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Characterization and Functional Studies on Rat Liver Fat-Storing Cell Line and Freshly Isolated Hepatocyte Coculture System

Marcos Rojkind,[*†‡§] Phyllis M. Novikoff,[*‡]
Patricia Greenwel,[*‡] Joshua Rubin,[||]
Luz Rojas-Valencia,[*†]
Antonio Campos de Carvalho,[*||]
Richard Stockert,[*†] David Spray,[*||]
Elliot L. Hertzberg,[*¶||] and Allan W. Wolkoff[*†¶]

From the Marion Bessin Liver Research Center[*] and the Departments of Medicine,[†] Pathology,[‡] Neurosciences,[||] and Anatomy,[¶] Albert Einstein College of Medicine, and the Department of Orthopedic Surgery,[§] Bronx-Lebanon Hospital Center, Bronx, New York

We developed and characterized a coculture system composed of a fat-storing cell clone (CFSC-2G) and freshly isolated hepatocytes that can reproduce* in vitro *some of the physical and functional relationships observed* in vivo. *Hepatocytes in the coculture are polarized, are smaller in size than hepatocytes plated on plastic, maintain a cuboidal shape, and have a tendency to form cords. Fat-storing cells, which are initially extended, retract and leave spaces that resemble liver sinusoids. Both cell types in the coculture system are functional for at least two weeks as determined by the expression of high levels of liver-specific protein mRNAs as well as by the production and secretion of liver-specific proteins into the culture medium. The hepatocytes maintain relatively high levels of asialoglycoprotein receptor on their cell surface and form functional gap junctional complexes with fat-storing cells. Hence, this coculture system retains a number of differentiated functions of hepatocytes, making it a useful model to study cell-cell interactions in culture and to analyze regulation of hepatocyte functions. (Am J Pathol 1995, 146:1508–1520)

When hepatocytes are plated on a tissue culture plastic surface, they lose the capacity to express various liver-specific genes.[1–3] For example, transcription of the albumin gene is significantly reduced[3] and expression of the hepatocyte plasma membrane asialoglycoprotein receptor is lost within 48 hours (Richard Stockert, personal communication). In contrast to these findings, when hepatocytes are plated on plastic coated with complex extracellular matrices such as biomatrix[4] or matrigel,[3,5,6] they retain these differentiated functions for several weeks in culture. When specific extracellular matrix components, such as heparans, are added to hepatocytes cultured on plastic, these cells partially regain their capacity to transcribe some liver-specific genes.[7]

Alternative procedures have been developed to maintain liver-specific gene expression of hepatocytes in culture. These consist of preparing cocultures with other liver epithelial cells, possibly derived from the canal of Hering,[8,9] or to use irradiated fibroblasts as feeder layers on which the hepatocytes are plated.[10] Cocultures with endothelial cells[11] or formation of hepatocyte spheroids have also been used.[12,13] Although liver-specific functions are partially restored by these experimental procedures, they neither resemble the normal cellular organization nor contain cell-matrix interactions observed in intact tissue. Preliminary data from our laboratory suggested that hepatocytes maintained in coculture with the fat-storing cell (FSC) clone CFSC-2G[14] retained some liver-specific functions for at least 2 weeks.[15,16] More recently, cocultures of hepatocytes and FSC were used to study changes in the expression of extracellular matrix components[17] and the role of hepatocyte injury in collagen production by FSC.[18] However, neither the survival of hepatocytes nor the expression of liver-specific proteins was investigated.

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Address reprint requests to Dr. Marcos Rojkind, Liver Research Center, U-625, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

Hepatocytes *in vivo* are in close contact with projections emitted by FSCs as well as with extracellular matrix components present in the space of Disse.[19] These extracellular matrix components are produced in part by FSCs,[20,21] sinusoidal endothelial cells,[22,23] and hepatocytes[18,21,24–27] with their composition varying upon the interactions established by the various cell classes.[28] The extracellular matrix components play an important role in providing attachment sites for the hepatocytes and other sinusoidal cells. In addition, they are important in sustaining their function. Cell-matrix interactions are established via specific cell surface receptors.[28] Hepatocyte receptors for fibronectin and laminin have been particularly well characterized, including determination of specific amino acid sequences recognized by these receptors for their extracellular matrix substrates.[28]

We have developed a coculture system with hepatocytes and FSCs that reproduces *in vitro* some of the physical and functional relationships observed *in vivo*. Because of the intrinsic problems of isolating simultaneously FSCs and hepatocytes, cocultures of freshly isolated hepatocytes were established with a FSC cell line developed[29] and cloned in our laboratory.[14] The FSC clone used (CFSC-2G) resembles the phenotype of freshly isolated FSCs with respect to the expression of various transcripts coding for extracellular matrix components.[14] In this communication we describe the characteristics of the coculture and demonstrate that hepatocytes and FSCs in coculture establish functional gap junctions between themselves and each other and retain a number of functions associated with well differentiated hepatocytes.

Materials and Methods

Establishment of Cocultures

Frozen stocks of FSC clones CFSC-2G, CFSC-8B, CFSC-3H, and CFSC-5H[14] were thawed and maintained in culture with minimal essential medium (GIBCO BRL, Gaithersburg, MD) containing nonessential amino acids (Gibco) and 10% fetal bovine serum (HyClone, Logan UT). Confluent dishes were trypsinized as previously described,[14] and approximately 1.0×10^6 CFSC-2G were plated in 75-cm^2 Falcon culture flasks (Becton Dickinson, Lincoln Park, NJ) and maintained in culture for 48 hours. Cells in one culture dish were trypsinized and counted to establish the approximate number of viable FSCs. In several experiments we found that the number of cells was 1.5×10^6 to 2×10^6. In preliminary experiments we determined that the best ratio of hepatocytes to FSCs needed for the hepatocytes to maintain their function was between 3:1 and 5:1. Therefore, we plated on top of the CFSC-2G, 10×10^6 freshly isolated hepatocytes[30] with minimal essential medium supplemented with 5 mg/L insulin (Sigma Chemical Co., St. Louis, MO) and 5% fetal bovine serum. Two hours after plating, culture medium was removed and replaced by a serum-free, hormonally defined culture medium (HDM).[31] The cells were maintained in culture for 2 weeks. HDM was replaced every other day. Cells were harvested at various times after plating the hepatocytes and used for the experiments described below. Initially, cocultures were prepared with freshly isolated hepatocytes and the various FSC clones developed in our laboratory.[14] The culture media obtained after 2 weeks in culture were tested by Western blot for the presence of albumin, with an antibody to rat albumin that did not cross-react with bovine serum albumin (kindly provided by Drs. J. and N. Roy-Chowdhury, Albert Einstein College of Medicine). As results suggested that secretion of albumin was greater when hepatocytes were plated on CFSC-2G (See Table 1), all additional cocultures were prepared with this FSC clone.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blots

Cell cultures were scraped from dishes into cold phosphate-buffered saline (PBS) containing 2 mM phenylmethylsulfonylflouride (Sigma Chemical Co.) freshly added from a 100 mM stock in isopropanol, washed, resuspended in a minimum volume of this buffer and lysed by brief sonication. A total of 75 µg of cellular protein (Bradford assay, Sigma Chemical Co.) was resolved by SDS-PAGE (10% gels) and transferred to nitrocellulose as described.[32] Blots were probed with a rabbit anti-Cx43 antibody[33] or an anti-asialoglycoprotein receptor antibody.[34] Subsequent to washes, blots were incubated with ^{125}I-labeled protein A (New England Nuclear, Boston, MA) and washed. Antibody binding was visualized by autoradiography.

