Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration

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ABSTRACT

Adult rat hepatocytes cultured in a collagen sandwich system maintained normal morphology and a physiological rate of albumin secretion for at least 42 days. Hepatocytes cultured on a single layer of collagen gel essentially ceased albumin secretion within 1 wk but could recover function with the overlay of a second layer of collagen gel. This culture configuration more closely mimics the hepatocytes' in vivo environment and provides a simple method for their long-term maintenance.—DUNN, J. C. Y.; YARMUSH, M. L.; KOEBE, H. G.; TOMPKINS, R. G. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J.* 3: 174–177; 1989.

Key Words: extracellular matrix • hepatocyte • collagen • artificial liver • cell polarity

THE ROLE OF EXTRACELLULAR MATRIX in the control of development and differentiation has been the subject of intense research for many years (1–4). In several culture systems, the addition of extracellular matrix induces both cellular polarity and tissue organization (5–9). For example, when a monolayer of mammary epithelial cells cultured on a flat sheet of collagen is further overlaid with a second layer of collagen, these cells reorganize to form tubular structures (10), thereby orienting their basal surface adjacent to the collagen and their apical surface adjacent to the lumen.

Hepatocytes are highly differentiated cells that perform many complex functions. There has been considerable interest in the control of growth and differentiation of hepatocytes in vitro; however, cultures of functional, differentiated adult hepatocytes have proved difficult to establish. Early attempts to culture adult hepatocytes invariably led either to overgrowth of contaminating cell types or to the dedifferentiation or fetalization of the cultured hepatocytes (11). More recently, several techniques have been explored to establish long-term cultures of functional hepatocytes. These include cultures: *1*) in arginine-free media (12), *2*) on floating collagen membranes (13), *3*) on various types of extracellular matrix materials (14, 15), *4*) together with other liver cell types (16, 17), and *5*) in the presence of dimethyl sulfoxide (18, 19). In each system, liver-specific functions have been maintained for periods ranging from 2 to 7 wk.

Although the use of various components of the extracellular matrix has been explored in hepatocyte culture, no attempt has been made to induce the formation of their rather specialized polarity by manipulating the extracellular matrix configuration. Hepatocytes are epithelial cells with distinct apical (bile canalicular) and basal (sinusoidal) surfaces that serve different functions. For example, bile acids are excreted into the bile duct by traversing the apical surface, whereas albumin is secreted into the circulation by traversing the basal surface. Unlike the classical epithelium, as typified by the intestinal absorptive cells, hepatocytes have a belt of apical surface dividing two basolateral surfaces that are in contact with the extracellular matrix (**Fig. 1**). Typically, hepatocytes have been cultured in systems that allowed cell attachment on one surface and medium on the opposite surface. In this report, we describe the effects of sandwiching hepatocytes between two layers of collagenous matrix, thereby providing an environment that more closely resembles the in vivo geometry for the cells.

EXPERIMENTAL PROCEDURES

Preparation of hepatocytes

Hepatocytes were isolated from 2-month-old female Lewis rats by a modified procedure of Seglen (20). Animals were anesthetized with ether. The liver, weighing roughly 8 g, was perfused with 300 ml of calcium-

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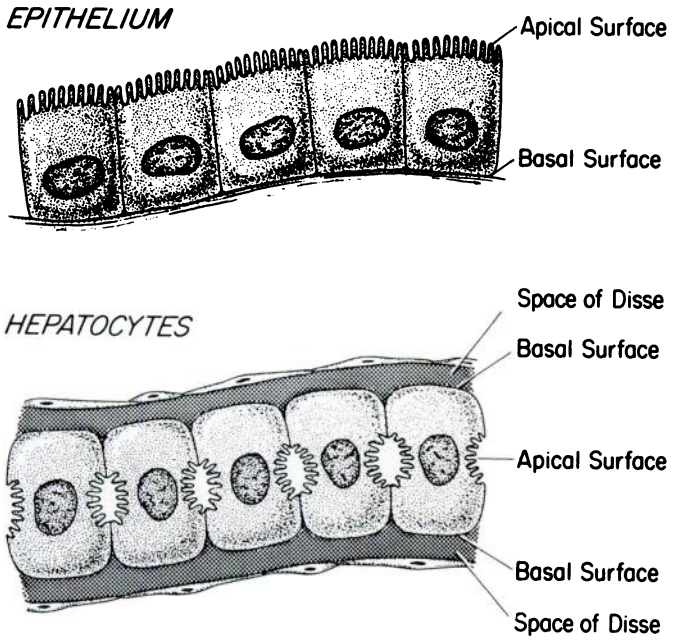

Figure 1. Schematic drawing of the histological organization of hepatocytes compared with that of the intestinal epithelium. Basal surfaces are adjacent to the extracellular matrix, and apical surfaces are adjacent to the lumen.

