Sudhakaran, P. R., Stomatoglou, S. C., and Hughes, R. C. (1986), *Exp. Cell Res.* **167,** 505–516.
11. Grisham, J. W. (1980), *Ann. NY Acad. Sci.* **349,** 128–137.
12. Bucher, N. L. R. (1987), in *The Isolated Hepatocyte: Use in Toxicology and Xenobiotic Biotransformations,* Rauckman, E. J. and Padilla, G. M. eds., Academic, New York, pp. 1–19.
13. Naughton, B. A., Kaplan, S. M., Roy, M., Gordon, A. S., and Piliero, S. J. (1977), *Science* **196,** 301–302.
14. Pertoft, H. and Smedsrod, B. (1987), in *Cell Separation: Methods and Selected Applications,* vol. 4, Pretlow, T. G., II, and Pretlow, T. P., eds., Academic, New York, pp. 1–24.
15. Naughton, B. A., San Román, J., Sibanda, B., and Weintraub, J. P. (1994) *Biotechnol. Bioeng.* **43,** 810–825.
16. Enat, R., Jefferson, D. M., Ruiz-Opazo, N., Gatmaitan, Z., Leinwand, L. A., and Reid, L. M. (1984), *Proc. Nat. Acad. Sci. USA* **81,** 1411–1415.
17. Miller, A. G. (1983), *Anal. Chem.* **133,** 46–57.
18. White, I. N. H., Green, M. L., and Legg, R. F. (1987), *Biochem. J.* **247,** 23–28.
19. Naughton, B. A., Birnbach, D. J., Liu, P., Kolks, G. A., Tung, M., Piliero, S. J., and Gordon, A. S. (1979), *Proc. Soc. Exp. Biol. Med.* **160,** 170–174.
20. Naughton, B. A., Sibanda, B., Triglia, D., and Naughton, G. K. (1991), *Toxicol. In Vitro* **5,** 389–394.
21. Hayner, N. T., Braun, L., Yaswen, P., Brooks, M., and Fausto, N. (1988), *Cancer Res.* **48,** 368–378.
22. Ben-Ze'ev, A., Robinson, G. S., Bucher, N. L., and Farmer, S. R. (1988), *Proc. Natl. Acad. Sci. USA* **85,** 1–6.
23. Clayton, D. F., Harrelson, A. L., and Darnell, J. E. (1985), *Mol. Cell Biol.* **5,** 2623–2632.
24. Landry, J., Bernier, D., Ouellet, C., Goyette, R., and Marceau, N. (1985), *J. Cell Biol.* **101,** 914–923.
25. Bernuau, D., Feldman, G., and Rogier, E. (1981), *Biol. Cell.* **40,** 17–22.
26. Guillouzo, A., Beaumont, C., LeRumeur, M., Rissel, Latinier, M-F., Guguen-Guillouzo, C., and Bourel, M. (1982), *Biol. Cell* **43,** 163–171.
27. Schreiber, G., Lesch, R., Weinssen, U., and Zahringer, J. (1970), *J. Cell Biol.* **47,** 285–290.
28. Araki, H., Ueda, H., and Fujimoto, S. (1992), *Acta Anat.* **143,** 169–177.
29. Maurice, M., Feldmann, G., Druet, P., Laliberte, F., and Bouige, D. (1979), *Lab. Invest.* **40,** 39–45.

30. Tonomura, A., Sawada, N., Sattler, G. L., Kleinman, H. K., and Pitot, H. C. (1987), *J. Cell Physiol.* **130,** 221–227.
31. Matsumara, T. and Thurman, R. G. (1983), *A. J. Physiol.* **244,** G656–659.
32. Martinez-Hernandez, A. and Amenta, P. S. (1993), in *Extracellular Matrix: Chemistry, Biology, Pathology,* Zern, M. and Reid, L., eds., Marcel-Dekker, New York, pp. 255–327.
33. Arber, N., Zajicek, G., and Ariel, I. (1988), *Liver* **8,** 80–87.
34. Sato, Y., Ochiya, T., Yasuda, Y., Matsubara, K. (1994), *Hepatology* **19,** 1023–1028.
35. Barnes, D. and Sato, G. (1980), *Cell* **22,** 649–655.
36. Tsao, M-S., Smith, J. D., Nelson, K. G., and Grisham, J. W. (1984), *Exp. Cell Res.* **154,** 38–52.
37. Farber, E. (1956), *Cancer Res.* **16,** 142–149.
38. Firminger, H. I. (1955), *J. Natl. Cancer Inst.* **15,** 1427–1441.
39. Fausto, N., Thompson, N. L., and Braun, L. (1987), in *Cell Separation: Methods and Selected Applications,* vol. 4, Pretlow, T. G., II, and Pretlow, T. P., eds., Academic, London, pp. 45–78.
40. Goulet, F. (1989), Doctoral thesis, Université Laval, Québec.
41. Parry, E. W. (1978), *J. Comp. Pathol.* **88,** 481–487.
42. Naughton, B. A., San Román, J., Sibanda, B., and Weintraub, J. P. (1994), *Hematol. Rev.* **8,** 37–49.
43. Naughton, B. A., Dai, Y., Sibanda, B., Scharfmann, R., San Roman, J., Zeigler, F., and Verma, I. M. (1992), *Somat. Cell Mol. Gen.* **18,** 451–462.
44. Asonuma, K., Gilbert, J. C., Stein, J. E., Takeda, T., Vacanti, J. P. (1992), *J. Ped. Res.* **27,** 298–301.
45. Demetriou, A. A., Whiting, J. F., Feldman, D., Levenson, S. M., Chowdhury, N. R, Moscioni, A. D., Kram, M., and Chowdhury, J. R. (1986), *Science* **233,** 1190–1992.
46. Dixit, V., Darvasi, R., Arthur, M., Brezina, M., Lewin, K., and Gitnick, G. (1990), *Hepatology* **12,** 1342–1349.
47. Matas, A. J., Sutherland, D. E. R. and Steffes, M. W. (1976), *Science* **192,** 892–894.
48. Langer, R. and Vacanti, J. P. (1993), *Science* **260,** 920–926.
49. Borel-Rinkes, I. H. M., Bijma, A. M., Kappers, W. A., Sinaasappel, M., Hoek, F.J., Jansen, P. L. M., Valerio, D., and Terpstra, O. T. (1992), *Transplantation* **54,** 210–214.
50. Uyama, S., Kaufmann, P-M., Takeda, T., Vacanti, J. P. (1993), *Transplantation* **55,** 932–935.
51. Vacanti, J. P., Morse, M. A., Saltzman, W. M., Domb, A. J., Perez-Atayde, A., and Langer, R. (1988), *J. Pediatr. Surg.* **23,** 3–9.
52. Nyberg, S. L., Shatford, R. A., Hu, W-S., Payne, W. D., and Cerra, F. B. (1992), *Crit. Care Med.* **20,** 1157–1168.