Dye Transfer Analysis

Dye transfer between hepatocytes and FSCs was analyzed by microinjecting 5% Lucifer yellow CH (Sigma Chemical Co.) in 150 mmol/L LiCl into single cells through microelectrodes. The spread of dye was directly observed and photographed within 1 minute of injection.

Light, Confocal Laser Scanning, and Electron Microscopic Studies

FSCs were plated on sterile No. 2 coverslips placed in sterile 100-cm bacteriological dishes (Falcon). After 48 hours in culture, when cells were subconfluent, 1×10^6 hepatocytes were added to the bacteriological dishes. Two hours after plating, culture medium was removed by aspiration, the cells were washed twice with PBS and the medium replaced by HDM as described above. Cells were maintained in culture for 24 to 48 hours after which time they were processed as follows. Cocultures were fixed in cold 4% paraformaldehyde-2.5% glutaraldehyde for 10 minutes. Cells were stained with methyl green pyronine (Sigma Chemical Co.) to determine the overall morphology and presence of mitosis.[35] They were examined for ATPase activity to detect bile canaliculi[36,37] and for catalase activity to determine the presence of peroxisomes.[38] Cocultures were rinsed for 1 minute with MEPS buffer (2 mmol/L $Mg^{2+}SO_4$, 5 mmol/L EGTA, 35 mmol/L K plus piperazine-*N,N'*-bis-(2-ethane sulfonic acid), and 0.2 mol/L sucrose, pH 7.0) that contained 0.5% Triton X-100. Cells were fixed for 5 minutes at 37 C with 0.25% glutaraldehyde in MEPS buffer containing 0.5% Triton X-100 and used for the immunocytochemical localization of β-tubulin to detect microtubule distribution; this was performed by sequential exposure of cocultures to mouse monoclonal antibody to β-tubulin (Sigma Chemical Co.) and to goat anti-mouse immunoglobulin G-fluorescein isothiocyanate (Sigma Chemical Co.). For ultrastructural studies, cocultures were postfixed in 1% osmium tetroxide and processed as previously described.[39] For confocal microscopy studies of cocultures, optical sections were scanned at 1-μ intervals over a depth of 31 μ with a Bio-Rad MRC 600 laser confocal microscope fitted with a Nikon 60× objective (numerical aperture 1.40). Volumetric reconstructions of cocultures were performed with the Voxel View program running on a Silicon graphics workstation.

Effect of Interleukin- (IL)-6 on Albumin, Collagen, and Fibrinogen mRNA Expression by Cocultures

Cocultures maintained for at least 10 days were washed twice with PBS after aspirating the culture medium. Fresh HDM containing 20 ng/ml recombinant IL-6 (kindly provided by Dr. T. Hirano, Osaka, Japan) was added, and the cells were harvested 6 hours later as described above. Total RNA was extracted as described below.

Northern Blot Analysis

Total RNA was extracted from the harvested cells as described by Chomczynski and Sacchi[40] with slight modifications.[14] Approximately 10 μg of RNA were electrophoresed on 1% agarose gels and transferred to a GeneScreen filter sheet (New England Nuclear), as described by the manufacturer. The following ^{32}P-labeled probes were used for hybridization: The rat cDNA probe for fibronectin (500-bp *Eco*R1 fragment) was provided by Dr. R. Hynes,[41] rat cDNA for α1(I) procollagen (1.6-kb *Pst*I fragment) was provided by Dr. D. Rowe,[42] rat albumin cDNA (700-bp *Pst*I fragment) provided by Dr. D. Shafritz,[43] and the rat fibrinogen probe (1.2-kb *Pst*I fragment) was provided by Dr. G. R. Crabtree.[44] The probes were radiolabeled by primer extension, with [^{32}P]dCTP with a specific activity of 3000 Ci/mmol (Amersham Corp., Arlington Heights, IL). The specific activity of the labeled probes ranged from 2×10^7 to 6×10^7 cpm/μg DNA. Hybridizations and washings of the blots were performed under stringent conditions as previously described.[14,29] All filters were exposed to Kodak X-Omat film at −70 C with intensifying screens.

Incorporation of [^{35}S]Methionine into Immunoprecipitable Albumin, Ceruloplasmin, and Fibrinogen

Cocultures sustained for 2 weeks were incubated with a methionine-free culture medium that contained 5 μCi/ml [^{35}S]methionine (Amersham Corp.) for 24 hours. The culture medium was harvested, and 1-ml aliquots were incubated overnight with protein G-agarose beads (GammaBind G, Genex Corp., Gaithersburg, MD) that had been previously incubated with one of the following polyclonal antibodies: anti-albumin (kindly provided by Drs. J. and N. Roy-Chowdhury, Albert Einstein College of Medicine), anti-fibrinogen (Accurate Chemical and Scientific Corp., Westbury, NY), or anti-ceruloplasmin, kindly provided by Dr. Michael Schilsky (Albert Einstein College of Medicine). Agarose beads were collected by centrifugation for 5 seconds at 16,000 rpm in a microcentrifuge. They were washed several times with PBS, once with Tris-buffered saline, and once more with PBS. After adding 100 μl of Laemmli buffer[45] to the agarose beads and boiling the samples for 2 minutes at 100 C, 50-μl aliquots were electrophoresed on 10% SDS-PAGE gels. Gels were incubated with Enhance (NEN Research Products, Boston, MA) and dried. The presence of the immunoprecipitated proteins was established by fluorography with Kodak X-Omatic film.