free Krebs Ringer bicarbonate buffer, containing 5.5 mM glucose and 20 mM HEPES, pH 7.4, at 50 ml/min. The perfusate was maintained at 37°C and equilibrated with 95% O_2 and 5% CO_2. The liver was subsequently perfused with 0.05% collagenase (Type IV, Sigma, St. Louis, Mo.) solution containing 5 mM Ca^{2+} for 10 min in a recirculating circuit. The resulting cell suspension was filtered through two nylon meshes (Small Parts, Miami, Fla.) with grid sizes of 250 and 62 μm. The cell pellet was collected by centrifugation at 50 *g* for 5 min. Cells were further purified by a modified procedure of Kreamer et al. (21). The cell pellet was resuspended to 50 ml, and 12.5 ml of cell suspension was added to 10.8 ml of Percoll (Pharmacia, Piscataway, N.J.) and 1.2 ml of 10 × concentrated Dulbecco's modified Eagle medium (DMEM, 4.5 g/liter glucose, Hazleton, Lenexa, Kans.). The mixture was centrifuged at 500 *g* for 5 min, and the cell pellet was washed twice with DMEM. Routinely, 200–300 million cells were isolated from an 8-g liver, with viability ranging from 90% to 98%. Nonparenchymal cells, as judged by their size and morphology, were less than 1%.

Preparation of rat tail tendon collagen

Type I collagen was prepared from rat tail tendon by a modified procedure of Elsdale and Bard (22). Four tendons were dissected from each rat tail and stirred into 200 ml of 3% acetic acid overnight at 4°C. The solution was filtered through four layers of cheesecloth and centrifuged at 12,000 *g* for 2 h. The supernatant was precipitated with 40 ml of 30% NaCl, and the pellet was collected by centrifugation at 4000 *g* for 30 min. After two rinsings with 5% NaCl and 0.6% acetic acid, the pellet was dissolved in 50 ml of 0.6% acetic acid. The solution was dialyzed against 5 × 500 ml of 1 mM HCL and sterilized by evaporating 0.15 ml of chloroform through the solution. A 5-ml aliquot was lyophilized and weighed to determine the concentration. Generally, 100 mg was isolated from one rat tail.

Hepatocyte culture

Hepatocytes were cultured on gelled rat tail tendon collagen. Plates were prepared by distributing 1 ml of collagen gel solution (mixture of 1 part 10 × concentrated DMEM and 9 parts collagen solution at 1.11 mg/ml) evenly over a 60-mm tissue culture dish at least 1 h before use. Two million viable cells were seeded in 4 ml of complete medium, consisting of DMEM, supplemented with 10% fetal bovine serum (Hazelton), 0.5 U/ml insulin (Squibb, Princeton, N.J.), 0.007 μg/ml glucagon (Lilly, Indianapolis, Ind.), 0.02 μg/ml epidermal growth factor (Collaborative Research, Bedford, Mass.), 7.5 μg/ml hydrocortisone (Upjohn, Kalamazoo, Mich.), 200 U/ml penicillin, and 200 μg/ml streptomycin (Hazelton). The second layer of collagen gel was spread over the cells after 1 day of incubation with 5% CO_2. Thirty minutes were allowed for gelation and attachment before the medium was replaced. Culture medium was changed daily.

Albumin and DNA assays

Collected media were analyzed for rat serum albumin content by enzyme-linked immunosorbent assay (ELISA). Chromatographically purified rat albumin and peroxidase-conjugated antibody to rat albumin were purchased from Cappel (Cochranville, Pa.). The absorbance was measured at 490 nm with a Dynatech (Chantilly, Va.) MR600 microplate reader. The DNA content of cultures was analyzed with Hoechst dye 33258 (Calbiochem, LaJolla, Calif.) (23). Cultures were rinsed twice with phosphate buffered saline (PBS), and 4 ml of 0.05% collagenase solution was added to the dish. After 30 min of incubation, sheets of cells detached from the dish and were collected by centrifugation. The cell pellets were rinsed twice with PBS and resuspended in a 4-ml solution containing 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 0.1 M Tris, pH 7.4. Forty microliters of sample was added to 2 ml of solution containing 0.1 μg/ml Hoechst dye, 1 mM EDTA, 2 M NaCl, and 10 mM Tris, pH 7.4. Fluorescence at 365 nm excitation and 458 nm emission was measured on an SLM (Urbana, Ill.) SPF 500C spectrofluorimeter, with lamp voltage set at 1025 V. Calf thymus DNA standard was purchased from Sigma.