Results

The morphology of hepatocytes in coculture is well preserved. Although variable in size (range of 20 to 31 μ measured at the cell center), on the average, these cells are smaller in size than those plated on plastic or on collagen-coated dishes.[3] They are characteristically cuboidal and form cords composed of two or three rows of cells. Figure 1, a-c shows the overall morphological appearance of the FSC-hepatocyte cocultures. Linear groups of hepatocytes are evident with FSCs in close proximity to one surface of the hepatocytes, and mitotic figures in both hepatocytes and FSCs are commonly found (Figure 1a). Figure 1b shows the localization of ATPase to the apical surface of contiguous hepatocytes. ATPase activity is enriched at the bile canaliculus pole in hepatocytes from rat liver; its presence indicates that bile canaliculi have formed between contiguous cells[37] and suggests that hepatocytes in the cocultures are polarized. Figure 1c shows the localization of catalase in peroxisomes, a hepatocyte-specific organelle.[38] ATPase activity or catalase-positive peroxisomes are not observed in FSCs. Figure 2, a and b, show the distribution of tubulin in microtubules in FSCs and in hepatocytes the microtubules appear as elongated tubules in both cell types; they are distributed throughout the cytoplasm and are concentrated in the centrosomal region close to the nucleus of FSCs and hepatocytes (Figure 2b) and near the bile canaliculus in hepatocytes.

Confocal microscopy also reveals important topographic relations between FSC-hepatocyte cocultures. In Figure 2a only a small area of the hepatocyte surface is contacted by the FSC. Furthermore, FSCs send out long projections and make contact with hepatocytes that are some distance away. In Figures 3, a-d, *y* axis views reveal that the contact region is over the centriole/nuclear region; in this region, microtubules are seen radiating from the centriole to the region beneath the site where the FSC extension touches the hepatocyte surface. Actin distribution in hepatocytes is found at the periphery of the cells and is concentrated near the bile canaliculi; in the FSC, actin is distributed at the cell periphery (not illustrated). Ultrastructural studies (Figure 4A) reveal typical hepatocyte and FSC subcellular structures and corroborates the establishment of hepatocyte polarity. Microvilli are seen at the hepatocyte basal plasma

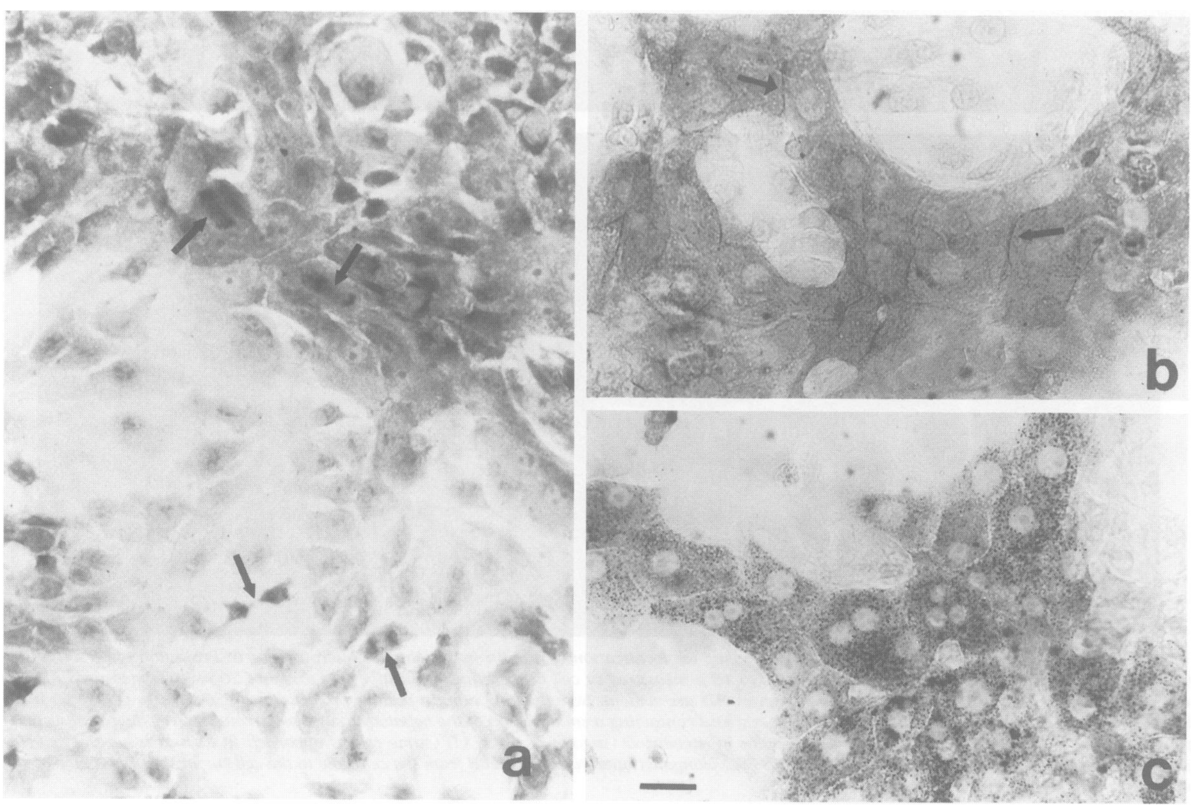

Figure 1. *Cytochemistry of cocultures.* **a:** *Methyl green pyronine staining. Several hepatocytes and FSCs are seen in mitosis (arrows).* **b:** *ATPase localization. ATPase activity is distributed between contiguous hepatocytes in bile canaliculi (arrows).* **c:** *Catalase localization. Catalase activity is found in hepatocyte cytoplasmic spherical structures (black dots) that correspond to peroxisomes. Bar, 50 μ.*