RESULTS AND DISCUSSION

Two million viable hepatocytes were cultured on a layer of gelled rat tail tendon collagen. After 1 day, hepatocytes clustered into cords that covered roughly half of the surface and exhibited polygonal morphology typical of hepatocytes. The addition of a second collagen gel layer did not appear to disturb the hepatocytes. Their distinct poly-

gonal morphology was maintained throughout the 7-wk period. Hepatocytes cultured without the collagen overlay, on the other hand, flattened rapidly and spread into a confluent monolayer by the third day. These hepatocytes appeared to die and detached continuously. Contaminating cell growth was negligible in the sandwich system, whereas in the single gel system, its overgrowth dominated the culture after 2 wk. **Figure 2** shows the deterioration that was invariably observed in the single gel system and the preservation of morphology observed in the sandwich system.

Hepatocytes cultured in the sandwich system also maintained albumin secretion, a liver-specific marker, significantly better than those cultured on a single layer of collagen gel (**Fig. 3**). For the single gel system, albumin secretion dropped to less than one-tenth of the initial rate at the end of 1 wk and remained essentially zero for the next 5 wk.

The decrease in albumin secretion observed for hepatocytes cultured on a single layer of collagen gel could be due to either a malfunction in the albumin synthetic/secretory system within the cells or to cell death. To determine whether the observed changes were reversible, hepatocytes cultured for as long as 14 days on a single layer of collagen were further overlaid with a second layer of collagen. As is shown in **Fig. 4**, recovery of albumin secretion by the overlay of a second collagen gel could be achieved even after 1 wk of culture on a single layer of collagen; only a partial recovery in albumin secretion was seen after 2 wk of culture on a single layer of gel. Within a few days after addition of the second layer, the morphology and albumin secretion rate of the recovered cells were comparable to those observed with hepatocytes

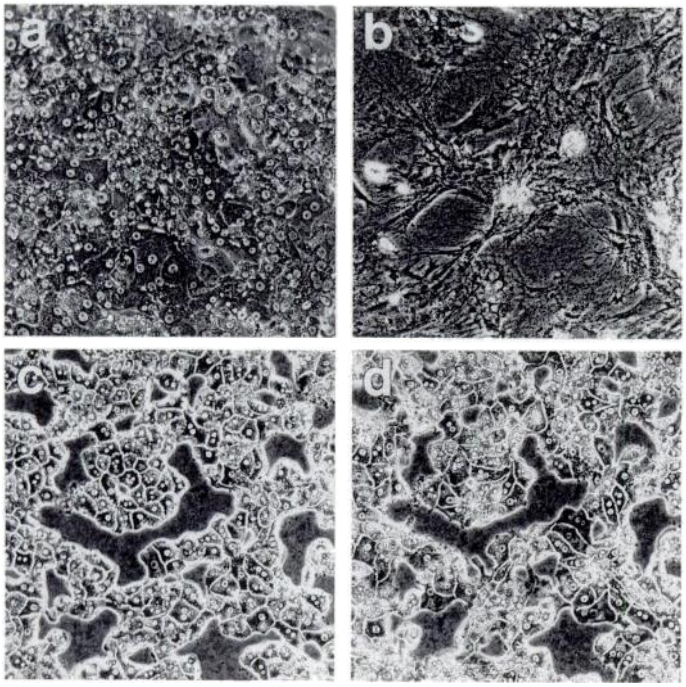

Figure 2. Phase-contrast photomicrographs of hepatocytes cultured for 1 wk on a single layer of collagen (*a*), 2 wk on a single layer of collagen (*b*), 1 wk in the sandwich system (*c*), and 2 wk in the sandwich system (*d*), same position on the culture dish as that in *c*. ×240.