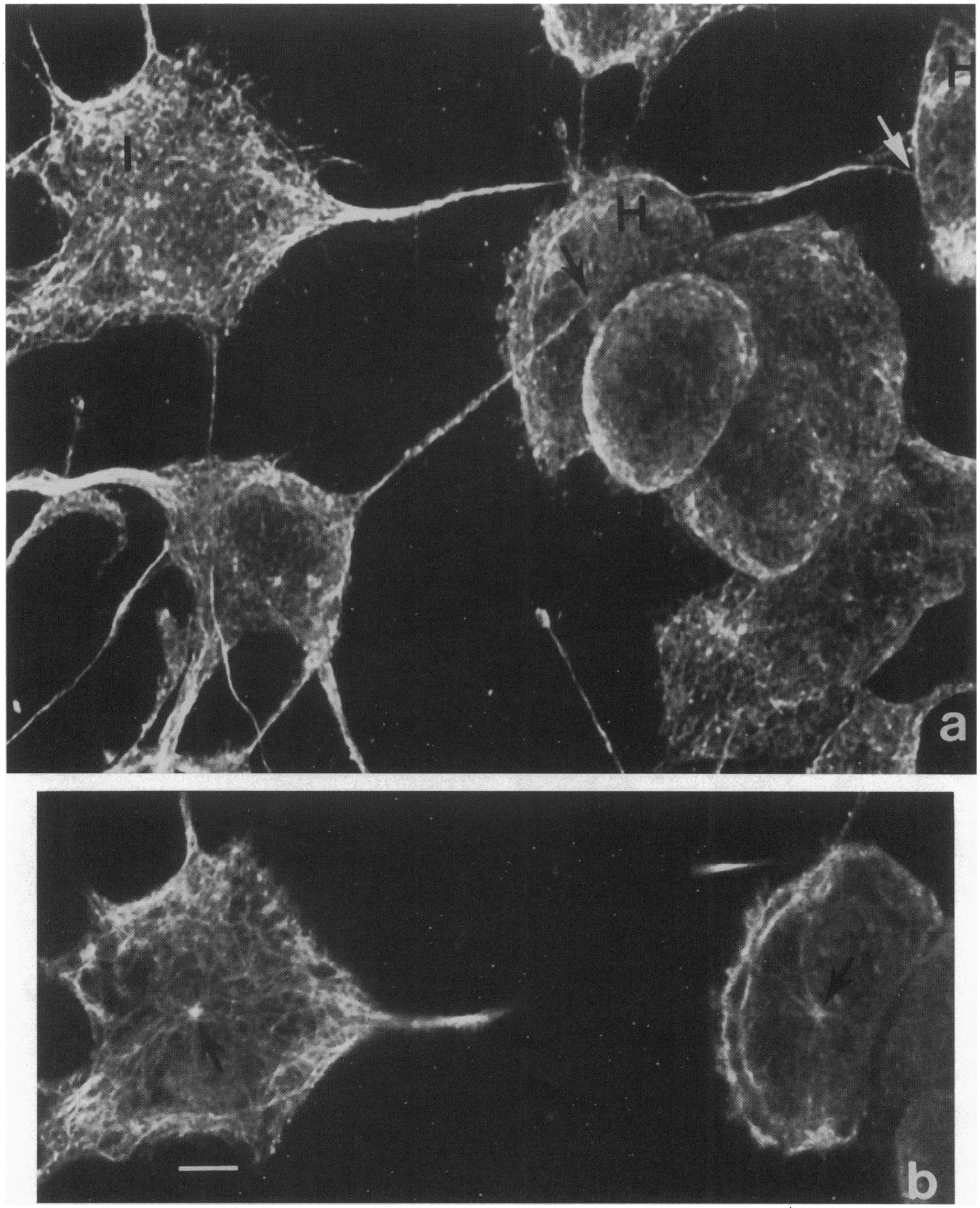

Figure 2. *Confocal microscope images of cocultures showing the localization of tubulin in cytoplasmic microtubules.* **a**: *Projection of 31 serial optical sections of the entire depth of FSCs (range of 12 to 18 μ measured at cell center) and of hepatocytes (range of 20 to 31 μ measured at cell center). The spatial relations of FSCs (I) and hepatocytes (H) are evident. Note the long tubulin-positive projections of FSCs (range of 8 to 14 μ in depth and 5 to 9 μ in length) extending to the hepatocyte and contacting a small region of the hepatocyte surface (arrows).* **b**: *A 1-μ optical section within the above projection of sections showing tubulin in a centriole (arrow) of a FSC (I) (same cell as upper left in* **a**) *and a hepatocyte (H) (same cell as upper right in* **a**). *In the hepatocyte and FSC, elongated microtubules extend from the centriole to the cell surface; in the FSC, microtubules are also seen within its projections. Bar, 10 μ.*

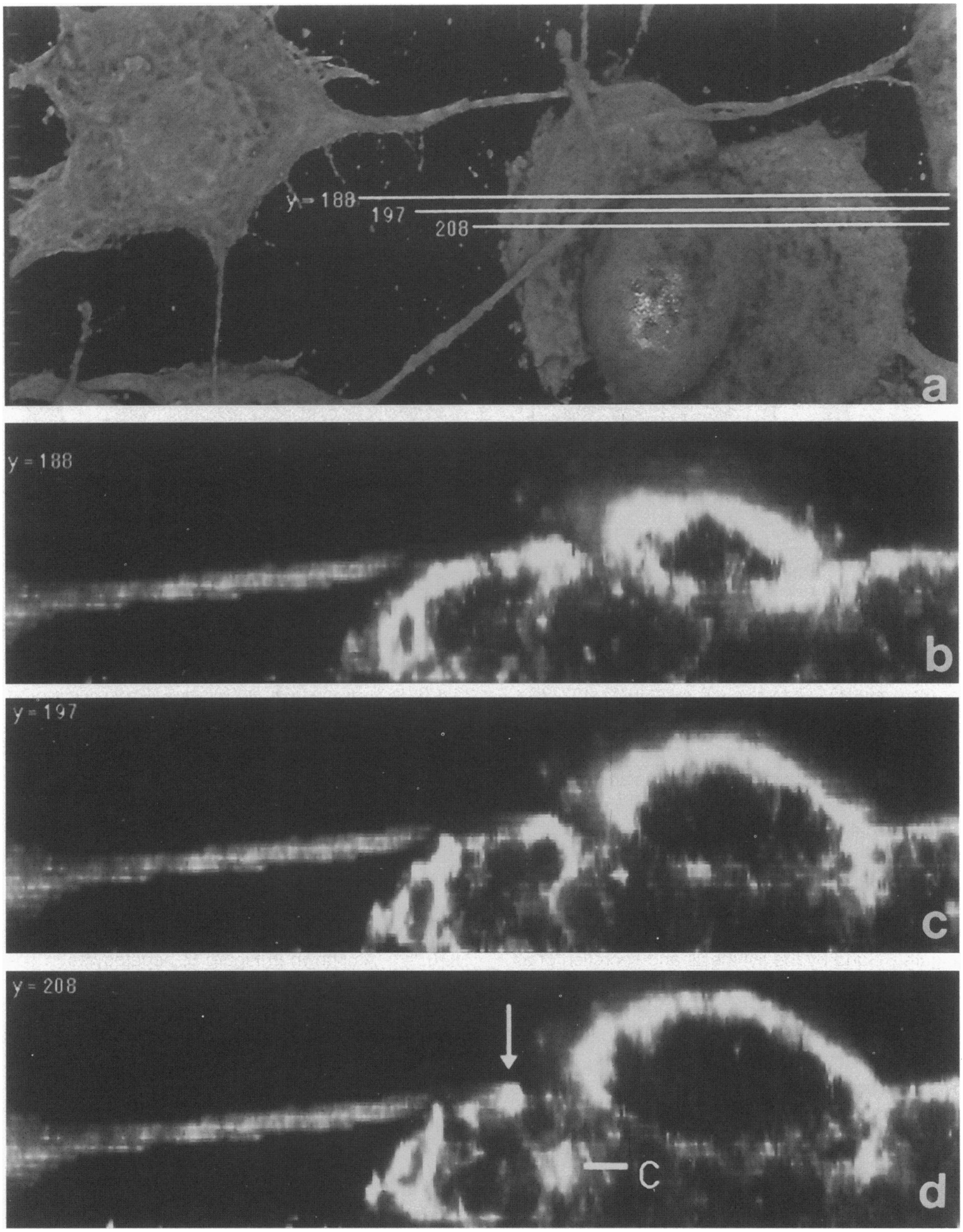

Figure 3. *Confocal microscope images of cocultures immunostained for tubulin.* **a**: *Volume of FSCs and hepatocytes rendered with Voxel View.* **b** *to* **d**: y *axis views examined at three different points in the area of contact between a FSC extension and a hepatocyte. Cytoplasmic microtubules appear white; note the presence of microtubules beneath the area of contact (arrow). In* (**d**), *the area of contact is above a centriole region (C). Microtubules appear to extend from the centriole to the surface beneath the contact area. Bar, 10 μ.*

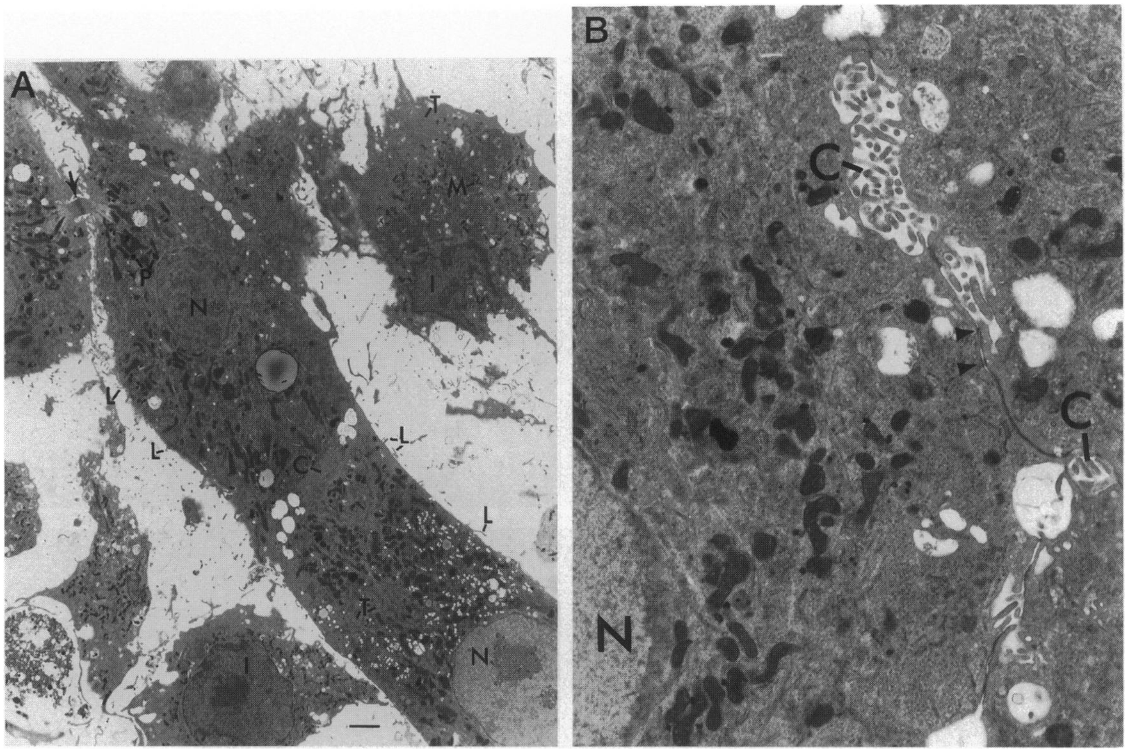

Figure 4. **A:** *Ultrastructural appearance of FSC and hepatocyte cocultures incubated for catalase activity with a diaminobenzidine, pH 9.7, cytochemical method. Hepatocyte nuclei are indicated by N and FSC nuclei by I. Hepatocytes are attached to each other and, between contiguous hepatocytes, a bile canaliculus (C) is evident. Note the presence of a midbody (arrow) connecting two separating hepatocytes at the end of mitosis. Microvilli (L) are seen at the basal surfaces of hepatocytes and at the bile canaliculus. Microtubules (T) are evident in both cell types. Also labeled are peroxisomes (P) in hepatocytes and mitochondria (M) in FSCs. Bar, 2.5 μ.* **B:** *Microvilli are evident on bile canaliculi between contiguous hepatocytes. Note the similarity of their ultrastructure to that of adjacent hepatocytes in intact liver. Arrowheads indicate the presence of desmosomes and junctional complexes on the opposing lateral membranes. Also labeled is the nucleus (N). Magnification, $\times$10,000.*

membrane surface and microvilli at the apical plasma membrane between contiguous hepatocytes form bile canaliculi bounded by tight junctions (Figure 4B).

Cocultures express a number of hepatocyte-specific functions as determined by the expression of asialoglycoprotein receptor protein (Figure 5), high levels of mRNAs coding for albumin and fibrinogen (Figure 6), and by the synthesis and secretion of albumin, ceruloplasmin, and fibrinogen (Figure 7). As illustrated in Figure 5, levels of asialoglycoprotein receptor in cocultures maintained for 10 days is approximately 30% of that of freshly isolated hepatocytes. Hence, in these cocultures in which FSCs contribute a significant amount of protein but do not express receptors, the amount of asialoglycoprotein receptor present in the plasma membrane of the hepatocytes is likely to be even higher than estimated. In contrast to these results, hepatocytes cultured on plastic for 4 days contain no detectable asialoglycoprotein receptor protein (not shown).

Figure 6 demonstrates that, after an initial decrease in mRNAs coding for albumin and fibrinogen, steady-state levels of these mRNAs increase and remain elevated over the 2-week period of the experiments. The initial drop in mRNA expression is associated, in part, with a decrease in the total number of hepatocytes plated, mainly as a result of detachment and death of hepatocytes that piled up on top of other hepatocytes.