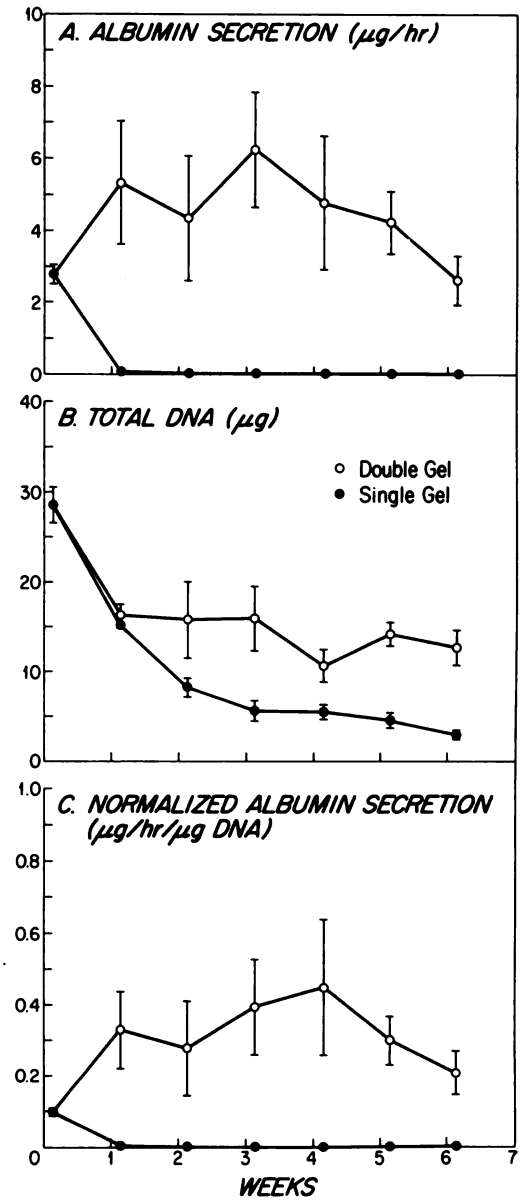

Figure 3. *A*) Mean rates of rat serum albumin secretion for 2 million hepatocytes seeded on a 60-mm dish with 4 ml of media in the sandwich configuration (double gel) and on a single layer of collagen (n = 6). The peak albumin secretion rate ranged from 6 to 12 μg/h between experiments. *B*) Total DNA remaining in the dish after different periods of culture. Total DNA decreased over the first wk followed by a stabilization over the remaining test period. *C*) Rates of serum albumin secretion normalized by the total DNA content.

overlaid with collagen after 1 day of culture. These results indicate that the decrease in albumin secretion for cells cultured on a single layer of collagen is likely to result from reversible loss of function. Preliminary studies in our laboratory showed that the levels of total RNA for albumin are preserved in both the single gel and the sandwich systems after 1 wk. These results suggest that albumin RNA transcripts in the single gel system are being produced but are either not being processed or are translated incorrectly.

The data presented demonstrate that hepatocyte morphology and albumin secretion can be maintained long-

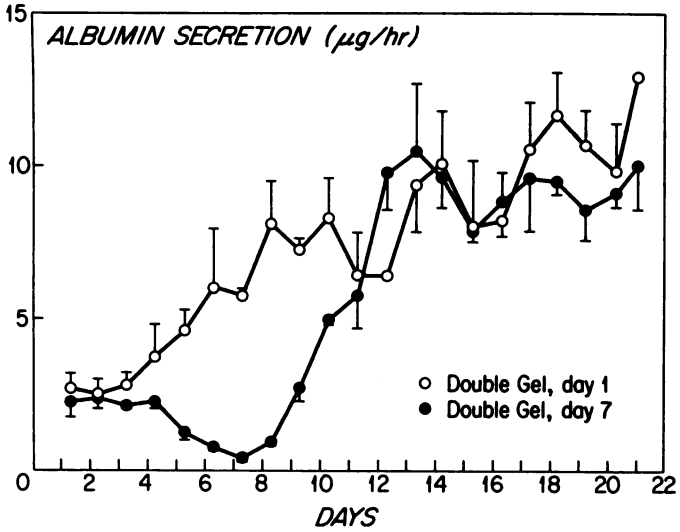

Figure 4. Mean rates of serum albumin secretion for 2 million hepatocytes seeded on a 60-mm dish with 4 ml of medium (n = 6). Cells were overlaid with a second layer of collagen after 1 or 7 days of culture on a single layer of collagen. Maximal rates of albumin secretion were similar in both systems.

term in a culture system that more closely mimics the normal histological organization of the liver. The mechanism for maintenance is unclear but is likely due to the matrix-induced polarization of the hepatocytes. With the proper matrix configuration, hepatocytes may properly orient their cytoskeleton so that the integrity of the cell is maintained and normal cell-cell communication is established.

There are many potential applications for hepatocytes cultured in the collagen sandwich configuration. From the standpoint of the basic science, this system will provide a simple, stable culture environment for studies of: *1*) growth and differentiation, *2*) cell polarity, *3*) cell-extracellular matrix interactions, *4*) hepatocyte-sinusoidal cell interactions, *5*) hepatocyte cryopreservation, and *6*) hepatocyte transplantation. From an applied science standpoint, this configuration might serve as the basis of a bioreactor for the treatment of liver failure or the production of therapeutically valuable proteins. FJ

We would like to thank Laura M. Sterling, Michael D. Harmon, and Toussaint Smith for their technical assistance. This work was supported by National Institute of General Medical Sciences grant GM-21700 and by a grant from the George Link, Jr. Foundation, New York, N.Y. R. G. Tompkins is an American Surgical Association Foundation Fellow and the recipient of National Institute of Diabetes and Digestive and Kidney Diseases Clinical Investigator award DK-01746. M. L. Yarmush is a Lucille P. Markey Scholar.

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Received for publication August 22, 1988.
Accepted for publication October 19, 1988.