It has been established that increased expression of fibrinogen mRNA is transcriptionally regulated by IL-6.[46,47] As shown in Figure 8, hepatocytes in coculture respond to IL-6 with increased expression of fibrinogen mRNA. As this gene is transcriptionally regulated, the results would suggest that hepatocytes in coculture are transcribing genes. Cocultures also express high levels of α1(I) procollagen and fibronectin mRNAs. The addition of IL-6 to cocultures induced the expression of α1(I) procollagen mRNA (Figure 9). As FSCs and not hepatocytes respond to recombinant IL-6 with increased expression of α1(I) procollagen mRNA,[14,48] these results suggest that FSCs are also functional. IL-6 also induced the expression of fibronectin mRNA (Figure 8). However, the actual cellular source of α1(I) procollagen and fibronectin transcripts in the coculture remains to be determined as both cell types used for the coculture are known to express both mRNAs.[23–27,49–51]

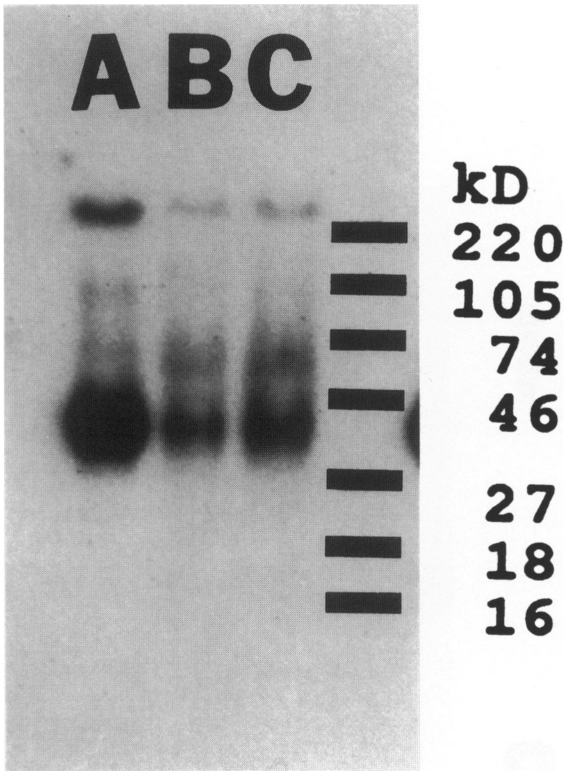

Figure 5. *Western blot analysis of proteins extracted from cocultures, electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose*[32] *(see Materials and Methods). The blot was probed with a rabbit anti-asialoglycoprotein receptor antibody.*[34] *Bound antibody was visualized after incubation with* ^{125}*I-labeled protein A and autoradiography.* **Lane A** *corresponds to 20 µg of protein extracted from freshly isolated hepatocytes.* **Lanes B** *(20 µg of protein) and* **C** *(40 µg of protein) represent samples obtained from cocultures maintained for 10 days. The horizontal lines represent the position of the various molecular weight markers.*

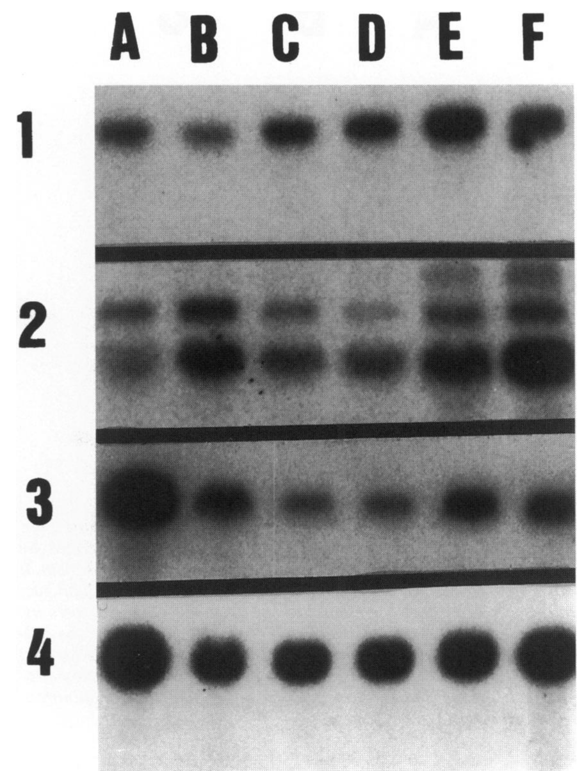

Figure 6. *Northern blot analysis of total RNA extracted from cocultures of hepatocytes and FSCs at 24* (**A**), *48* (**B**), *72* (**C**), *and 96* (**D**) *hours and at 7* (**E**) *and 14* (**F**) *days after plating the hepatocytes on the FSCs (see Materials and Methods). The same blot was sequentially probed with cDNAs for fibronectin* (**1**), *α1(I) procollagen* (**2**), *albumin* (**3**), *and fibrinogen* (**4**). *Blots were exposed for various time periods that varied from 15 minutes for albumin to 24 hours for α1(I) procollagen.*

mRNA levels may not always reflect the capacity of cells to produce and secrete proteins. Therefore, it was important to determine whether the hepatocytes retained the capacity to synthesize and secrete liver-specific proteins. [^{35}S]Methionine labeling experiments demonstrate that cocultured hepatocytes produce and secrete a number of plasma proteins (see Figure 7). Experiments performed in duplicate showed that 2-week cocultures incorporated 3.0×10^6 and 2.8×10^6 cpm of the label into trichloroacetic acid-precipitable protein; of this, 5.48×10^4 and 4.98×10^4 cpm were incorporated into secreted proteins and 2.5×10^6 and 2.3×10^6 cpm were incorporated into cellular proteins. Of the total radioactivity in the culture medium, approximately 8.4% was recovered in albumin, 3.7% in fibrinogen, and 1.8% in ceruloplasmin immunoprecipitates.

FSCs express the gap junction protein connexin (Cx)43 and form functional gap junctions in culture.[14] Among the types of interactions possible between FSCs and hepatocytes *in vivo* and *in vitro* is gap junction-mediated direct intracellular communication. As shown in Figure 10, injection of Lucifer yellow into hepatocytes resulted in transfer of the dye within 1 minute to other hepatocytes and FSCs. We had earlier shown that FSCs express and phosphorylate the gap junction protein Cx43 and form functional gap junctions in culture.[14] Interestingly, in experiments designed to determine which of the FSC clones developed in our laboratory[14] was most capable of sustaining hepatocyte function, we found a direct correlation between this capacity and expression of Cx43 (Table 1).

Discussion

The cocultures described in this communication have cellular structures and interactions similar to those in hepatocytes and FSCs *in vivo.* In the coculture system, the hepatocytes establish polarity with characteristic apical and basolateral surfaces, including formation of bile canaliculi and tight junctions. The FSCs

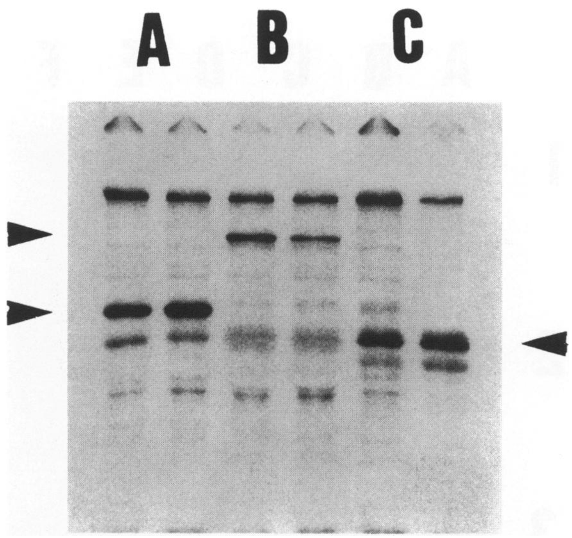

Figure 7. *Fluorography of [^{35}S]-methionine-labeled albumin* (**A**), *ceruloplasmin* (**B**), *and fibrinogen* (**C**) *produced and secreted by cocultures of hepatocytes and FSCs sustained for 2 weeks with a serum-free HDM. These experiments were performed in duplicate, and each pair of lines correspond to one protein. Cocultures were incubated for 24 hours with [^{35}S]methionine, and aliquots of the culture medium were immunoadsorbed to protein G-agarose beads that had been precoated with a polyclonal antibody to the respective protein. The arrows indicate the position of ceruloplasmin, albumin, and fibrinogen.*

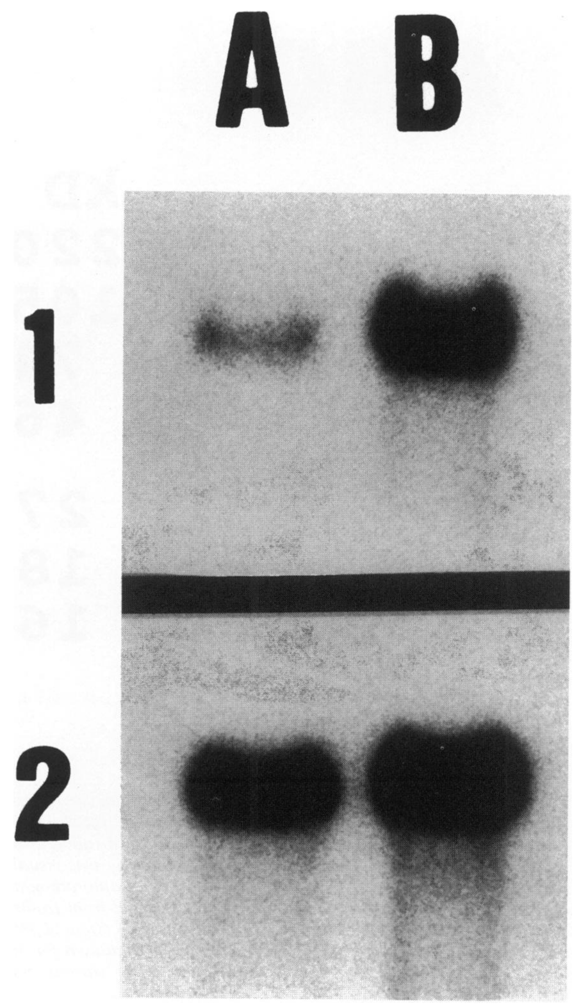

Figure 8. *Northern blot analysis of total RNA extracted from cocultures of hepatocytes and FSCs maintained for 10 days in culture with a serum-free HDM* (**A**). *Sample* (**B**) *was obtained from cocultures maintained as described in* (**A**), *except that 20 ng/ml recombinant IL-6 were added 6 hours before harvesting the cells and extracting RNA. The same blot was sequentially probed with a fibrinogen* (**1**) *and an albumin* (**2**) *cDNA, respectively. Note the induction of fibrinogen, whereas albumin shows little change after the cells were exposed to recombinant IL-6.*

establish long extensive projections, some of which contact the basal surfaces of hepatocytes. The site of contact between hepatocytes and FSCs occurs in a restricted area of the hepatocyte surface that appears to be over the centrosome/nuclear region of the hepatocyte. In this region, concentrations of microtubules are found that radiate out to the basal and apical surfaces of the hepatocyte. Although the significance of the interaction between FSCs and hepatocytes in this region is unknown, it may play an important role in cell-to-cell signaling and in sustaining the functional capacity of both cell types. We may speculate that functional gap junctions between FSCs and hepatocytes, described in this paper, may also occur in the centrosome/nuclear region and could play a role in establishing direct communication between the two cell types. The functional role of this region has been demonstrated in short-term cultures of rat hepatocytes grown on collagen in the absence of FSCs; we have found that the centrosome-associated microtubules play a role in receptor-mediated endocytosis of asialoglycoproteins (Novikoff et al., manuscript in preparation).

Hepatocytes cocultured with CFSC-2G cells have maintained a number of diverse differentiated functions. They synthesize and secrete liver-specific proteins (see Figures 5 to 7) and they respond to IL-6 with increased expression of fibrinogen mRNA. As previously shown,[46] fibrinogen is transcriptionally activated by IL-6, thus suggesting that hepatocytes in coculture retain the capacity to transcribe genes.

Although CFSC-2G cells have a doubling time of approximately 24 to 36 hours when cultured with 10% FBS,[14] they do not proliferate when cultured with the serum-free HDM developed by Reid and Jefferson.[31] However, when placed in coculture with hepatocytes, CFSC-2G cells proliferate (see Figure 1a). These findings indicate that hepatocytes are producing a growth factor that induces the proliferation of FSCs. Indeed, the presence of such a factor in hepatocyte-conditioned medium has been recently suggested.[52] The hepatocytes also modify the capacity of

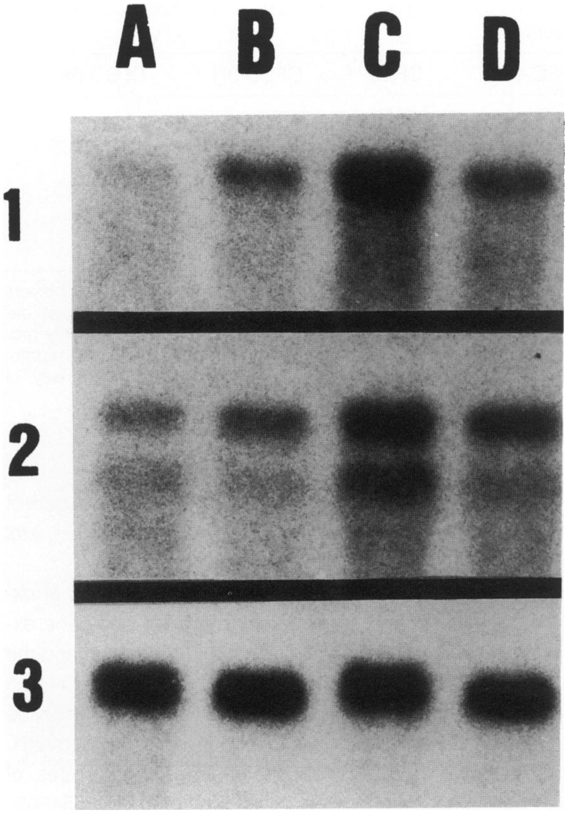

Figure 9. *Northern blot analysis of total RNA extracted from cocultures of hepatocytes and FSCs as described in Figure 8. Cocultures were incubated with either 10* (**B**), *20* (**C**), *or 40* (**D**) *ng/ml recombinant IL-6. RNA from untreated controls is shown in* (**A**). *Blots were sequentially probed with a fibronectin* (**1**), *an α1(I) procollagen* (**2**), *or an albumin* (**3**) *cDNA probe. Note that IL-6 induced the expression of α1(I) procollagen and fibronectin mRNAs in a dose-dependent manner. However, 40 ng/ml IL-6 was already inhibitory. As also shown, IL-6 had no effect on the expression of albumin mRNA.*

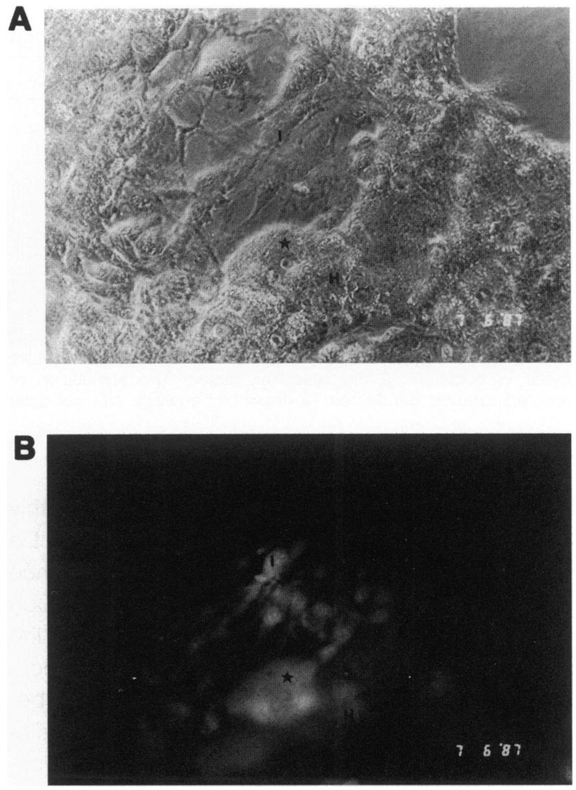

Figure 10. *Dye coupling between cocultured FSCs and hepatocytes.* **A:** *Phase contrast photograph of cocultured hepatocytes (H) and FSC (I).* **B:** *Fluorescent photograph of the same field as pictured in* (**A**). *Lucifer yellow (5%) was microinjected into a hepatocyte (star) and within 1 minute had spread to other hepatocytes and to cells in the FSC layer. Hepatocytes and FSCs were easily distinguished by their characteristic morphology.*

CFSC-2G cells to express α1(I) procollagen mRNA. As previously shown by Greenwel et al,[14] CFSC-2G cells express very low levels of α1(I) procollagen mRNA under basal conditions. However, basal expression of α1(I) procollagen in cocultures is drastically increased. Although we have not yet determined the expression of cytokines and growth factors by cocultures, it is possible that hepatocytes produce and secrete transforming growth factor-α, one of the growth factors produced during liver cell regeneration.[53,54] This cytokine is known to induce hepatocyte[53] and FSC proliferation[21] and also induces the expression of α1(I) procollagen by cultured FSCs.[21] In addition, we have shown that transforming growth factor-α induces a change in morphology in FSCs, similar to that observed in cocultures.[55]

An unexpected finding was that the ability of FSC lines to assist in maintaining differentiated hepatocyte function in cocultures correlated with their level of expression of the gap junction protein Cx43. Furthermore, we found fluorescent dye transfer, mediated by gap junctions, between hepatocytes and FSCs in cocultures. Although close contacts between the two cell types is known to occur *in vivo*,[56,57] the presence of gap junctions has not been reported. In this context, it is notable that FSCs express Cx43[14] whereas hepatocytes express Cx32 and Cx26.[58–61] Our three-dimensional studies have demonstrated that the contact site between FSCs and hepatocytes occupies an area equal to the width of the tip of a FSC projection and is, indeed, a very small area of the hepatocyte surface. The chances of finding such a contact by two-dimensional analysis (eg, conventional light and electron microscopy) are extremely low and would explain why contact sites have not been seen previously. Studies are in progress to perform three-dimensional reconstructions and volume rendition of cocultures in which tubulin and connexins 43, 32, and 26 are immunolocalized. These studies should permit a detailed analysis of the contact site and the possible interrelations between centrosome-associated microtubules and gap junctions.

Table 1. *Correlation between the Expression of Cx43 by FSC Clones and Their Capacity to Sustain Hepatocyte Survival and Expression of Liver-Specific Proteins by Hepatocytes in Coculture*

FSC clone	CFSC-2G	CFSC-8B	CFSC	CFSC-3H	CFSC-5H
Cx43 content	22.8	11.9	5.5	3.0	1
Collagen type I	+	+	+++	++++	+++++
Collagen type III	+	+	+	+++	+++
Fibronectin	++++	+++++	+++	++++	++++
Laminin	++	++	++	+++	+++
Coculture (*survival*)	++++	ND	++	+	+
Coculture (*albumin mRNA synthesis*)	4.8	ND	2.4	ND	1.0

The relative expression of Cx43 was derived from the relative density of autoradiographic bands from Western blot analysis of monolayers of FSCs. Approximately 75 µg of cellular protein were electrophoresed on 7.5% SDS-PAGE and transferred to nitrocellulose paper as described by Yamamoto et al.[32] Blots were probed with an anti-Cx43 antibody.[33] The relative levels of mRNA for extracellular components produced by monolayers of FSC clones are indicated by pluses. Likewise, the ability of different FSC clone lines to promote survival of hepatocytes in coculture is indicated by pluses. The stimulation of hepatocyte albumin synthesis was quantified by relative density of autoradiographic bands from Northern blot analysis. ND, not determined.

In summary, the coculture system described in this communication has unique features that make it a useful model in which the functional interdependence of hepatocytes and FSCs can be studied. Although we have investigated only the effect of FSCs on the functional capacity of the hepatocytes, it is also possible to explore how the hepatocytes modify the behavior of FSCs. As FSCs are the main producers of extracellular matrix components in normal and cirrhotic livers,[20,21] it is possible to use the coculture system to explore how ethanol, CCl_4, or other toxins that induce hepatocyte injury may affect collagen gene expression by FSCs. Indeed, preliminary results from our laboratory have indicated that the coculture system appears to be a useful model to study liver fibrosis *in vitro*.[62] Finally, the unexpected demonstration of functional gap junctions between hepatocytes and FSCs suggests that such communication may exist *in vivo* and may have a role in the development and/or maintenance of hepatocyte function.

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